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A novel ammonia lowering strategy for the treatment of intracranial hypertension in acute liver failure

Experimental studies in pigs

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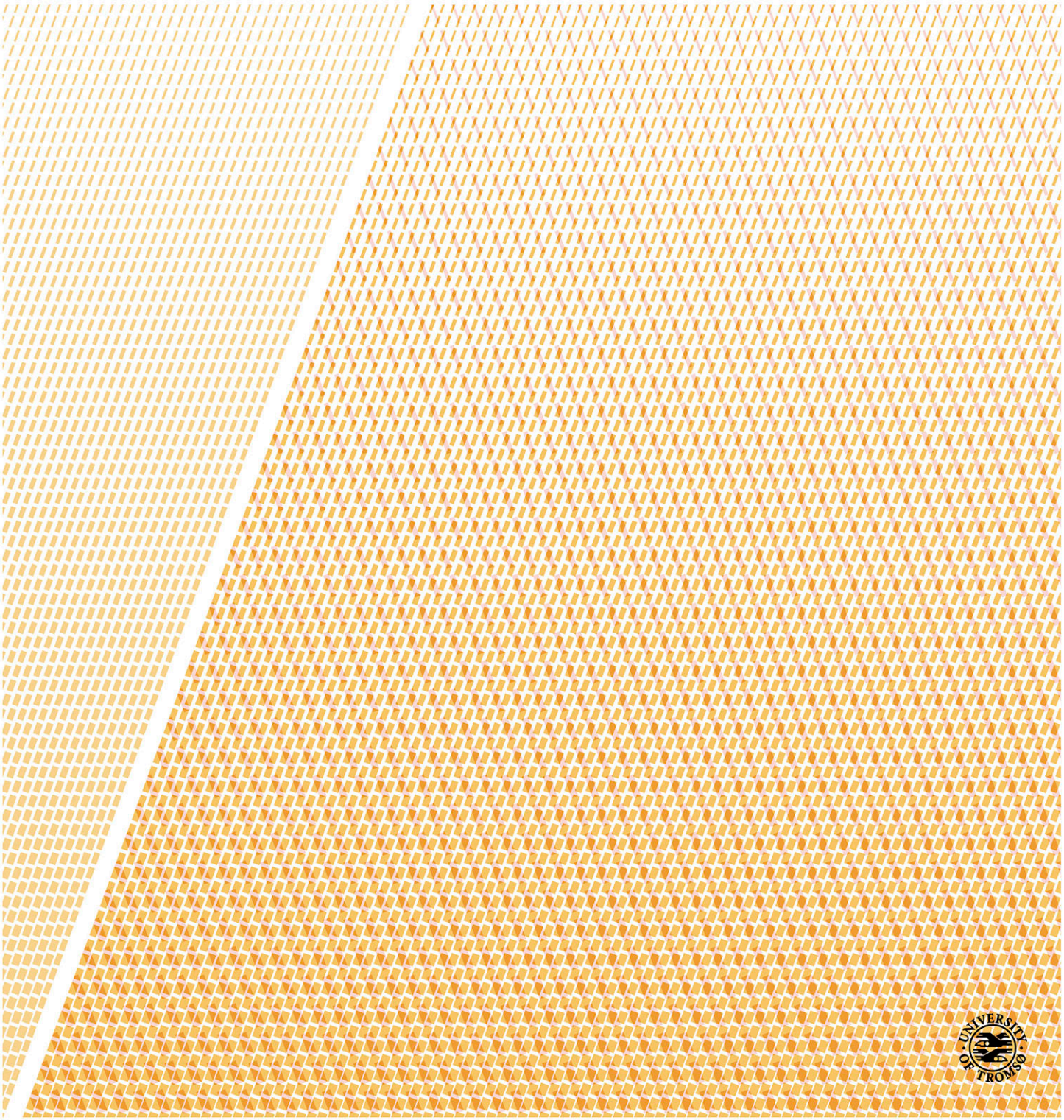
A dissertation for the degree of Philosophiae Doctor – January 2016



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1. BACKGROUND

Acute liver failure

Acute liver failure (ALF) is a clinical syndrome characterized by an acute insult to the liver leading to severely impaired liver function with coagulopathy due to hepatocyte necrosis and the development of hepatic encephalopathy (HE) (1, 2). This occurs with no preexisting liver disease in contrast to patients suffering from acute on chronic liver failure. ALF was initially described in 1946 and further defined by Trey and Davidson in 1970 as a "potentially reversible condition, the consequence of severe liver injury, with an onset of hepatic encephalopathy within eight weeks of the appearance of the first symptoms and in the absence of pre-existing liver disease" (3). Today, the preferred categorization introduced by O`Grady et al. in 1993 (4) takes into account the time interval between onset of symptoms, defined as the appearance of jaundice, and the development of encephalopathy (HE) (table 1). Clinically this association has important prognostic implications. This time interval can also provide indications on the etiology of hepatic failure (i.e paracetamol more often causes hyperacute liver failure while idiosyncratic drug reactions often cause sub-acute liver failure (5)).

	Hyperacute	Acute	Subacute
Prognosis	Moderate	Poor	Poor
Duration of jaundice	0-7 days	8-28 days	> 28 days
Cerebral edema	Common	Common	Infrequent
Encephalopathy	Yes	Yes	Yes
Prothrombin time	Prolonged	Prolonged	Least prolonged
Bilirubin	Least raised	Raised	Raised

Table 1 Categorization of ALF.

ALF is a rare disease with incidence of less than 10 cases per million per year in the industrialized world (6, 7). Intoxication with paracetamol (acetaminophen) has been the single most frequent etiological factor for the development of ALF in the Western world (5, 8, 9), while viral hepatitis type A and E are the major causes in the developing world (6, 10). Intoxication caused by paracetamol has decreased in the UK since 1998, and the overall survival from ALF has increased from 17 % in the time period 1973 – 1978 to 62 % in the time period 2004 – 2008. This has mainly been due to early referral to tertiary centers, improved intensive care treatment and increased use of emergency liver

transplantations (7). Overall survival from ALF in the USA now exceeds 65 %, with a spontaneous recovery rate of 40 % (5).

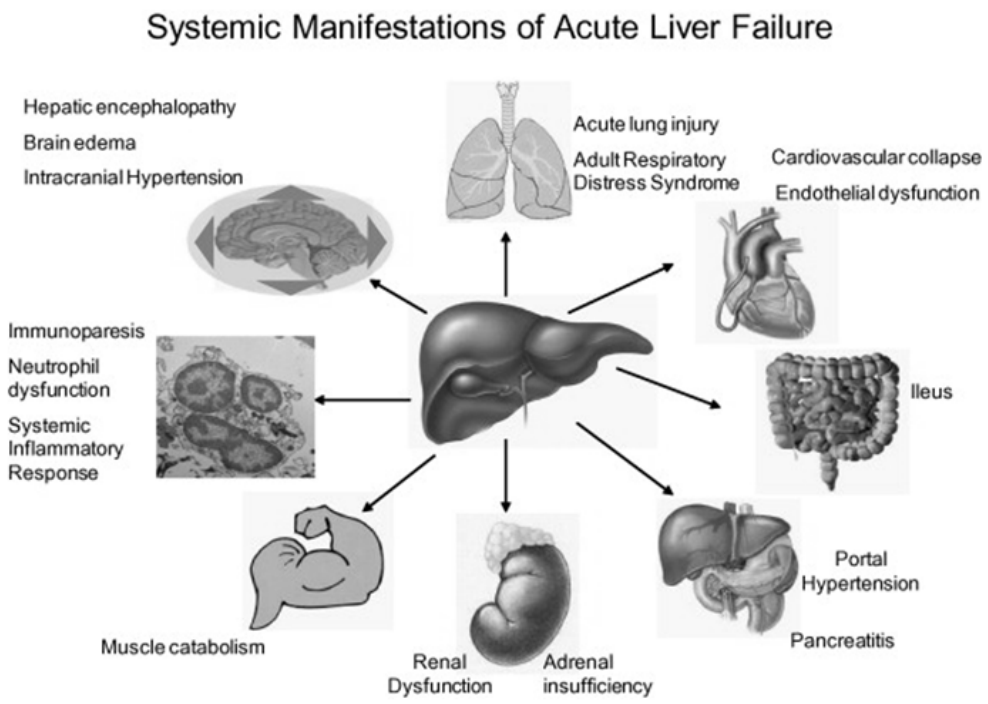

Figure 1 Systemic manifestations of ALF.

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ALF leads to multiorgan failure (MOF) (6), making the treatment challenging (figure 1). Today the major cause of death is the development of MOF triggered by a systemic inflammatory response syndrome (SIRS) with or without manifest infection (5). The acute liver necrosis leads to release of pro-inflammatory cytokines, and exceeds the regenerative capacity of the liver precipitating the development of the clinical ALF syndrome (11). Fulfilled SIRS criteria worsens the prognosis and are strongly linked to the progression of encephalopathy (12).

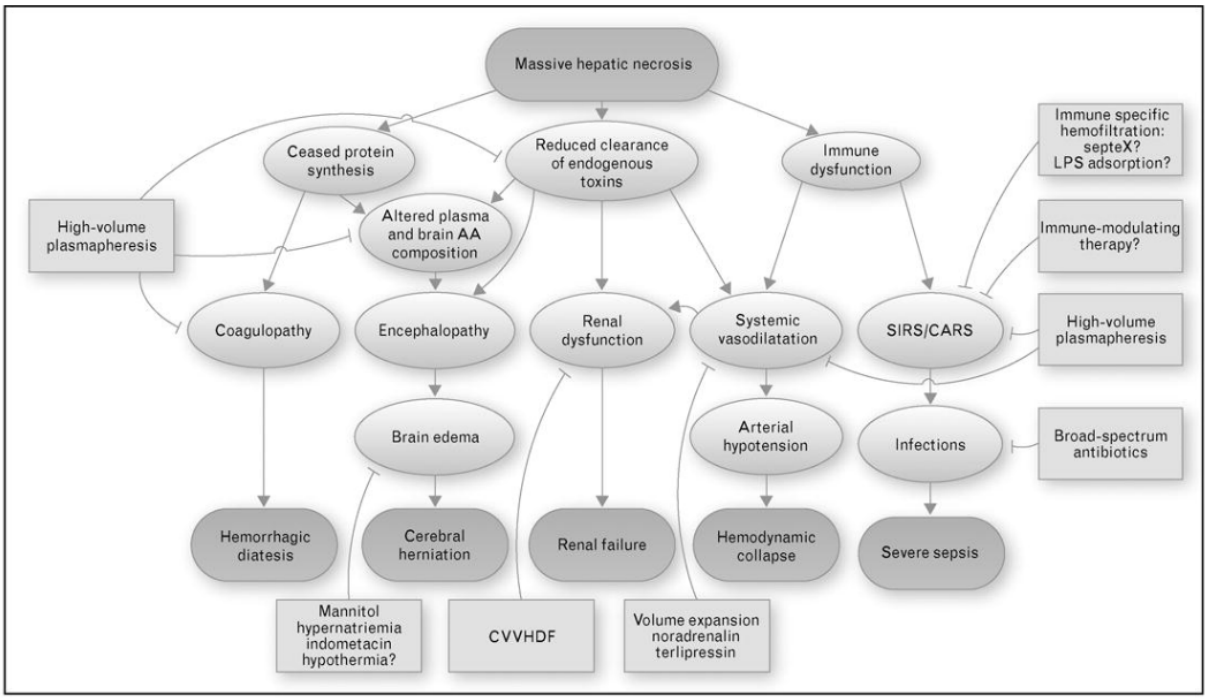

Figure 2 Representation of how MOF develops in ALF.

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Patients with ALF develop a hyperdynamic circulatory state with systemic arterial vasodilation due to reduced precapillary sphincter tone leading to low peripheral resistance. Cardiac output is increased causing a high output low resistance state (13) (figure 2). Hemodynamic changes also include increased portal pressure, splanchnic sequestration of blood and decreased venous return, although not as pronounced as in patients with liver cirrhosis (5, 8, 14).

ALF patients develop disturbances in the coagulation system due to reduced production of coagulation factors and accompanying loss of platelets (5, 15), and altered fibrinolytic activity (16, 17). An increased international normalized ratio (INR) is also a determinant of prognosis.

Failure of the liver also leads to hormonal and metabolic disturbances such as adrenal insufficiency, hypoglycemia and reduced lactate and ammonia clearance (18, 19). Glucose homeostasis is impaired due to impaired gluconeogenesis. Lactate levels are increased due to both increased production (impaired microcirculation) and decreased clearance (Cori cycle). Lactate also serves as a prognostic marker in patients with paracetamol-induced ALF (20, 21). General muscle catabolism is seen in ALF and can have impact on ammonia removal by skeletal muscle. An association between ALF and acute pancreatitis has also been reported (22).

Furthermore, impaired immune function shown by increased susceptibility to infections and dysfunction of neutrophils have been found in ALF patients (23). The presence of infection is associated with progression of encephalopathy and increased mortality (12, 24).

Renal failure due to hypotension and acute tubular necrosis (13) is common in ALF (25), with an incidence near 50 % (5, 26). The development of renal dysfunction is associated with worsened prognosis (27), and the presence of SIRS has been found to be predictive for the development of acute renal dysfunction in patients with non-paracetamol induced ALF (28). In ALF caused by paracetamol intoxication, the toxicity of the drug itself can also contribute to the development of renal dysfunction (5, 13).

Respiratory dysfunction is uncommon in the early phase of ALF, but acute lung injury can develop and often associated with sepsis (6). Severe lung injury can also present as adult respiratory distress syndrome (ARDS) (13), with a prevalence of 21 to 30 percent in patients with ALF (5, 29).

Ventilatory support can, however, be required due to high grade (III-IV) HE rather than respiratory failure itself, especially in the early phase of the disease (26). Close monitoring of the conscious level in ALF is mandatory as the development of HE is the key element defining its progression from acute liver injury to acute liver failure and defines prognosis (7, 30). In the setting of ALF progressing to HE grade III and IV, Glasgow Coma Scale can provide more clinically relevant information regarding the neurological status of the patient, and is often preferred for continuous evaluation (31). The development of HE grade III and IV necessitates endotracheal intubation to protect the airways and secure optimal oxygenation and ventilation (5).

Historically, the development of intracranial hypertension (ICH) due to brain edema has been present in around 80 % of patients with ALF (32), and represented the main cause of death (33). A retrospective study by Jalan et al. from 2003 found the mortality due to brain herniation to be 35 %, frequently in relation to multi organ failure (30). Recently, the incidence of ICH was found to be reduced to 20 % in a large study from Kings College. Number of deaths due to cerebral herniation (CH) was substantially reduced, which may in part explain the improvements in overall survival (7). However, a mortality rate of 55 % in patients developing ICH warrants the development of new treatments for reducing brain edema.

Pathophysiology of hepatic encephalopathy in acute liver failure

Intracranial hypertension in ALF

HE, originally described by Sherlock et al. (34), is graded from I to IV and encompasses a wide spectrum of neurological symptoms ranging from mild cognitive impairment and confusion, to stupor and coma (35). The development of HE of any degree represents a major deterioration in the clinical state and defines the progression from acute liver injury (ALI) to ALF (36). It is a prognostic factor (37-39) and its grade correlates with outcome (13). HE is categorized in three different types (A-C) based on the underlying pathology, with encephalopathy associated with ALF defined as type A (35). HE in ALF is characterized by a potentially rapid progression to ICH due to brain edema.

Brain edema has been demonstrated to be closely linked with ICH in ALF since an increase in brain water tissue has been observed in both animal models and in patients (32). The development of cerebral swelling in the setting of acute hepatic necrosis was initially described by Ware et al. in 1971 and further evaluated in animal models (40, 41). Brain edema is found in both ALF and acute-on-chronic liver failure (42), but studies in patients with cirrhosis suggest a low grade edema with the clinical picture of rapid developing edema and death from ICH being far less frequent than in ALF (43, 44). Furthermore, patients presenting with hyper acute liver failure more often develop brain edema compared to acute and sub-acute liver failure (5), emphasizing the relevance of the time course in this disease.

ICP is dependent on brain tissue volume, cerebrospinal fluid (CSF) volume and cerebral blood volume. Any change in one of these factors will lead to a compensatory change in either of the two other factors, known as the Monro-Kellie doctrine (45). As disturbances in any of these factors can result in increased ICP, all three factors are closely regulated to maintain an ICP within 7 – 15 mmHg. There is a non-linear relationship between brain volume and ICP that allows the brain volume to increase to a certain point before ICP begins to rise (figure 3B). At this point exponential relationship exists between brain volume and ICP due to the non-compliant and rigid scull (46). ICP between 20 and 25 mmHg has been shown to affect outcome in patients with head injuries (47), and with ICP above 25 compensatory mechanisms do not longer provide sufficient protection to avoid substantial elevations in ICP.

Although the incidence of CH has decreased, the development of ICH remains a major clinical challenge, especially in the absence of liver transplantation as definitive treatment. Interventions to prevent and treat brain edema in patients with HE is still an unmet clinical need (36, 48). Brain tissue comprises 80 percent of the intracranial volume (figure 3A), and elucidating the mechanisms behind the development of brain edema is important (32).

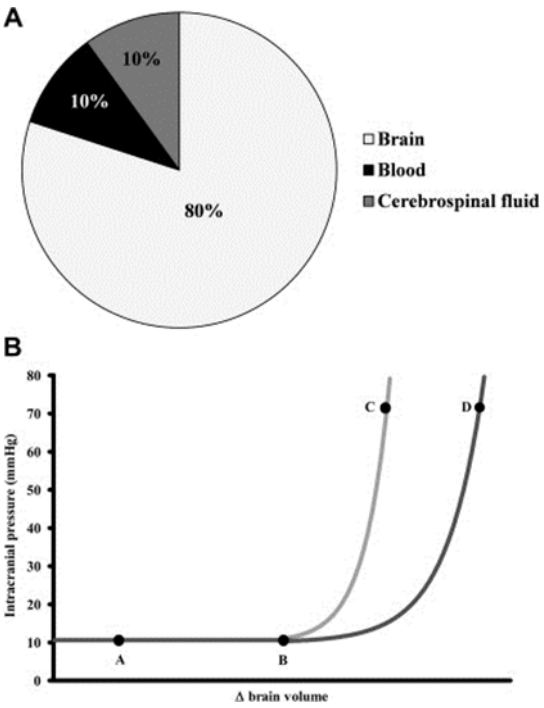

Figure 3 A) Cerebral constituents and their contribution to intracranial volume. (B) Relationship between brain volume and intracranial pressure: points A (healthy individuals) B: a certain increase in brain volume does not lead to an increase in ICP; points B-C: exceeding the brain volume capacity leads to an increase in ICP as observed in ALF; points B-D: due to brain atrophy or alterations in brain volume constituents, a further increase in brain volume is required in order to initiate an increase in ICP.

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Vasogenic and cytotoxic brain edema

Klatzo defined brain edema as "an abnormal accumulation of fluid associated with volumetric enlargement of the brain" and divided it into two different forms; vasogenic and cytotoxic brain edema. This categorization was based on the presence of vessel injury leading to escape of water and plasma constituents into the surrounding brain tissue, or undisturbed vascular permeability (49). Brain edema represents a final common path for several pathological states in the brain, including metabolic encephalopathies such as HE (50). This results in a net increase of brain water content and can consequently affect the overall brain volume.

Cytotoxic edema is caused by metabolic disturbances primarily in astrocytes (soma and processes), which are important cells in brain water regulation (46, 51). These alterations lead to water transfer across the cellular membrane due to osmotic forces, possibly through aquaporins (51, 52). This results in an intracellular swelling of the astrocytes due to shift of water from extracellular space to

intracellular space. This can be induced by metabolites such as ammonia and lactate (51), and can affect both grey and white matter (46).

Vasogenic edema is a result of increased permeability of the vascular cells comprising the blood brain barrier (BBB). This can lead to increased extravasation of water and osmolytes to the extracellular space due to hydrostatic pressure forces (51).

Blood brain barrier

The blood brain barrier is a barrier between the vasculature of the brain and the microenvironment of the neurons. It is of vital importance in order to make a stable microenvironment for the neurons to allow them to function properly (53, 54), and prevents blood constituents such as amino acids, hormones, H+/K+ and cytokines from disturbing neurotransmission.

The neurovascular unit of the CNS consists of neurons, endothelial cells, astrocytes and pericytes with the basal lamina. This unit is involved in cerebral blood flow regulation and can influence the permeability of the BBB (53, 55). The endothelial cells lining cerebral micro-vessels are wrapped upon themselves constituting a network through tight junctions (figure 4). An intercellular barrier is made, preventing free para-cellular diffusion of molecules except for gases and lipid soluble compounds. This leaves much of the cellular transport of substances as glucose, amino acids and other charged molecules dependent on trans-cellular routes and specific transporter systems in the absence of fenestrations (53). Important constituents of tight junctions are the transmembrane proteins occludin, claudin-3 and 5 and junction adhesion molecules (JAMs) (56). These proteins have recently attracted attention as important modulators of permeability of the BBB.

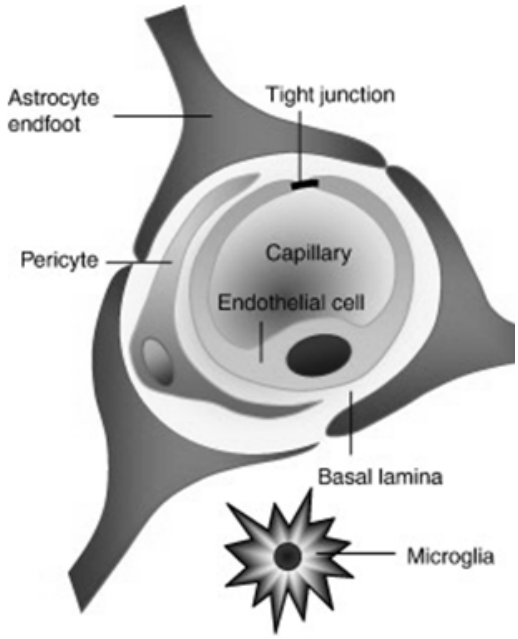

Figure 4 The cell associations at the BBB. The cerebral endothelial cells form tight junctions at their margins which seal the aqueous paracellular diffusional pathway between the cells.

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Glia cells consist of fibrous (white matter) and protoplasmic (grey matter) astrocytes, oligodendroglia and microglia (resident macrophages in the brain). Astrocytic end feet form a network that attaches on to the endothelial lining of the vessels (figure 4). They have an important regulatory role of the BBB through induction of new tight junctions and by modulating the transport of substances across the BBB. Additionally they provide the cellular link to the neurons (51, 53).

BBB breakdown enables water and solutes as plasma proteins freely to move into the brain parenchyma (extracellular space) driven by the systemic blood pressure (51).

Brain edema seen in ALF is characterized by astrocytic swelling and has therefore been considered mainly to be of cytotoxic origin. Permeability changes have been observed, but the BBB has been found histologically intact (57, 58). One of the few histopathological studies performed looking for BBB changes in ALF was a post mortem study showing primarily an intact BBB with no convincing signs of BBB breakdown (59). Increased permeability for large molecules in ALF has however been observed, and ammonia was shown to be a possible cause for this increase (60, 61).

We have learned from earlier work with our pig model that ICP is raised with evidence of brain edema revealed by brain water measurements (62). Furthermore, several other studies have

confirmed the presence of regional differences of brain edema (62-64). However, ultrastructural changes in different brain regions in a large animal model had, to our knowledge, not been investigated before.

Ammonia

The development of brain edema in ALF is today considered to be multifactorial, displaying a synergistic effect of ammonia, systemically and brain derived inflammation and impaired cerebral auto-regulation (55, 65, 66). However, the important role for ammonia in the pathophysiology of ALF remains undisputed and has been the focus for this thesis.

In aqueous solutions ammonia (NH_3) forms a reversible conjugation pair with the ammonium ion (NH_4^+) with pK_a 9.3. At physiological pH about 98% is kept in the ionized form NH_4^+ (67). Ammonia (NH_3) diffuses freely across cell membranes and the ammonium (NH_4^+) with similar ionic characteristics as K^+, can be transported across membranes via K^+ - transporters and channels (68, 69).

A correlation between ammonia and brain edema has been substantially proven in several animal models of ALF (62, 70-72). Clemmesen et al. were the first to correlate arterial ammonia to the incidence of cerebral herniation (CH) in humans. They found arterial ammonia levels of more than 146 μM within 24 hrs after the development of HE grade III to be predictive of CH, and observed increased cerebral ammonia uptake with increasing arterial ammonia levels (73). The relation between ammonia on admission and later cerebral complications was confirmed by Bernal et al. with ammonia concentration being an independent risk factor for the development of HE and ICH. They found that arterial ammonia levels of 200 μM were predictive of ICH in patients with established ALF and HE (38). In a study of ALF from mainly viral causes and with no ammonia lowering therapy or use of liver transplantation, a cut-off value of 124 μM measured on admission to hospital was found to be a predictor for outcome and the development of cerebral edema (74). Kumar et al. found persistent hyperammonemia to predict higher mortalilty and incidence of cerebral edema (75), and Kundra et al. found plasma ammonia levels to correlate with severity of HE and ICH (76). Furthermore, patients with urea cycle enzyme deficiency can develop cerebral edema without having liver insufficiency other than impaired detoxification of nitrogen (77).

Brain ammonia is derived from diffusion from blood and endogenous pathways from glutamine, glutamate and aspartate metabolism (78). Arterial blood ammonia is the major determinant of brain ammonia uptake (79, 80), and net ammonia uptake from blood to brain has been shown in several human studies both in normal state and hyperammonemic state (73, 81-84).

Nitrogen homeostasis

Nitrogen containing molecules are under strict regulation in the body. Nitrogen is important in a number of synthetic pathways and reactions, but its metabolic product ammonia is toxic to the brain necessitating a strict regulation to maintain homeostasis (67, 85). Ammonia is transported from peripheral organs to the liver as glutamine or alanine. The liver is the only organ containing the complete enzymatic machinery for urea production and thereby net excretion of nitrogen (86). Ammonia is furthermore derived from the gut by bacterial degradation and catabolism of amino acids, proteins and nucleic acids.

Ammonia can be fixed into metabolic molecules, such as amino acids, through the reactions of different enzymes including glutamate dehydrogenase (GDH), glutamine synthetase (GS) and carbamoyl phosphate synthetase (87). The latter captures ammonia for processing in the urea cycle, producing urea as the non-toxic excretion product of nitrogen. However, in the setting of diminished urea synthesis capacity the first two pathways are of pivotal importance.

GDH incorporates ammonia by direct amination of α-ketoglutarate yielding glutamate (figure 5). GS amidates glutamate; taking up free ammonium for the production of glutamine (figure 6). GS is a cytosolic enzyme located in perivenous hepatocytes and in several other organs such as muscle, kidney and brain. In the liver it functions as an ammonia scavenger for any ammonia escaping the periportal urea synthesis. Both reactions can potentially incorporate ammonia in the presence of hyperammonemia (67) and therefore are important in liver failure.

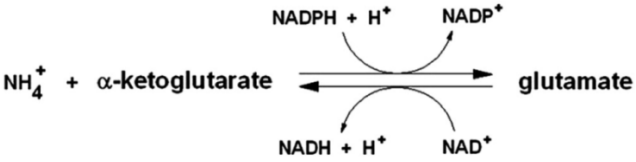

Figure 5 Glutamate dehydrogenase reaction.

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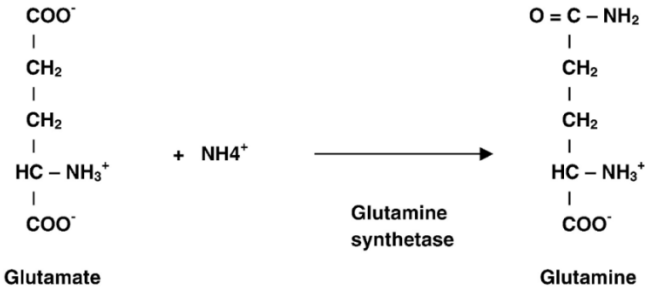

Figure 6 Glutamine synthetase reaction (GS).

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The enzyme glutaminase is responsible for the removal of the amide group from glutamine yielding glutamate and ammonium (figure 7). This is a mitochondrial enzyme that is expressed in several tissues including liver, brain, kidney and small intestines (67).

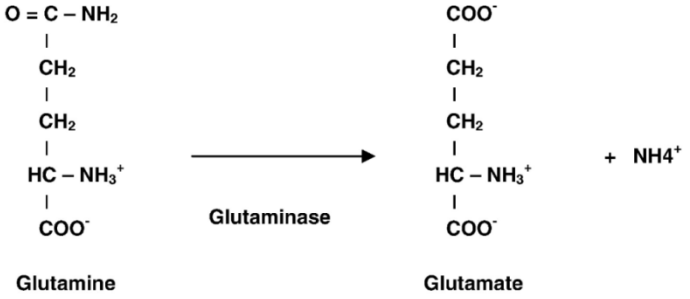

Figure 7 Glutaminase reaction.

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GS and glutaminase reactions play important roles in interorgan nitrogen homestasis.

Amino acids and ammonia metabolism

Muscle turnover and breakdown of ingested amino acids are the primary sources of free amino acids. The body has no storage for amino acids and the supply for metabolic reactions must constantly be adjusted to the demands or degraded and excreted (88). The biggest pool of free amino acids is confined to the intracellular compartment (89). Plasma amino acid concentrations therefore do not necessarily provide information on the overall amino acid balance in disease states. However, the plasma free amino acid content is important in ensuring a continuous availability of free amino acids for nitrogen containing reactions.

Transport of amino acids across cell membranes is mediated by Na+ coupled active transport mechanisms against a concentration gradient (87). Therefore, the overall energy state of the cell can influence on the amino acids concentration.

In transamination reactions the amino group is transferred from an amino acid to an α-ketoacid catalysed by a transaminase. These reactions are readily reversible, and do not change the overall balance of free ammonium since the amino groups are only switching to another carbon skeleton.

Oxaloacetate-aspartate, pyruvate-alanine and branched chain amino acids (BCAA) –branched chain keto acids switch amino groups in a coupled reaction with α-ketoglutarate-glutamate (figure 8). Glutamate then acts as a donor of amino groups for further synthetic and excretions pathways. Oxaloacetate and α-ketoglutarate are both intermediates in the tricarboxylic acid cycle (67).

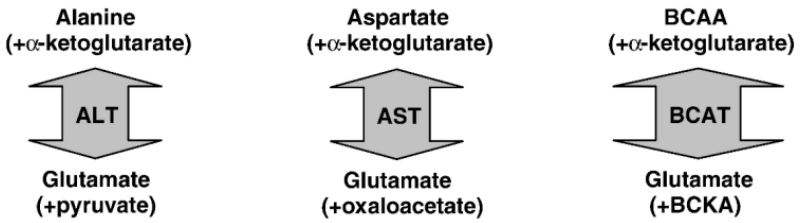

Figure 8 Aminotransferases reaction. ALT: alanine aminotransferase; AST: aspartate aminotransferase; BCAA: branched-chain amino acids; BCAT: branched-chain amino acids aminotransferase; BCKA: branched-chain keto acids.

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Glutamate has an important position in the general amino acid metabolism as a receiver of amino groups from other amino acids, through transamination, for further metabolism in the urea cycle (86). Furthermore, it serves an important role in interorgan transport through the synthesis of glutamine (GS reaction) and transamination to alanine. In normal urea cycle α-ketoglutarate in the cytosol of periportal hepatocytes receive amino groups from amino acids forming glutamate. Glutamate is then transported into the mitochondria where the ammonia enters the urea cycle. The second ammonium molecule is derived from aspartate. In the periphery glutamate is amidated by GS forming glutamine that acts as transporter of the ammonia to the liver, preventing an increased plasma ammonia concentration.

Glutamine is the most abundant amino acid in the body (88). It serves many important functions as energy provider for rapid renewed cells like enterocytes and immune cells, and functions as nitrogen donor for synthetic reactions. It is also important in the regulation of acid-base balance in the kidneys. Glutamine furthermore serves as a non-toxic carrier for ammonia from peripheral organs such as skeletal muscle to the liver for nitrogen disposal through the urea cycle. Glutamine is a non-essential amino acid and is synthesized in the GS reaction (87).

Alanine is important in the interconnection between carbohydrate metabolism and amino acid metabolism through the glucose-alanine cycle. It serves as a transporter of amino groups from BCAA metabolism to the liver for further metabolism in the urea cycle. Alanine is formed in the intestines by conversion of other amino acids in the enterocytes (87).

Glycine is a one carbon amino acid that takes part in the synthesis of several molecules such as porphyrines and glutathione. It is an inhibitory neurotransmitter in the spinal cord and acts as a modulator at the N-methyl –D-aspartate (NMDA) receptor in the frontal cortex (90, 91). It can arise through three reactions; the glycine cleavage system, synthesis from serine and trough transamination with alanine from glyoxylate (87). Serine is synthesized from 3-phosphoglycerate, an intermediate from glycolysis. It undergoes transamination with glutamate as amino group donor. Serine can be further converted to pyruvate by serine hydratase.

Interorgan metabolism of ammonia

The plasma concentration of ammonia is held within narrow limits (below 65 μM) to prevent toxic effects on the brain (67, 92). Interorgan metabolism of ammonia is dictated by the distribution of GS and glutaminase (93).

In the normal state ammonia is being transported to the liver from the intestines and peripheral organs for disposal through the urea cycle. The urea cycle is a low affinity-high capacity system for detoxification of ammonia through several steps, initialized by carbamoyl-phosphate synthetase in periportal hepatocytes. GS in perivenous hepatocytes removes any ammonia escaping from urea synthesis. In addition to this, periportal hepatocytes contain glutaminase which liberates ammonium (94). Hence, the liver has the ability to regulate the balance between glutamine breakdown and synthesis in an intercellular glutamine cycle within the liver by these two simultaneously active reactions (95).

In the normal state about 50 % of ammonia is generated from intestinal blood derived amino acids (96), the rest from colonic bacteria. A substantial ammonia production in germ free animal models (absence of colonic bacteria) has been shown, highlighting the importance of amino acid derived

ammonia (97). Enterocytes have a predominantly glutaminase activity as the primary energy source for these cells is the deamination of glutamine (98). Ammonia is liberated and glutamate can be further transaminated yielding alanine. Portal drained viscera is thus considered to be net ammonia liberating organs.

The kidneys are glutaminase (kidney type) abundant organs, and take up glutamine for energy utilization (99) and for pH-regulation releasing about 30 % of the liberated ammonia into the urine. Renal uptake of glutamine constitutes about 10-15 % of the body glutamine flux (100). Kidneys contain both glutaminase and GS and are capable of both synthesizing and degrading glutamine (101). They are considered to be predominantly ammonia producing organs, but can under certain conditions shift from net ammonia release into the renal vein to net ammonia excretion by reversing to 70 % excretion in urine (100).

Active muscle produces ammonia through metabolism of amino acids and degradation of proteins. Muscle GS produces glutamine for transport of ammonia to the liver, where it is detoxified through the urea cycle. Due to its mass relative to other GS containing organs, muscle is probably the main glutamine synthesizing organ (100).

Brain contains both glutaminase and GS. The enzymes are compartmentalized to neurons and astrocytes respectively, and their interplay is important in brain energy metabolism and neurotransmitter production (78).

Nitrogen metabolism in acute liver failure

Circulating level of ammonia in ALF is regulated by the interplay between the failing liver, muscles, kidneys, and intestines (102). Acute liver failure leads to abrupt changes in the overall balance of amino acids and ammonia in plasma, causing an increased concentration of most amino acids (83, 103-105). The capacity for urea synthesis is significantly decreased because of diminished amount of viable hepatocytes. Furthermore, nitrogen containing blood from the portal vein is partly shunted to the systemic circulation, thereby escaping hepatic metabolism. This is in contrast to the situation in cirrhotics with acute-on-chronic liver failure where a net removal of ammonia across the liver is seen (73). These factors together make the removal of ammonia dependent on alternative routes.

By virtue of its mass, skeletal muscle becomes the most important organ for removal of excess ammonia in hyperammonemia through the action of GS (102, 106). Increased ammonia uptake by muscle has been found in patients with ALF (81, 82, 107), although conflicting results have been reported in animal models of ALF (108). Furthermore, conflicting observations have been reported on whether glutamine efflux from muscle is increased. In a human ALF study ammonia uptake across

the muscle was quantified to 100nm/100g/min which correlated with glutamine production (107). Ammonia uptake and glutamine release was also observed in cirrhotic patients with induced hyperammonemia (109). Furthermore, an increased GS activity in muscle has been found in ALF providing increased capacity for ammonia removal (110). However, increased release of glutamine across the muscle can also be due to increased muscle catabolism.

The kidneys contain both glutaminase and GS making them capable both synthesizing and degrading glutamine (102). They can therefore adapt to the body`s acid-base balance and plasma ammonia levels. In hyperammonemia a shift from systemic release to an increased excretion of ammonia has been observed both in animal models and patients (111, 112). However, in the setting of ALF this protective mechanism seems to be overwhelmed (113). The combination of rapidly increasing hyperammonemia and decreased renal function results in net systemic release, making the kidneys net ammonia producing organs in ALF (96).

Also the intestines can contribute to the ammonia release in hyperammonemia due to glutaminase containing enterocytes. In a study of cirrhotic patients ammonia was released by the portal drained viscera and glutamine was taken up (109). However, during a simulated upper gastrointestinal bleed the increased arterial ammonia was found to be caused by increased ammonia release by the kidneys and not the intestines (112). In ALF, the increased ammonia contribution from portal drained viscera is considered to be due to intra and extra-hepatic shunting and not altered ammonia production by the gut (102). Taken together, this underlines the important role for kidney glutamine metabolism in hyperammonemia.

These observations have been confirmed in the model of ALF used in this study (114). Hepatic devascularisation induced hyperammonemia, and the kidneys were shown to be important contributors. Furthermore, portal drained viscera ammonia release did not increase in ALF and consumption of glutamine was not affected by devascularisation. Ammonia was not found to be taken up by muscle, in accordance to other animal studies (108). However, this model shows increased arterial levels of glutamine and decreased levels of glutamate, making it suitable and relevant for the present studies (114).

Due to low tissue mass the brain is quantitatively of minor importance in ammonia removal (102, 115), but plays a qualitative important role as hyperammonemia may lead to the development of brain edema.

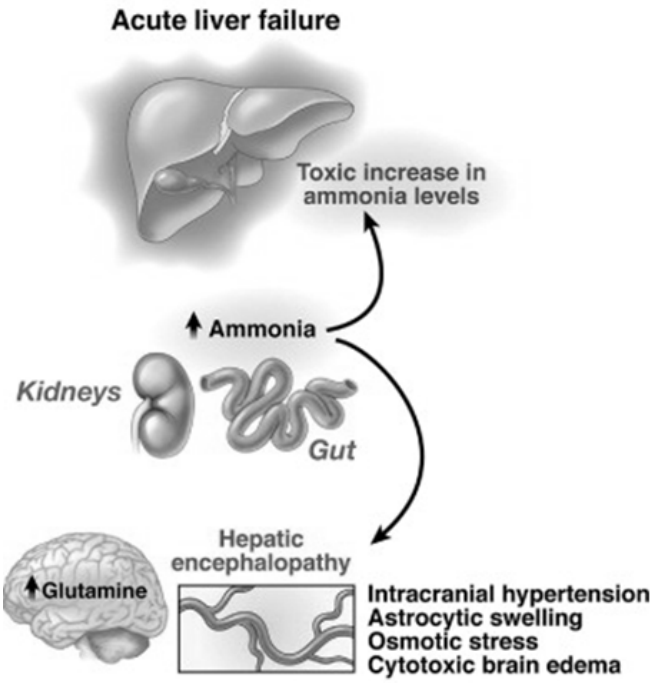

Figure 9 In ALF, reduced capacity of urea synthesis leads to hyperammonemia. Gut and kidneys can contribute to this via GS activity, which liberates ammonia. Increased circulating ammonia increases glutamine production in the brain, leading to astrocytic swelling and brain edema. This can lead to ICH.

Reprint with permission: Jalan R, Lee WM, Treatment of hyperammonemia in liver failure: a tale of two enzymes, Gastroenterology 2009;136(7):2048-51

Ammonia and its effect on the astrocyte

Under physiological conditions ammonia is transferred between the neuron and the astrocyte via the glutamate-glutamine cycle for energy and neurotransmitter production (glutamate, GABA) (85, 116). A distinct compartmentalization for glutaminase and GS exists. GS is a predominantly, if not solely an astrocytic enzyme (117). GS produces glutamine through the amidation of glutamate. The glutamine is then taken up by the neurons and converted to glutamate by glutaminase, which replenishes the glutamate neurotransmitter for release from the neuron. Neuron released glutamate is taken up by the astrocyte and used to generate glutamine and remove ammonia.

In hyperammonemia GS becomes an ammonia removal pathway in order to compensate for the increased ammonia load to the brain. The brain does not contain the enzymes for urea cycle, leaving it dependent on the GS reaction to remove excess ammonia (85). Astrocytic GS is not up-regulated in hyperammonemia, as is the case for muscle GS (118). Therefore, this pathway has very little excess capacity rendering the brain particularly vulnerable to the adverse effects of hyperammonemia (119). The removal of ammonia and generation of glutamine by astrocytes leads to increased

intracellular osmolality and hence an obligate shift of water from extracellular to intracellular compartment resulting in astrocytic swelling (cytotoxic edema) (77).

Increased brain glutamine production is seen in brain edema due to hyperammonemia (71, 120), and a correlation between brain extracellular glutamine and ICP in persistent hyperammonemia has been found in patients with ALF (121). Furthermore, inhibition of GS has been shown to prevent increased cortical glutamine and brain water in hyperammonemic animals (122-124). Astrocytes compensate for the increased glutamine production by expelling K^+ and osmolytes such as glutamate, taurine and myo-inositol (57, 125). In accordance with this, studies have shown decreased myo-inositol concentration in astrocytes in hyperammonemia and an inverse relation to glutamine. This compensation is, however, likely to be insufficient in ALF with rapidly increasing arterial levels of ammonia (126).

Despite the fact that increased intracellular glutamine and astrocytic edema seems to be closely related, several studies have shown a lack of correlation between brain glutamine and brain edema in ALF suggesting that other potential mechanisms could also be involved (57, 125, 127-129). Rather than a steady increased intracellular production, the newly formed glutamine may be prevented from leaving the astrocyte due to defect glutamine transporters across the cell membrane, which might further increase the osmotic burden on the astrocyte (127). Another hypothesis suggests that the pathogenic effect of glutamine is mainly due to a mitochondrial metabolism of glutamine yielding ammonia ("Trojan horse-hypothesis"). This induces oxidative stress that can initiate the mitochondrial permeability transition reaction leading to cell swelling and energy failure (130). The role of oxidative stress induced by ammonia in ALF is well acknowledged (131, 132), even though the Trojan horse hypothesis remains debated (77, 133). Either way, a connection between osmotic and oxidative stress is indicated, creating a self-amplifying loop triggering downstream consequences (134).

Studies have furthermore indicated that increased intracellular glutamine may act as the signal for increasing cerebral blood flow (CBF) in hyperammonemia, linking two major pathophysiological mechanisms behind brain edema in ALF (135).

An increased extracellular glutamate concentration in the brain is also found in several models of ALF. This could be due to diminished expression of GLT-1, a glutamate transporter on the astrocyte, that can result in a relative NMDA receptor overactivity (136).

Another study found increased ICP to correlate with increased extracellular glutamine level and increased lactate-pyruvate ratio in the brain, indicating an accelerated glycolysis and impaired energy

supply in ALF (137). Increased brain and CSF lactate concentrations have consistently been shown in hyperammonemia (119), and was found to precede surges of high ICP in patients with ALF (138). Furthermore, an association between reduced ICP and attenuated extracellular ammonia and lactate was observed in the current model used in this thesis (139). In a rat model of ALF impaired glucose metabolism shown by altered lactate metabolism was found to be the major cause of brain edema (128, 140).

In conclusion, the exact mechanisms by which ammonia disrupt the astrocytic homeostastis is not yet fully elucidated. Astrocytic swelling is multifactorial and additional mechanisms such as upregulated peripheral type benzodiazepine receptor, increased neurosteroid production and increased glutathione concentration may also be implicated (123).

Inflammation and brain edema

Infection and inflammation evident by the presence of two or more SIRS criteria, increase the risk for ICH in ALF (12, 24). ALF is a generalized inflammatory state initiated by the release of damage associated molecular patterns (DAMPs) from dying hepatocytes leading to an increased pro-inflammatory cytokine production (TNF-α, IL-6) and leukocyte recruitment (11, 141). A case report describing uncontrollable ICP in a patient with ALF, reported that hepatectomy awaiting the new liver reduced ICP and improved hemodynamic parameters. Plasma pro-inflammatory cytokine level decreased, while ammonia levels remained elevated (142). Patients with ALF are functionally immunosuppressed. Immuno-paresis during the resolution phase of an inflammatory response predisposes to secondary infections (11, 143) which can aggravate ICH. Also, a dysfunction of neutrophil leukocytes, which correlates with peak ammonia levels, has been found in ALF similar to what has been observed in patients with sepsis (23). The association between inflammation and increased ICP in hyperammonemia has also been shown in animal models. In a porcine ALF model increased ICP was observed when lipopolysaccharides were administered together with a hepatotoxin, but without changing ammonia levels (144). Another study found increased brain edema in cirrhotic rats administered lipopolysaccharides. This increase was unrelated to ammonia levels (145).

Both locally (brain) and systemically derived pro-inflammatory mechanisms seem to be involved in the development of brain edema (146). Evidence for activation of microglia with increased production of pro-inflammatory cytokines has been shown in an animal model (147), and increased pro-inflammatory cytokine production in the brain has also been found in patients with ALF (148).

The impact of inflammation on the BBB has therefore gained interest in explaining the synergisms between ammonia, inflammation and cerebral blood flow alterations in ALF (149).

Cerebral blood flow

Cerebral blood flow (CBF) is increased in ALF. This is caused by gradual cerebral vasodilation and impaired cerebral autoregulation, and has been reported in both humans and animal models (66, 150-152). With impaired autoregulation cerebral blood flow fluctuates in parallel with arterial blood pressure. Increased CBF has been shown to be associated with brain swelling in hyperammonemia (135) and increased ICP in models where inflammation has been induced in the presence of hyperammonemia (153). Cerebral blood flow is tightly regulated to avoid fluctuations in cerebral perfusion pressure (CPP), and is connected with the metabolic demands of the brain. In the case of ALF this coupling seems to be interrupted, resulting in increased blood flow that exceeds the metabolic needs of the brain. This has been termed luxury perfusion and is prominent in ALF (154). Furthermore, increased CBF has been observed to precede surges in ICP in patients with high grade HE. Interestingly, studies indicate that the loss of autoregulation is not caused by ammonia or the increased ICP itself, suggesting other factors such as the effects of hepatic necrosis and reduced liver mass (155). Increased CBF has been associated with maximal glutamine accumulation in the astrocytes, suggesting that other factors than ammonia are important (156). Interestingly, moderate hypothermia has been shown to restore cerebral autoregulation in ALF (157).

In conclusion, a two hit model for the development brain edema and ICH in ALF has been proposed. Increased ammonia levels represent the first insult to the brain followed by a synergistic effect of inflammation and increased CBF (158, 159).

Treatment of acute liver failure

General treatment

The only definitive treatment for ALF is liver transplantation (160). However, improved supportive therapy awaiting spontaneous recovery of liver function has resulted in a survival rate of one third to half of the patients without the need for transplantation (21). Medical treatment of ALF seeks to provide metabolic and hemodynamic stability to optimize the conditions for spontaneous hepatic regeneration and minimize the risk for complications (6). This includes ventilatory support for improved oxygenation and airway protection, renal replacement therapy, the use of fluids and vasopressors to provide hemodynamic stability and prevention of hypoglycemia. Early administration of N-acetylcysteine (NAC) is important to reduce the hepatotoxic effect of paracetamol (161, 162). Furthermore, liberal use of empirical broad spectrum antibiotic therapy to patients developing SIRS or increasing encephalopathy grade is recommended (163).

Treatment of intracranial hypertension

Current therapeutic interventions for HE in ALF includes mannitol for ameliorating ICP surges, strict control of serum sodium and the use of sedatives such as propofol (164) and ammonia clearance using hemofiltration (165). Liver replacement therapy has been extensively studied. MARS (Molecular Adsorbent Recirculating System) can possibly reduce HE grade and act as a bridge to transplantation (166, 167). The use of moderate hypothermia has been studied as a bridge to transplantation with encouraging results (48, 84) showing a favorable effect on multiple pathophysiological mechanisms. A recent retrospective multicenter study found a positive effect for young patients with paracetamol intoxication although there was no difference in overall 21-day survival (168). The mechanisms behind ICP reduction during hypothermia are not fully elucidated, but an effect on arterial ammonia and uptake of ammonia in the brain has been observed (84, 169).

Ammonia lowering treatment

Ammonia is closely linked to the development of HE and ICH in ALF (38). However, current ammonia lowering strategies have not proven to be effective in patients suffering from ALF (26). The classical approach to reduce ammonia in liver failure has been directed toward reducing the production of ammonia in the gut with non-absorbable disaccharides and non-absorbable antibiotics. The rationale for the first intervention is to decrease the transit time and change the pH in order to decrease uptake of ammonia across the intestines. Poorly absorbable antibiotics will reduce the urea production from urease containing bacteria in the large bowel (170).

A meta-analysis from 2004 concluded that there was a lack of evidence for the routine use of these strategies for the treatment of hepatic encephalopathy (HE) in patients with cirrhosis (171), but later studies have shown potential beneficial effect in certain clinical settings (172). In ALF no clinical trial has been performed, but preliminary results of a retrospective study from the U.S. liver failure group showed no effect of lactulose in ALF (173). It has been shown that a considerable part of intestinal derived ammonia is due to increased glutamine breakdown in the small intestines yielding free ammonia, contrary to bacterial production in the colon (101). The routine use of lactulose has therefore been questioned (37).

The current understanding of hyperammonemia has led to therapies trying to increase the ammonia removal as opposed to decreasing ammonia production by exploiting other organs that are of importance, with muscle being the main target. Ornithine and aspartate (LOLA-treatment) increase the overall provision of glutamate, thereby providing an increased substrate for glutamine production by GS and extra substrate for the remaining urea cycle function (174). LOLA was shown to lower plasma and CSF ammonia and prevent brain edema in a rat model of ALF (72).

Hyperammonemia, induced by portacaval anastomosis, has been shown to increase GS activity in muscle (118, 175). In the study by Rose et al. LOLA treatment further increased GS activity in muscle, revealing the important role of skeletal muscles in treating hyperammonemia (72). LOLA was also shown to protect against ammonia precipitated coma in a study of portacaval shunted rats (174).

In ALF ammonia removal is dependent on extrahepatic organs. However, organs containing glutaminase, such as the intestines and kidneys are able to re-metabolize glutamine to glutamate and ammonia thus creating rebound hyperammonemia. This may occur as the formed glutamine is not being removed from the circulation. In 2009 a randomized, controlled study of LOLA vs placebo showed no beneficial effect on morbidity or mortality. Also, the mean reduction in ammonia levels over 6 days was not significantly different between LOLA and placebo treated groups (176). Furthermore, ammonia did not decrease at any time point in this study, which was explained as a futile cycling of glutamine between muscle and intestines in the absence of a definitive removal by the liver (176). Clemmesen et al. provided further evidence for this relationship as they were able to decrease systemic ammonia by removing glutamine using high-volume plasmapheresis (177).

Treatment of hyperammonemia in children with urea cycle disorders (UCD), by providing non-urea dependent pathways for nitrogen removal was initially discovered in 1979 by Brusilow (178). The administration of phenylacetate and benzoate increased the removal of glutamine and glycine, respectively (179). This has significantly increased survival and decreased morbidity for children with UCD (180, 181). Alternative pathway therapy has also previously been studied in liver failure. Sodium benzoate was found to be as effective as lactulose for the treatment of acute portosystemic encephalopathy (182). However, the risk of depleting glutamate stores in liver failure led to the hypothesis of combining ornithine from LOLA-treatment with alternative pathway removal of ammonia. This hypothesis was put forward in 2007 by Jalan et al. and initiated the current studies (183). It was proposed that the concomitant administration of L-Ornithine and Phenylacetate (OP) in acute liver failure would provide a sustained reduction in arterial ammonia concentration through;

1. Increased provision of glutamate by transamination of ornithine for detoxification of ammonia to glutamine

2. Excretion of the glutamine thus formed by conjugation with phenylacetate as phenylacetylglutamine in the urine.

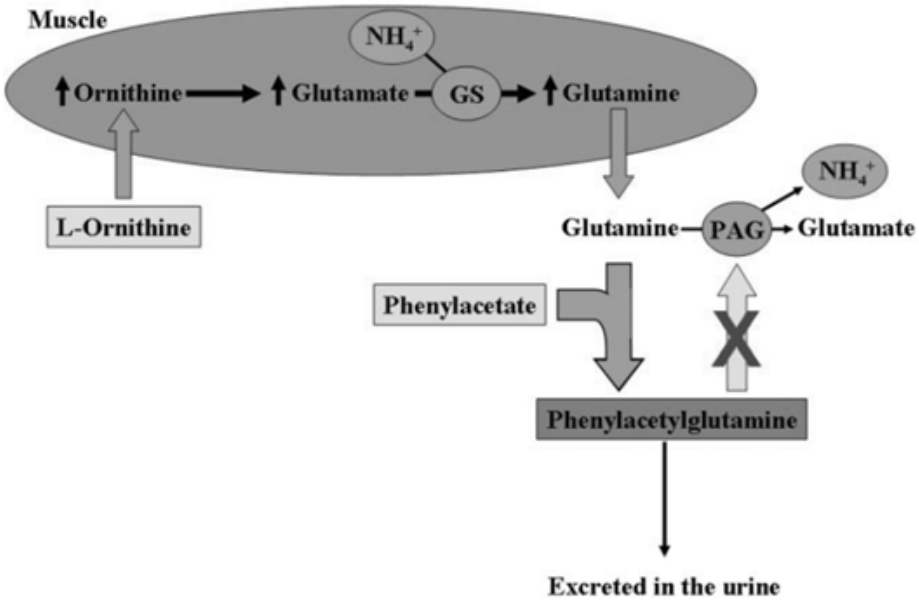

Figure 10 The co-administration of L-ornithine and phenylacetate to pigs with ALF stimulates ammonia removal by increasing glutamate in the muscle (transamination of ornithine to glutamate) and increasing glutamine production through GS. Newly formed glutamine is thus conjugated with phenylacetate and excreted as phenylacetylglutamine through the kidneys, preventing a glutamine-induced ammonia rebound effect.

Reprint with permission:, Ytrebø LM, Kristiansen RG, L-ornithine phenylacetate attenuates increased arterial and extracellular brain ammonia and prevents intracranial hypertension in pigs with acute liver failure, Hepatology 2009;50(1):165-74

As glutamine contains two nitrogen atoms, the conjugation with phenylacetate would remove two waste nitrogen atoms by each phenylacetate molecule, providing a net removal rate equal to the urea cycle (184).

2. AIMS

Study I

The aim of this study was to describe ultrastructural changes in different parts of the brain in pigs with acute liver failure.

Study II

We wanted to determine whether administration of the newly invented drug Ornithine Phenylacetate (OP) could attenuate ammonia in blood and the brain (extracellular fluid) through the hypothesized phenylacetylglutamine conjugation pathway, and whether this could modulate the increased ICP observed in this model of acute liver failure.

Study III

Based on the results from study II we wanted to investigate whether other conjugation pathways for ammonia could be involved in the ammonia removal reported in study II. We specifically wanted to study the impact of OP treatment on the phenylacetylglycine pathway as an additional ammonia-lowering pathway in ALF.

3. METHODS

Methods are described in detail in each of the individual papers included in this thesis. The aim of the current section is to provide the reader with a general introduction to the methods applied.

Pig model

The study was performed in the Surgical Research Laboratory at UiT - The Arctic University of Norway. Animal experiments were approved by the Norwegian Experimental Animal Board. The pigs were kept in the animal department for at least 2 days before the experiments. The animals were looked after by the veterinarian care service and the general health conditions were continuously monitored prior to the experiments. The conditions in the animal room were strictly controlled to reduce stress for the animals.

A well-characterized and highly reproducible large animal model which recapitulates the cardinal features of human ALF was used (185). The model is a hepatic devascularized model of acute liver failure (portacaval shunt + hepatic artery ligation). We induced a hepatic insult mimicking the initial (hyper acute) phase of ALF. It provides a therapeutic window of 8 hours which makes it suitable for testing interventions which could potentially modulate the early course of disease. We have focused our experiments on studying end organ dysfunction in acute liver failure and both *in vivo* and *in vitro* methods have been applied.

We defined T = - 30 minutes as the time when all catheters were in place, but before ALF induction. T = 0 hour was defined as the time of ALF induction or completion of sham surgery. All *in vivo* experiments were terminated at T=8 (animals sacrificed) and samples harvested for further *in vitro* studies.

Pigs with acute liver failure induced by hepatic devascularization develop a hyperdynamic circulation with increased cardiac index and decreased systemic vascular resistance index. Hyperammonemia and ICH (186), together with increased liver enzymes and signs of coagulopathy develop rapidly (185).

Animal preparation

The pigs were anesthetized with soidum thiopenthal, fentanyl and midazolam. The anesthesia was stopped after the induction of liver failure. Level of anesthesia was regularly examined and boluses given as required. All animals were tracheotomised and ventilated using a volume controlled modus adjusted by means of repeated arterial blood gas analyses. They were kept normothermic, defined as 38.5 ± 1˚C (187). ALF was induced with an end-to-side portacaval shunt followed by ligation of the

hepatic arteries at T = 0 h and monitored for 8 hrs (185). All animals received NaCl 9 mg/ml containing glucose as a preoperative load, and a continuous infusion with normal saline during the experiment. After ALF induction, glucose and human albumin (200 mg/ml) were continuously infused, with sham-operated animals receiving only half the amount of glucose in order to render the glucose levels comparable between the groups. All animals received equal amounts of intravenous fluids during the experiments.

The animals were invasively monitored by means of a pulmonary artery catheter and a femoral artery catheter. A central venous catheter was inserted for the administration of fluids and study medications. An intraparenchymatous ICP-transducer was used for continuous measurement of intracranial pressure. A second burr hole was made for a microdialysis catheter. Cardiac output, cardiac index and cerebral perfusion pressure were calculated based on continuous measurements. Arterial and venous blood was drawn every second hour and frozen for later analysis. Venous arterial differences were calculated as the difference between venous and arterial concentrations of the actual substance. Microdialysate was collected every hour and frozen for later analysis. The urine bladder was drained via a cystotomy and urine was collected hourly.

Methods related to paper I

Brain biopsies

A craniectomy was performed and brain biopsies were obtained from frontal cortex, brain stem and cerebellum at T=8. Samples were fixated on McDowell fixation liquid for further analysis by light microscopy and electron microscopy.

Electron microscopic examination of brain biopsies

We performed a semi-quantitative evaluation of ultrastructural changes of the brain samples. Prior to ultrastructural examination, semi-thin sections for light microscopic examinations were performed and areas of interest for further studies by transmission electron microscopy were subsequently defined. Areas with visible artifacts were excluded prior to ultrastructural examination. The severity of ultrastructural changes was graded according to an established scoring system based on well acknowledged ultrastructural criteria (Tables I–III, paper I) (188-190). The more pronounced changes in each biopsy specimens were selected for each scoring. All sections on electron microscopic pictures were blinded for the investing pathologist. From each pig we selected eight micrographs from each region, which were examined and scored.

Methods related to paper II and III

Administration of study medication

L-ornithine was administered intravenously at a dose of 0.07 g/kg/hour. Phenylbutyrate (pro-drug for phenylacetate) was administered at a dose of 0.05 g/kg/hour via an oro-duodenal catheter. Correct catheter position was confirmed during the laparotomy. Medication was administered as a continuous infusion for the duration of the experiment, and normal saline was used as vehicle in both the ALF and sham groups.

Arterial ammonia

Ammonia was analyzed according to the method described by Neeley et al. (191) using an ammonia assay reagent kit (AA0100) supplied by Sigma Aldrich (St. Louis, MO, USA). The method is based on an enzymatic reaction between ammonia in the sample, α-ketoglutarate and reduced nicotinamide adenine dinucleotide phosphatase (NADPH) in the presence of L-glutamate dehydrogenase. The end products in the reaction are L-glutamate, water and oxidized nicotinamide adenine dinucleotide phosphatase (NADP+). The formation of NADP+ decreases the sample absorbance at 340nm and this decrease is proportional to the concentration of ammonia in the sample.

Extracellular ammonia in brain microdialysate

Microdialysis is based on dialysate sampling of molecules of interest by a semipermeable membrane induced by concentration differences across the membrane. It can be used for measuring extracellular concentrations of endogenous and administered molecules, and it allows for continuous measurements over several hours (192). We applied this method to measure the extracellular concentration of ammonia in the brain. Ammonia in microdialysate was analyzed with the same method as for arterial ammonia measurements.

Biopsies from brain and muscle

Brain biopsies and muscle biopsies were harvested at T=8 and immediately frozen (freeze clamp technique at -80˚C) for the measurement of amino acids and urea.

Amino acids and urea in blood and tissue from brain and muscle

For the identification and quantification of amino acids in blood, brain microdialysate and brain tissue we applied a Biochrome 30 amino acid analyzer. It is a standardized, automated method based on separation by ion exchange chromatography followed by post column derivatization using ninhydrin. The ninhydrin-amino complex formed was detected by UV/light-absorbance. Chromatography uses the different physiochemical properties of the molecules to distinguish the compounds based on differences in their distribution between mobile phase and a stationary phase

through the column. The compounds are eluted in a specific order, and the time between application and elution, called retention time, is used for identification of the compound. The method can be applied for different physiological fluids as plasma, CSF and urine (193) and for tissue samples.

Phenylacetylglutamine, Phenylacetylglycine and Hippuric acid in urine

For the measurement of phenylacteylglutamine, phenylacetylglycine and hippuric acid we applied liquid chromatography tandem mass spectrometry (LC/MS-MS). A mass spectrometer is an instrument that can separate charged atoms or molecules according to their mass-to-charge ratio *(m/z).* Ionized molecules flow through a magnetic field and create a fragmentation of ions establishing a pattern that serves as a fingerprint of the compound (194, 195). This can be compared to known patterns to identify a compound. The use of stable isotope internal standards and tandem MS (MS-MS) enhances the sensitivity and accuracy. LC/MS-MS is used for detecting and identifying endogenous and exogenous molecules of low concentration in physiological fluids.

A mass spectrometer system generally consists of 5 main parts:

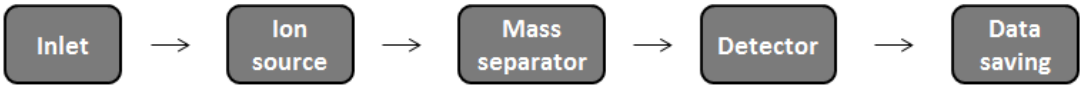

Figure 11 Mass spectrometry system

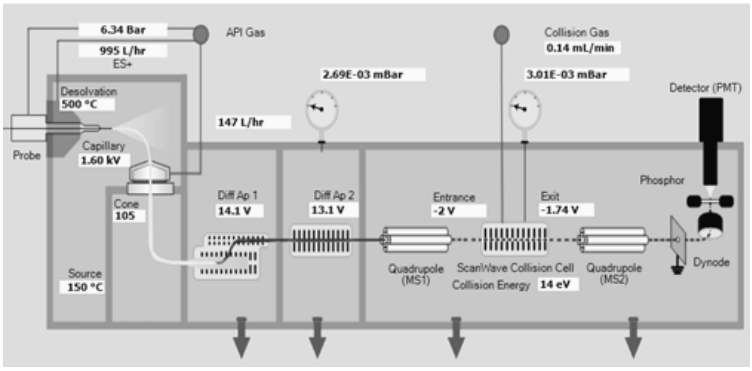

Figure 12 Consol for system - MS display Xevo TQ-S Screen.

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Glutamine synthetase activity in muscle biopsies

Glutamine synthetase activity was measured by the calorimetric method as described by Minet et al. (196). It is a well validated method for the measurement of GS activity in muscles. For further details regarding preparation and calculation see supplementary material to paper II.

4. SUMMARY OF RESULTS

Study I

In this study our focus was to investigate ultrastructural changes in the brain of pigs with ALF induced by hepatic devascularization. We found macroscopic differences between the sham and ALF group as the ALF group had signs of increased brain water and swelling, flattening of the gyri and narrowing of the sulci. Furthermore, electron microscopic evaluation of the three regions frontal cortex, cerebellum and brain stem revealed a significantly increased degree of pathological findings with regard to edema, neuronal and astrocytic damage and oligodendrocytes/myelin changes in the ALF group compared with the sham group. Few inflammatory cells were found, except for focal areas with polymorphic nuclear granulocytes around necrotic cells in the ALF group. Signs of disrupted blood-brain barrier were seen in the ALF-group.

Study II

In this study we intended to explore the effect of Ornithine Phenylacetate (OP) on arterial and brain extracellular ammonia and its potential effect on ICP in this model of ALF. We found a significant increase in arterial and brain extracellular ammonia in the placebo-treated ALF group that was significantly attenuated in the OP-treated ALF group. This effect was seen in both arterial blood and brain extracellular fluid. We also observed a significant attenuation in ICP in the OP-treated group. The decrease in ICP correlated to the decrease of ammonia in both arterial blood and brain extracellular fluid. We found a significant increase in phenylacetylglutamine excreted in urine in the OP-treated group compared to placebo-treated ALF pigs, although the increase in phenylacetylglutamine in urine did not correlate stoichiometrically to the decrease in arterial ammonia.

Study III

In this study we conducted a secondary analysis to explore the lack of stoichiometric correlation between the decrease in arterial ammonia and the increase in phenylacetylglutamine excreted in urine. We found an increase in arterial glycine in the ALF placebo-treated group that was significantly attenuated in the OP-treated group. Arterial glycine significantly correlated to arterial ammonia and ICP levels published in paper II. Furthermore, the tissue concentration of glycine in brain frontal lobe was significantly decreased in the OP treated group compared to the ALF group. Together, these findings indicate a possible role for glycine in the ammonia metabolism in this model of ALF. We also found a nearly significant increase in the glycine release across the kidneys, which was significantly attenuated in the OP treated group, indicating an important role for the kidney in ammonia

metabolism. Finally, we found a highly significant increase in phenylacetylglycine in urine in the OP treated animals. This suggests that conjugation of phenylacetate to glycine is an important metabolic pathway in this model of ALF. This finding provides a mechanistic explanation for the net removal of ammonia in OP treated pigs.

5. DISCUSSION

Model

A well characterized porcine devascularization model of ALF has been used for this thesis. This model presents the cardinal features of human ALF with development of hyperammonemia with typical amino acid disturbances, increased ICP, hyperdynamic circulation and coagulopathy within 8 hrs after ALF induction (185). It has been validated as a suitable model for the study of interventions in the initial phase of ALF as it provides a time frame of 8 hrs for studying the effect of potential new treatment concepts (62, 197).

ALF is a rare disease emphasizing the importance of having reproducible and clinically relevant animal models. A large animal model provides a clinical relevant setting for studying the underlying pathophysiology, and provides an opportunity to perform intensive care monitoring and provide supportive therapy to resemble the clinical setting (198, 199). The use of a large animal model is furthermore recommended by the criteria put forward by Terblanche and Hickman, as it enables the use of therapies applicable to man (200). Compared to small animal models, the possibility of taking multiple blood and extracellular fluid samples allowed us to study the temporal pattern in the course of the disease.

Three strategies for inducing ALF have traditionally been applied – hepatotoxic drugs, different degrees of devascularisation and hepatectomy. A devascularization model precludes the investigation of potential reversibility, but is on the other hand highly reproducible. In the case of acetaminophen models, maintaining a suitable plasma level of drug to induce the liver failure without causing complications such as methemoglobinemia is challenging (201). For the study of ALF the presence of a disturbed nitrogen metabolism is of pivotal importance as one of the major pathophysiological factors in ALF is the development of hyperammonemia. Hyperammonemia is highly reproducible in this model in contrast to the paracetmol model proposed by Dabos et al. showing an unaffected urea cycle (202). We found an abrupt decrease in urea levels which implies that the urea cycle is impaired making the metabolism dependent on alternative pathways. A devascularization model is therefore suitable for the study of liver independent metabolism. An important notion is, however, the potential effect of residual hepatic vein circulation in this model. Although ALF induces an inflammatory response, circulating inflammatory markers have not been found in this model. This could be due to the relatively short time frame of the experiment (62).

Our main focus has been brain dysfunction and interorgan metabolism of ammonia and amino acids in ALF. These pigs develop intracranial hypertension within 8 hrs. Increased ICP has also been observed in earlier studies of ALF in pigs induced by devascularization (41). The use of a hepatotoxin could potentially confound histological examination of the brain due effects on the BBB (203). In a study comparing devascularisation and hepatectomized models, devascularisation models were shown to be superior to hepatectomy models in creating an amino acid pattern which resembles human plasma amino acids profile (204).

The current studies included 32 animals. 8 animals (O and P) were included for hypothesis generating experiments, leaving 24 animals for the main studies. Due to technical reasons three, one in each group, were excluded. Accordingly 21 animals were included in the final data analyses. This is a small cohort for performing formal statistics which in turn increases the risk of both type 1 and type 2 errors. Avoiding type 1 errors is important. Furthermore, the risk of not detecting physiological differences also increases with such a small cohort. The use of large animal models for studying ALF is of high value, but for ethical reasons the number of animals are reduced. However, less than 7 pigs in each study group would not allow us to draw confident conclusions. A substantial sample bank was made in order to perform different studies from the same experiment.

Paper I

The development of cerebral edema represents a feared complication in ALF, and cerebral herniation due to edema is still a major cause of death in ALF (7). Cerebral edema, defined by a net increase in brain tissue water content (51), is closely linked with increased ICP (32, 139). Current treatment for intracranial hypertension (ICH) includes infusion of hyperosmotic solutions, sedation and hypothermia as a promising therapeutic modality (48).

Brain edema can be of cytotoxic or vasogenic origin, the latter being indicative of a physical breakdown of blood brain barrier (BBB). The first studies on ultrastructural changes in coma induced by hepatic failure were performed by Martinez et al. and Livingstone et al. Martinez found swollen astrocytes in brain biopsies from two patients dying from hepatic coma (205). Livingstone found indications of an increased permeability across the BBB (substances such as inulin and trypan blue which normally do not cross the BBB) in a rat model of ALF. Furthermore, on electron microscopic examination swollen astrocytes especially in pericapillary locations were also found (206). They concluded that a vasogenic component contributed in the later stages of ALF. Other studies in different animal models have also found indications of increased permeability across the BBB for substances normally not entering the brain (60, 207, 208). Kato et al. found marked swelling of the perivascular astroglial foot processes, dilatation of the extracellular spaces and endothelial cells with

increased numbers of vesicles and vacuoles on electronmicroscopic examination in animals and patients with ALF (59, 209). However, in human post mortem frontal cortex biopsies from patients dying from ALF they found intact tight junctions between the brain capillary endothelial cells. They concluded therefore that brain edema developed primarily due to cytotoxic mechanisms with intact BBB (59). A later animal study concluded with edema of both cytotoxic and vasogenic origin (64). The type of brain edema seen in ALF has since been debated with studies indicating a mainly, if not solely cytotoxic origin (210) on one hand, and studies indicating a mixed mechanism with subtle changes in the BBB leading to increased permeability rather than a full breakdown (57, 211) on the other hand.

The present model of ALF has previously been shown to be a valid model for hyper acute liver failure with brain edema and increased ICP (185). Morphological studies of the brain have, however, not been performed. In light of the results from the study by Kato et al. we therefore wanted to provide a descriptive study by means of electron microscopic examination of brain edema in ALF compared to control animals. For the evaluation of the neuropathological changes we applied a semi-quantitative method introduced by Shaper et al. (190). We evaluated perivascular edema with endothelial cell morphology, glia cell morphology and oligodendrocyte morphology. We also looked at neuronal damage and the presence of inflammatory cells (microglia). The use of a semiquantiative evaluation system allowed us to perform statistical evaluation of the observed changes. We looked into the pathological changes in three different regions; frontal lobe, cerebellum and brain stem, and we found a significantly higher score for all factors and all regions except for oligodendroglia in frontal lobe and purkinje celles (neuronal celles) in cerebellum in the ALF animals compared to sham animals.

Biopsies from the different regions were primarily assessed by light microscopy and regions with artefacts were excluded. This initial assessment was not blinded for the investigating pathologist as the main purpose was to find representative regions for further evaluation. Regions showing the most pronounced changes were consistently selected for further investigation to ensure comparable examination between sham and ALF groups. Chosen areas were further prepared for electron microscopic examination and then blinded for the investigator.

We found evidence of severe brain injury including macroscopic edema, cellular swelling and necrotic cell death of both neurons and astrocytes. The observation of perivascular edema is in accordance with prior histopathological studies (59, 64, 206, 209). Furthermore, damaged endothelium with vacuoles and vesicles indicate vasogenic involvement (64). We also observed swollen astrocytes and broken projections radiating from the vessel wall ending into the perivascular space, presumably astroglial and pericytic processes.

This study was designed as a histological examination of brain edema. Electron-microscopic examination is a valid method for evaluating the integrity of the BBB. However, previous small animal studies have also applied markers as Evans blue, trypan blue, and inulin to assess functionally the permeability of the BBB. These substances are normally not transferred into the brain due to tight junctions between the brain capillary endothelial cells (BBB). When present in brain tissue, indications of increased permeability and potential vasogenic edema are given. Lv et al. found an increase in the concentration of Evans blue in brain tissue from T=2 in a model of ALF in mice (212). Livingstone found increased permeability for trypan blue, inulin, sucrose and glucose (206), a finding confirmed by Zaki (60). Performing such analysis undoubtedly would have added further information regarding the BBB permeability in our model. However, for technical reasons, we did not test for BBB breakdown by the use of permeability markers. In addition, apoptotic antibody staining could have provided more information regarding the mechanisms for injury to the endothelial cells.

A discrepancy has been observed between electron-microscopic findings and functional permeability measurements of the BBB. In general, a complete breakdown of the BBB has not been found in spite of increased permeability of markers. Nevertheless, with improving methods for investigating the BBB, subtle changes in tight junction proteins that can increase the permeability to small molecules have been found (211). Modulation of the expression of genes coding for tight junction proteins as occludin have been reported (213), and claudin-12 has was found to be altered in hyperammonemia (214). Increased activity of MMP-9 was found to induce degradation of occludin and claudin-5 in different models of ALF and in vitro models (215). Furthermore, transport mechanisms of molecules across cell membranes may be altered in hyperammonemia (32, 209, 216). The accepted tenet today for brain edema in ALF is therefore primarily a cytotoxic edema with potential vasogenic components due to subtle changes in tight junctions, but without a complete disruption of the BBB (57).

This model of ALF is characterized by a sudden increase in ammonia and brain edema shown by increased brain water (62). This part of our study did not allow us to draw conclusions about the cause of brain edema seen by histological examination. Still, in this model there is no doubt that ammonia is a major contributing factor to the brain edema, as changes in inflammatory state or cerebral blood flow have not been observed (62). Increased ammonia leads to compensatory increased glutamine production through GS in the astrocytes which culminates in swelling of the astrocyte and an edema of cytotoxic origin. Due to the sudden rise in ammonia, compensatory mechanisms as extrusion of other osmolytes and decreased CSF production may not manage to compensate in the short time aspect of this model. We observed increased ICP from T=2 to T=8 that correlated with the ammonia increase. At T=8 the mean arterial ammonia level was approximately 590 µM in the ALF group. Human studies have shown a correlation between arterial ammonia of

more than 150 μM and the incidence of cerebral herniation (CH) (73). The high ammonia level and rapid progression to brain edema in this model may not fully correspond to the clinical setting seen in human ALF or other animal models. Our histopathological findings could therefore represent changes that are attributable to vasogenic edema late in the course of ALF, when an established cytotoxic edema is present.

The histological examination was done at T=8 only. ICP measurements give indirect evidence of the presence of brain edema in this model, but our study does not provide any information about the type of brain edema at earlier time points than T=8. We therefore do not provide information to what time point the edema starts or to what type of edema is present at the different time points. For future experiments, a study design with termination points at T= 2, 4 and 6 would therefore be preferable to be able to study the temporal pattern of brain edema in this model. Conflicting results regarding the order and development of cytotoxic and vasogenic edema have been reported. Livingstone et al. interpreted their findings of vasogenic edema as possibly a late stage phenomenon (206). Dixit et al. found marked swelling of perivascular astrocytes already at HE grade I in a rat galactosamine model of ALF, and from grade III they observed increased permeability for Trypan blue, indicative of BBB breakdown (208). Cauli et al. found, using a galactosamine induced ALF rat model, vasogenic edema to be the primary event in certain brain regions, followed by a cytotoxic edema later in the course of the disease (217). Chavarria found mainly cytotoxic mechanisms using MRI in a devascularization model of ALF in the rat (218).

Earlier studies reporting BBB breakdown in ALF have been criticized for not accounting for the terminal phase of brain edema, suggesting that their findings could be affected by hypotension, hypoxia and alterations in CBF. A component of hypoxia at T=8 is difficult to completely rule out. However, factors such as cerebral perfusion pressure (CPP), arterial oxygen saturation, plasma glucose and sodium levels and temperature were all closely monitored to prevent potential confounding effects. Mean CPP in the ALF group was 50 mmHg at T=8 during the brain biopsy harvesting. This is at the lower limit of recommended CPP (163), and could potentially have caused ultrastructural alterations in itself.

Studies have shown different distributions of edema for different regions in ALF (63, 64). Gove et al. found regional differences between forebrain and hindbrain in rats with ALF, with vasogenic mechanism being the more important in the hindbrain. Additionally, they found the cerebellum to be more susceptible to edema formation (64). Traber et al. found increased brain water in cortical grey matter, but not subcortical of pontine white matter or in the cerebellum (63). In the present study we focused on ultrastructural changes, rather than brain water measurements. We examined frontal

lobe, cerebellum and brain stem. Interestingly we did not observe significant changes in white matter, studying purkinje fibers in the cerebellum and myelin in the frontal lobe. In accordance with this Horowitz et al. found no uptake of 14-alpha-aminoisobutyric acid in the white matter in rabbits with ALF (207). Relating our ultrastructural findings to brain water measurement for different regions will be a focus for further studies in this model. Different types of astrocytes are found in grey and white matter. This could also have implications for the development of brain edema in liver failure (46, 51). Moreover, a prior study in this model showed white matter more responsive to intervention (albumin dialysis) than grey matter (62), but further studies are needed to elucidate the mechanisms behind this finding.

Inflammation plays a major role in the development of increased ICP in ALF (12). Studies have also shown that proinflammatory cytokines can affect BBB permeability in ALF, and have linked this to decreased expression of tight junction proteins (212). Furthermore, brain derived inflammatory response, shown by microglia activation has been reported (219). In our study we did not find a significantly increased number of inflammatory cells (microglia) in the brains of ALF animals. This corresponds to previous studies in this model showing no increase in serum pro-inflammatory cytokines in pigs with ALF. Wang et al. showed on electron microscopic evaluation that tight junction disruption and increased permeability of the BBB could be prevented by TNF-α inhibitor (220). Takada et al. observed a further increased ICP in a hepatotoxin porcine model of ALF, after administration of lipopolysaccharides (LPS) (144). The lack of inflammatory response in our model could be due to a short time interval.

The presence of a potential BBB break down in the short time frame of this model makes it clinically relevant since treatment options such as hypertonic solutions are dependent on an intact BBB. The use of mannitol can be effective in treatment of increased ICP (221). However, patients who do not respond to this treatment may have BBB breakdown. Due to the high level and rapid increase of ammonia in this model, it may resemble a phase beyond reversibility of the edema, and compensatory mechanisms as extrusion of other osmolytes may not be sufficiently effective. This study was not designed to study reversibility of brain edema in ALF. Therefore, our data are not conclusive as to whether administration of mannitol is of benefit in ALF. An intervention-study looking at changes and possible reversibility at T=2, 4 and 6 would add knowledge to this question, and is warranted.

Questions have been raised regarding the total reversibility of hepatic encephalopathy as a metabolic syndrome (92, 222, 223). We find severe, neuronal damage on ultrastructural examination in this

model of ALF. A vasogenic component in brain edema present in ALF and the presence of neuronal damage would suggest a nonreversible factor that may be persisting after liver transplantation.

We found significant but small differences in the neuropathology analysis between the sham and ALF groups and therefore, with such small differences, we did not pursue to study differences between ALF and OP groups. The primary purpose of study I was to evaluate brain edema in this model of ALF with EM methodology.

In conclusion, we believe our findings support the concept that vasogenic mechanisms are important in the pathogenesis of brain edema in ALF.

Paper II and III

Today ammonia is accepted as a primary contributor in the pathogenesis of brain edema in ALF (38, 224). At present there is no definitive treatment for reducing the ammonia load on the brain, and the only definitive treatment option for ALF is emergency liver transplantation. By reducing ammonia, brain edema can potentially be prevented or sufficiently reduced giving the liver time to regenerate and recover sufficient function to maintain homeostasis and avoid the need for liver transplantation.

The main finding in the intervention study of Ornithine Phenylacetate (OP) in ALF covered in paper II and paper III was a reduction in arterial and brain ammonia that was accompanied by a significant reduction in ICP. This observation confirms a strong correlation between ammonia and ICH in ALF (38, 73-76). Furthermore, an exploration of potential pathways for ammonia removal in this model led us to discover a novel and potentially significant role of glycine in ALF.

We observed an increase in arterial ammonia reaching 590 µM in the ALF group at T=8. This increase was attenuated in the OP treated group, with a maximum ammonia concentration of 365 µM at T=8. This is the first study showing ammonia reducing effect of the OP-treatment in ALF. In addition, it shows the effectiveness of the treatment in the short time frame of this model displaying very high arterial ammonia levels. Together with arterial ammonia levels, we also measured ammonia in brain extracellular fluid using microdialysis. There, we found a significant attenuation of ammonia in the OP treated group (220 µM in OP treated group vs 500 µM in placebo treated group) confirming an ammonia lowering effect also in the brain. The attenuation of plasma ammonia significantly correlated with the change in brain extracellular fluid ammonia supporting the fact that systemic hyperammonemia is associated with increased concentrations of ammonia in the brain in ALF (83).

The correlation between increased arterial ammonia and increased ICP has been observed both in humans and animal models (70). In the ALF group ICP increased to an average of 18 mmHg at T=8.

This increase was prevented by the OP treatment, showing ICP levels not significantly different from the sham group. Increased brain water and ICP have previously been shown in this model (62). For the present study we did not do brain water measurement. None the less, in a cirrhotic rat model a significant reduction in brain water after OP treatment was shown (225). This reduction was associated with restored myoinositol/glutamine ratio indicating an adaptive response over time. Such response is rather unlikely in the short time frame of this and other models of ALF (126), and in the clinical setting of ALF. The exact mechanisms for ICP lowering effect of OP in this model therefore need further studies, focusing on repeated measure in the brain of the extracellular domain, using microdialysis.

This model displays a hyperdynamic circulation induced by ALF (185). Systemic hemodynamic measurements were undertaken to elucidate whether changes in ICP could relate to hemodynamic improvement due to the OP treatment. Significantly increased cardiac index and decreased cerebral perfusion pressure (CPP = MAP-ICP) were found in the ALF group compared to the sham group. Significant intergroup differences were not found between placebo treated and OP treated animals, excluding a cofounding effect of improved systemic and cerebral hemodynamics of OP treatment. This allowed us to conclude the attenuation of ICP is independent of CBF, in accordance with a previous study in this model (62).

Brain edema is believed to be mediated by a synergistic effect of ammonia and inflammation together with increased CBF in relation to ICP surges (65). Since studies in this model have not detected any increased levels of inflammatory markers (62), brain edema has been regarded as independent of inflammation. However, in this model the hepatic veins are left intact making a distribution of inflammatory cytokines from the damaged liver possible.

The strong correlation between ammonia and ICP in ALF suggests that ammonia lowering treatment is of pivotal importance in the treatment of ALF. Treatments aiming at reducing the production of ammonia in chronic liver failure, as non-absorbable antibotics and disaccharides, have shown beneficial effect for secondary prophylaxis in cirrhosis (226). In ALF the abrupt, complete failure of ammonia removal by the liver leads to an acute overload, and the ammonia production overwhelms the removal by treatments such as lactulose. Indeed, these treatments for hyperammonemia have not proved beneficial effect in ALF (173). Moreover, studies have shown that the gut derived ammonia in large part is due to glutamine breakdown in the short bowel instead of luminal content derived ammonia from the colon (96, 109). This would imply that modulating the glutamine level is important to decrease the overall ammonia level in patients with liver failure. Reducing ammonia by means of increasing removal rather than decreasing production has therefore gained renewed

interest. Giving L-Ornithine L-Aspartate has been shown to reduce arterial ammonia by providing increased glutamine for detoxification and increasing the residual capacity for urea synthesis in the liver in patients with cirrhosis (170). The results have, however, been conflicting and a number of studies have never been published. The immediate effect of LOLA is to increase the physiological ammonia carrying effect of glutamine by providing substrates for glutamine synthesis. Together with this, an increased residual effect of urea synthesis can remove more ammonia (174). In ALF, with little or no rest function of periportal urea synthesis or perivenous glutamine synthesis, the effect of LOLA is dependent on extrahepatic glutamine synthesis. As glutamine is not being removed from the circulation, concerns have been raised regarding a potential rebound hyperammonemia due to the action of the enzyme glutaminase primarily in the intestines and the kidneys, which has also been observed in patients treated with LOLA (183). Indeed the only randomized controlled trial for LOLA in ALF could not find any reduction in arterial ammonia or outcome (176). In ALF, with little or no remaining liver function, extrahepatic compensatory mechanisms for ammonia removal are needed. Net removal of glutamine from the circulation is pivotal to prevent a rebound hyperammonemia (183).

In our model we found highly significantly increased arterial ammonia together with a significant decrease in the urea levels indicating an abrupt and fulminant deterioration of the nitrogen handling in the liver. This makes this mode suitable for studying extrahepatic metabolism of ammonia. We focused our investigation on the role of the muscle (paper II) and the kidneys (paper III) in metabolism of ammonia and related amino acid.

Means of reducing arterial ammonia by modulating its interorgan metabolism and providing non-urea dependent pathways for removal have been explored both in liver failure (182) and in isolated enzymatic disorders like UCD (179). The use of alternative pathway therapy such as phenylacetate and benzoate has shown positive effect on morbidity and mortality in patients with UCDs (181). UCDs and ALF share one important feature - the presence of brain edema in hyperammonemia. UCD patients have, however, no liver insufficiency other than metabolism of nitrogen and ammonia, confirming the important role of ammonia in brain edema.

In cirrhosis glutamate deficiency has been reported (96). We observed a significantly lower glutamate level in ALF animals compared to sham animals. This was restored when given OP. Decreased glutamate has also been reported in patients with ALF and in prior studies in this model (107, 114) providing a rationale for giving ornithine. Ornithine provides a substrate for glutamate through transamination (227). Increasing the glutamate synthesis is reasonable in order to prevent a potential depletion of branched chain amino acids (BCAA) from muscle (228). We found increased

ornithine in both plasma and muscle showing an uptake across the muscle. A lack of glutamate transporters in muscle cells has been reported. Therefore, indirectly increasing muscle glutamate using ornithine was required.

Patients suffering from UCD, without intrinsic liver disease, have upregulated perivenous GS and therefore phenylacetate treatment alone in these patients is enough to lower blood ammonia levels. In our study ALF pigs demonstrated an increase in blood glutamine levels. However, in a pilot phase of this study phenylacetate given alone failed to lower blood ammonia. Neither did ornithine alone lower ammonia as might be suggested if the ammonia removal effect would be the glutamine production alone. Therefore the combination of ornithine and phenylacetate induces a synergistic effect that lowers and removes ammonia. Hence, the subsequent studies evaluated the effect of combined ornithine and phenyalacetate treatment (OP).

Interorgan metabolism of ammonia is given by the distribution of glutamine synthetase and glutaminase (93). The liver contains both enzymes and controls the ammonia homeostasis in the normal state resulting in no net increase in systemic ammonia in spite of varying nitrogen load from the portal blood (95, 96). In ALF, GS activity in muscle becomes of vital importance due to its overall mass. Increased glutamine synthesis can increase detoxification of ammonia in liver failure, and increased activity and expression of the GS enzyme has been found in animal models treated with LOLA (72). In accordance with this, we found a trend toward increased GS activity, although not significant, emphasizing the capacity of muscle GS activity increase in this short time frame. We did not find increased enzyme expression which would be unlikely due to the short time span of this model. Glutaminase is abundant in intestines and kidneys (102). By administration of phenylacetate a potential rebound hyperammonemia due to glutaminase activity in the kidneys and intestines was prevented. This was also confirmed in a cirrhotic model over five weeks (225).

In this study we found a significantly increased arterial glycine level in ALF animals that was significantly attenuated in the OP treated group. We also found a significant correlation between glycine and ammonia. Glycine is an important ammoniagenic amino acid (229), which is increased in ALF (107). Glycine metabolism can generate free ammonia through both glycine oxidase and the glycine cleavage reaction (230). Increased arterial glycine has been observed both in a paracetamol induced ALF animal model (202) as well as in patients suffering from fulminant hepatic failure (107). In addition, an increase in extracellular levels of glycine in the brain has been demonstrated in ALF rats (91, 231), supporting a role for glycine in the onset of hyperammonemia and HE. Increased glycine in the brain is associated with metabolic brain disturbances as hyperglycinemic

encephalopathy, a disease resulting in developmental abnormalities in children(232). Glycine is also an important inhibitory neurotransmitter in the central nervous system.

Our data do not provide a definitive explanation to the origin of the increased glycine in ALF. Glycine is produced through three different mechanisms as outlined in figure 14. Briefly, serine, alanine and the glycine cleavage complex can increase glycine. We did not find differences in serine that could confirm serine as origin of the increased glycine in this model. There is reason to discuss whether increased glycolysis could lead to increased serine used for glycine synthesis in this model. Plasma levels of serine do not necessarily reflect the overall balance of serine. This could be elucidated if studying serine in other compartments. Alanine can increase glycine through transamination with pyruvate. We do see increased alanine in the OP group, but not in the placebo treated ALF group, which adds further questions to the origin of the observed increased glycine. Alanine serves an important role for transport of ammonia between muscle and liver, and provides also a link between the nitrogen and carbohydrate metabolism (glucose-alanine cycle). Alanine has also been shown to be an alternative to glutamine synthesis for ammonia removal in the brain (233) . The glycine cleavage complex mainly works in a catabolic way, liberating ammonia (230). Therefore, minimizing the substrate for this reaction provides a reasonable explanation for the removal of ammonia.

OP treatment attenuated arterial glycine. Decreased arterial glycine and ammonia levels were accompanied by decreased brain tissue glycine from frontal lobe. Our results do not provide indications as to whether this is blood mediated or results from modulated production in the brain. Further microdialysis studies could shed light on this. We also found a significant correlation between arterial glycine and ICP measurement. Based on our results we cannot conclude whether glycine affects brain edema through ammoniagenesis or directly affects the osmolarity in the astrocytes. These pathophysiological pathways merit to be further investigated.

Our data suggest that the kidneys are important in the metabolism of glycine in this model of ALF. Both the increase in the ALF group and the attenuation in the OP treated group seem to be mediated by the kidneys when studying venous arterial differences across portal drained viscera, hind leg and kidneys. We found significant differences only across the latter. In this devascularized model of ALF it would be reasonable to assume that the kidneys are important metabolic organs in the absence of a functional liver. This necessitates available enzymatic machinery for both glycine production and the conjugation with phenylacetate. The conjugation enzyme is mainly found in the liver but is also present in the kidneys (234). We did not measure activity of enzymes in this study, which could have provided us with further mechanistic insight.

In the OP treated group we found no removal of glutamine. Glutamine and alanine can induce negative feedback inhibition on the GS-enzyme (235). In this model a systemic inhibition of GS is likely as the produced glutamine is not removed and an increase in arterial alanine is observed. This can potentially stimulate other ammonia removing pathways, including glycine mediated removal of ammonia. The removal of ammonia is therefore likely to have taken place in the ammonia fixating step of GDH producing glutamate instead of the GS reaction giving glutamine. The GDH reaction is reversible and its direction is set by the concentration of its substrates and products. This study sheds light on the potential ammonia removing capacity of glycine in liver failure, but further studies are warranted. Removal of glycine is already an accepted treatment in UCD patients by the use of benzoate (179). Benzoate has been evaluated in one clinical study in cirrhosis and was found to be as effective as lactulose in reducing hyperammonemia (182).

The lack of stoichiometry between ammonia removal and increased phenylacetylglutamine in urine in paper II prompted further investigation on alternative excretion pathways. Known pathways for non-urea dependent removal of nitrogen include phenylacetyglutamine, phenylbutyrylglutamine, phenylacetylglycine (236) and hippuric acid. As we did not administer benzoate, an induction of the hippuric acid pathway seemed unlikely. Indeed, we found no increase in the excretion of hippuric acid in the OP treated group. Further analysis showed uptake of phenylbutyrate from the intestines and conversion to systemic phenylacetate across the enterocytes (β-oxidation) (237). We did, however, not find sufficient amounts of phenylacetylglutamine in plasma or tissue that could explain our observations as purely a time related excretion deficit.

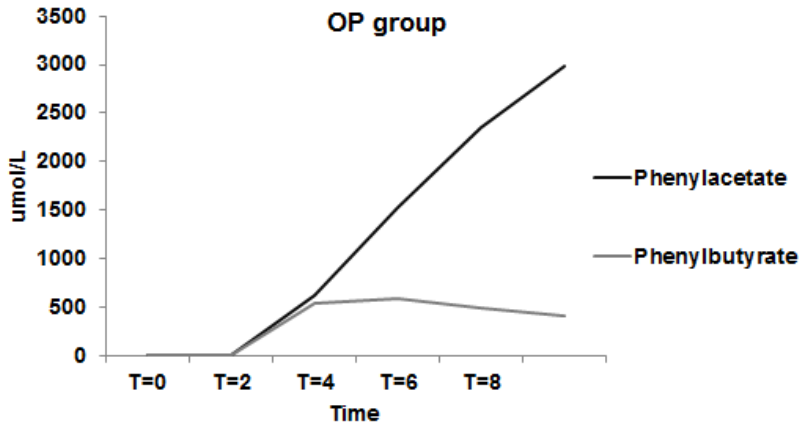

Figure 13 Arterial phenylbutyrate and phenylacetate. Phenylbutyrate is converted to phenylacetate by β-oxidation in enterocytes.

The lack of glutamine removal suggested another amino acid substrate for the conjugation reaction. In light of the results from paper III the proposed mechanism of ammonia reducing effect by OP seems to be confounded by the conjugation of phenylacetate to glycine. We found highly increased excretion of phenylacetylglycine in the OP treated group, and significantly more than phenylacetylglutamine. This finding could however represent a species specific effect.

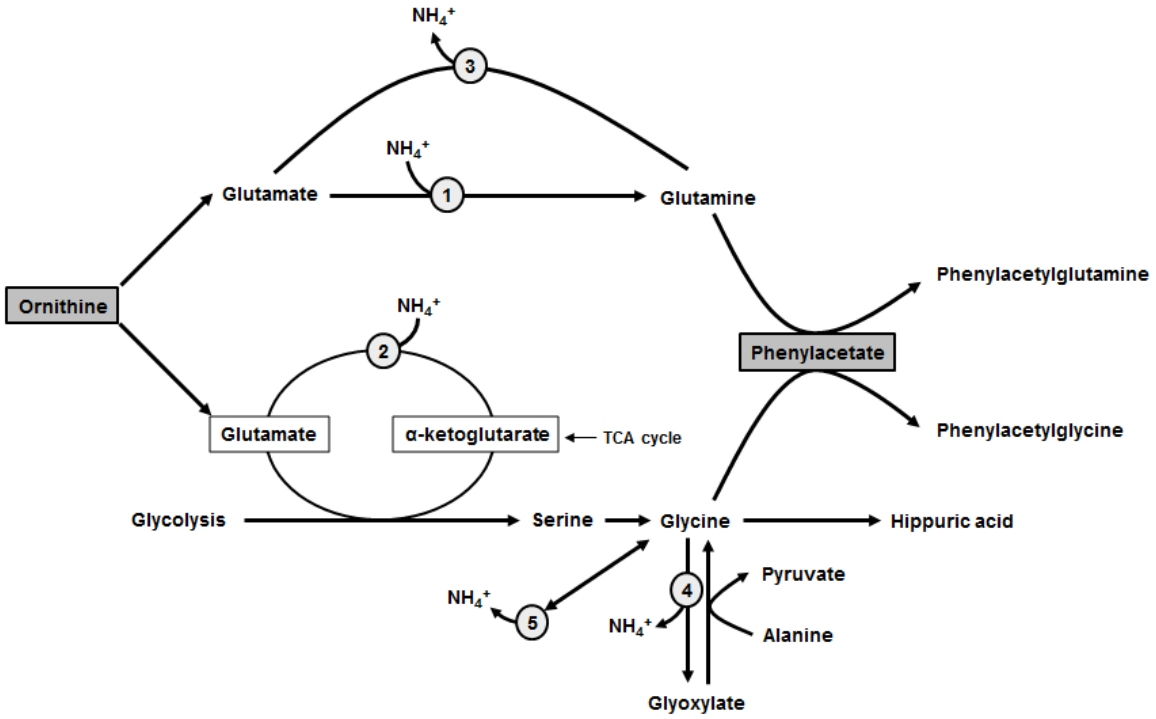

Figure 14 Influence of OP on glutamine and glycine metabolic pathways. Ammonia can be removed following reactions with glutamine synthetase (1) and glutamate dehydrogenase (2). Ornithine administration stimulates glutamate production which in turn generates glutamine (via glutamine synthetase) and glycine (via serine). Glutamine and glycine metabolism can lead to ammonia generation via glutaminase (3) and glycine oxidase (4) and glycine cleavage complex (5), respectively. Administration of phenylacetate leads to an increase in conjugation with both glutamine (generating phenylacetylglutamine) and glycine (generating phenylacetylglycine) precluding the generation of glutamine and glycine-derived ammonia and preventing an ammonia rebound.

Reprint with permission: Kristiansen RG, Rose CF, L-Ornithine Phenylacetate reduces ammonia in pigs with acute liver failure through phenylacetylglycine formation: a novel ammonia-lowering pathway, Am J Physiol Gastrointest Liver Physiol. 2014;307(10):G1024-31

Studies of OP treatment in liver failure have consistently shown ammonia reduction (238, 239), although further mechanistic studies are warranted (227). One human study has been conducted, showing OP to be well tolerated and able to decrease ammonia (240). A study using phenylbutyrate

alone in liver failure observed a reduction in ammonia and HE events in cirrhosis (241). However, amino acid profile or the presence of non-urea dependent urinary excretion products were not presented. Further mechanistic data is therefore warranted.

Today phenylacetate is given intravenously, and not intraduodenally as in this study. We did not conduct recovery analysis in order to account for the ornithine and phenylacetate given, as was done in the study by Dadsetan et al (227). Calculating recovery rate for ornithine and phenylacetate could improve the dosing of the drug in clinical studies and elucidate potential excretion delay that could result in adverse effects. Also, as ALF patients often present with accompanying renal failure, the excretion with impaired renal function needs to be evaluated. Studies have shown that plasma levels of phenylacetylglutamine are reduced by hemodialysis (242, 243).

6. CONCLUSIONS

ALF may cause severe, irreversible brain damage. Ultrastructural examination of different brain regions in pigs with ALF support the concept that vasogenic brain edema plays an important role in the development of intracranial hypertension in ALF.

A strong correlation between increased arterial ammonia and ICH exists, and definite treatment for hyperammonemia is still an unmet clinical need. The administration of OP to pigs with ALF successfully reduces arterial and extracellular brain ammonia levels and prevents any increase in ICP.

The ammonia removal is mediated through increased removal of glycine in this model. We provide experimental evidence for a novel ammonia-removing pathway via glycine that is active in the kidneys in pigs with ALF.

OP treatment might be a significant step forward in the treatment of patients suffering from ALF.

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