Université de Montréal

Implications of sex and extra-hepatic ammonia metabolism in chronic liver disease and development of hepatic encephalopathy

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Implications of sex and extra-hepatic ammonia metabolism in chronic liver disease and development of hepatic encephalopathy

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Résumé

Contexte et objectifs : L'encéphalopathie hépatique (EH) est un trouble neuropsychiatrique, une complication majeure de la maladie hépatique chronique (MHC). L'EH se manifeste par un large éventail de symptômes, allant d'un léger manque d'attention et de troubles de la mémoire à une léthargie sévère et un coma. L'hyperammoniémie est centrale dans la pathogenèse de l'EH puisque l'ammoniac est neurotoxique et que l'ammoniac dérivé du sang traverse facilement la barrière hémato-encéphalique (BHE). Cependant, d'autres facteurs pathogènes sont également impliqués dans l'EH, notamment le stress oxydatif. Au cours de la MHC, le muscle joue un rôle compensatoire essentiel dans l'élimination de l'ammoniac par l'action de l'enzyme glutamine synthétase (GS), qui transforme le glutamate en glutamine. Étant donné que les cellules endothéliales de la BHE sont l'interface entre le sang et le cerveau, il est plausible qu'elles métabolisent l'ammoniac pour protéger le cerveau de la neurotoxicité induite par l'ammoniac. Cependant, cela n'a jamais fait l'objet d'études. Les thérapies de réduction de l'ammoniac sont les traitements courants de l'EH. Cependant, les réponses des patients aux traitements sont hétérogènes, et les différences de sexe pourraient en être la cause.

Par conséquent, nos objectifs étaient 1) d'explorer le métabolisme de l'ammoniac dans les cellules endothéliales de la BHE par la présence de GS et 2) d'évaluer l'impact du sexe sur la MHC et ses complications, y compris la sarcopénie et l'EH.

Méthodes : Pour le premier objectif, nous avons évalué l'expression et l'activité de la protéine GS *in vitro* et *ex vivo* chez des rats naïfs. Nous avons également évalué l'impact de l'ornithine, du glutamate et du α-kétoglutarate sur l'activité de la GS dans les cellules endothéliales de la BHE via la génération de glutamine 5-^{13}C marquée. Pour le deuxième objectif, nous avons évalué l'impact du sexe sur le neurophénotype (anxiété, mémoire, coordination motrice et activité) chez des rats ligaturés des voies biliaires (BDL) (et contrôles respectifs) ainsi que sur le développement d'une EH sévère (léthargie/perte du réflexe de redressement). Nous avons également évalué les marqueurs des lésions hépatiques, l'hyperammoniémie, le stress oxydatif systémique, la masse et la fonction musculaire et la clairance de l'ammoniac musculaire.

Résultats : Nous avons trouvé l'activité et l'expression de la GS *in vivo* et *ex vivo* dans les cellules endothéliales de la BHE. L'analyse au microscope confocal a montré que la GS dans les cellules endothéliales est moins abondante que dans les astrocytes. L'exposition de cellules endothéliales cultivées à des substrats marqués a révélé que l'ornithine est la plus efficace pour générer de la glutamine. Chez les femmes, la chirurgie BDL a provoqué une MHC (augmentation des enzymes hépatiques circulantes et de la bilirubine) et de l'EH (altération de la coordination motrice et de l'activité nocturne) par rapport aux rats contrôles respectifs. De plus, le degré d'hyperammoniémie et la clairance musculaire de l'ammoniac étaient similaires entre les sexes. Contrairement aux mâles, les rats femelles n'ont pas développé de perte musculaire, d'œdème cérébral et de perte de mémoire à court terme. De plus, les femelles présentaient un stress oxydatif plus faible et étaient complètement protégées contre les EH sévères précipitées par l'ammoniac par rapport aux mâles BDL.

Conclusions : Nous concluons que la GS est exprimée dans les cellules endothéliales de la BHE, jouant peut-être un rôle dans l'atténuation ou le retard de l'entrée de l'ammoniac dans le cerveau et que la supplémentation en ornithine améliore l'activité de la GS en fournissant du glutamate pour la détoxification de l'ammoniac. De plus, nous concluons que le sexe a un impact sur les complications des maladies du foie, y compris la sarcopénie et l'EH, le stress oxydatif systémique jouant un rôle vital dans la susceptibilité à l'EH sévère induite par l'ammoniac.

Mots clés : glutamine synthétase, barrière hémato-encéphalique, ornithine, ligature des voies biliaires, sarcopénie, stress oxydatif.

Abstract

Background and aims: Hepatic encephalopathy (HE) is a neuropsychiatric disorder and a major complication of chronic liver disease (CLD). HE manifests with a wide range of symptoms, from mild lack of attention and memory impairments to severe lethargy and coma. Hyperammonemia is central in the pathogenesis of HE since ammonia is neurotoxic, and blood-derived ammonia easily crosses the blood-brain barrier (BBB). However, other pathogenic factors are also implicated in HE, including oxidative stress. During CLD, muscle plays an essential compensatory role in removing ammonia by the action of the enzyme glutamine synthetase (GS), which amidates glutamate into glutamine. Since the endothelial cells of the BBB are the interface between the blood and the brain, it is plausible that they metabolize ammonia to protect the brain from ammonia-induced neurotoxicity. However, this has never been investigated. Ammonia lowering therapies are the mainstream treatments for HE. However, patients' response to treatments are heterogeneous, and sex differences might be the cause.

Therefore, our aims were 1) To explore ammonia metabolism in BBB's endothelial cells through the presence of GS and 2) to assess the impact of sex on CLD and its complications, including sarcopenia and HE.

Methods: For the first aim, we assessed GS protein expression and activity *in vitro* and *ex vivo* in naïve rats. We also evaluated the impact of ornithine, glutamate, and α-ketoglutarate on GS activity in endothelial cells of the BBB via the generation of labeled 5-^{13}C glutamine. For the second aim, we assessed the impact of sex on the neurophenotype (anxiety, memory, motor coordination, and activity) in bile-duct ligated (BDL) rats (and respective SHAMs) as well as on the development of an ammonia-precipitated severe HE (lethargy/loss of righting reflex). We also assessed liver injury markers, hyperammonemia, systemic oxidative stress, muscle mass and function, and muscle ammonia clearance.

Results: We found GS activity and expression *in vivo* and *ex vivo* in endothelial BBB cells. The confocal microscope analysis showed that GS in endothelial cells is less abundant than astrocytes. Exposing cultured endothelial cells to labeled substrates revealed that ornithine is the most

efficient in generating glutamine. In females, BDL surgery caused CLD (increased hepatic enzymes and bilirubin) and HE (impaired motor coordination and night activity) vs. respective SHAMs. Furthermore, the degree of hyperammonemia and muscle ammonia clearance was similar between sexes. Contrary to males, female rats did not develop muscle loss, brain edema, and short-term memory loss. In addition, females had lower oxidative stress and were completely protected against ammonia-precipitated severe HE compared to male BDLs.

Conclusions: We conclude that GS is expressed in endothelial cells of the BBB, possibly playing a role in attenuating or delaying ammonia entry into the brain and, ornithine supplementation enhances GS activity by providing glutamate for ammonia detoxification. In addition, we conclude that sex impacts the complications of liver disease, including sarcopenia and HE, with systemic oxidative stress playing a vital role in the susceptibility to ammonia-induced overt HE.

Keywords: glutamine synthetase, blood-brain barrier, ornithine, bile-duct ligation, sarcopenia, oxidative stress.

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Abreviation list

HE : hepatic encephalopathy

CHE : covert hepatic encephalopathy

OHE : overt hepatic encephalopathy

ALF : acute liver failure

CLD : chronic liver disease

TIPS : transjugular intrahepatic portosystemic shunt

BBB : blood-brain barrier

GS : glutamine synthetase

AMPA : α-amino-3-hydro-methyl-4-isoxasole-propionic acid

NMDA : N-methyl-D-aspartate

GABA : γ-aminobutyric acid

ROS : reactive oxygen species

TCA cycle : tricarboxylic acid cycle

BDL: bile-duct ligation

MRI : magnetic resonance imaging

GDH : glutamate dehydrogenase

KCC : K^+, $2Cl^-$ transporter

TIPS : transjugular intrahepatic portosystemic shunt

HCC : hepatocellular carcinoma

MELD : Model for End-stage Liver Disease

INR : International normalized ratio

HBV : hepatitis B virus

HCV: hepatitis C virus

LPS : lipopolysaccharides

CSF : cerebro-spinal fluid

CNS : central nervous system

PFC : prefrontal cortex

ZO : zona occludens

UCD : urea cycle disorders

NASH : non alcoholic seteatohepatitis

PBC : primary biliary cholangitis

PSC : primary sclerosing cholangitis

GP : glycerol phenylbutyrate

SB : sodium benzoate

LOLA : L-ornithine L-aspartate

OP : ornithine phenylacetate

NO : nitric oxide

GFAP : glial fibrillary acidic protein

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Introduction

Hepatic encephalopathy

Hepatic encephalopathy (HE) is a major complication of liver disease, which affects up to 80% of patients with end-stage liver disease (Rose *et al.*, 2020). HE impacts patients' quality of life, affecting the ability to perform daily tasks such as working and driving a car, resulting in a substantial burden for affected patients (Wein *et al.*, 2004; Bajaj *et al.*, 2011). In addition, HE increases the risk of mortality by 2-fold in compared to patients without HE (64% with HE vs. 17-29% without HE) (Jepsen *et al.*, 2010). Among liver disease complications, HE has the highest impact on healthcare systems. In the USA, hospital admissions due to HE increased 30% from 2010 to 2014, generating a burden of US $ 11.9 billion per year (Hirode, Vittinghoff and Wong, 2019). Despite the burden of HE, optimal treatments for HE are lacking, and more research is needed to understand this devastating syndrome.

The liver

Anatomy

The liver is the body's largest solid organ, responsible for regulating systemic processes such as energy metabolism (by acting on the absorption, processing, and storage of nutrients), production of plasmatic proteins and coagulation factors, and the detoxification of toxins. The liver comprises hepatocytes, Kupffer cells, sinusoidal endothelial cells, and hepatic stellate cells organized as lobules. The liver has a unique vasculature, being perfused by both the hepatic artery and portal vein, the latter carrying blood directly from the intestines. Each hepatic lobule is perfused by a branch of the portal vein, hepatic artery, hepatic vein (central vein), and bile duct, forming the hepatic acinus, the functional unit of the liver. The hepatocytes are richly equipped with enzyme complexes, transporters, and molecules that will allow the liver to function successfully. In addition, hepatocytes are specialized according to their localization, with periportal hepatocytes expressing different enzymes and transporters than pericentral hepatocytes.

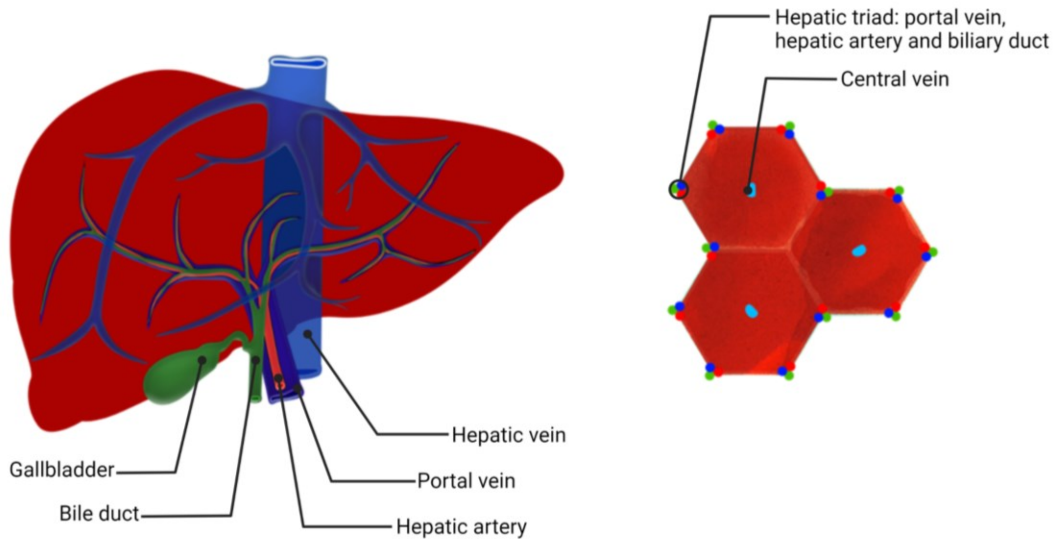

Figure 1. – Liver morphology with vasculature and hepatic lobule.

Energy metabolism

The liver is the main regulator of the energetic metabolism, a complex and highly regulated process. The energy metabolism is driven by circulating nutrients and pancreatic glucagon and insulin secretion (α and β-cells, respectively). In addition, the liver works in tight connection to extra-hepatic tissues, especially muscle and adipose tissue, for energy homeostasis. The liver balances blood glucose levels by storing energy postprandially and releasing glucose into circulation at a non-fed state, keeping blood glucose levels stable.

In the post-prandial state, blood glucose is stored in the liver (and muscle) through a series of reactions comprising glycogenesis. In addition, excess carbohydrates might be converted by lipogenesis (de novo synthesis) into fatty acids and sent to adipose tissue or stored as lipid droplets in the hepatocytes. Glucose can also generate energy or substrates for other processes via glycolysis by metabolizing glucose into pyruvate.

During fasting, when glucose levels are low, the increase of pancreatic glucagon secretion (and drop in insulin secretion) leads to the activation of glycogenolysis. Glycogen is then metabolized, and glucose is released into the circulation. In addition, during gluconeogenesis, the liver can form glucose and generate ATP from amino acids and lactate (from muscle) and fatty acids and glycerol (from adipose tissue), increasing fatty-acids beta oxidation. Since amino acids are an essential substrate for gluconeogenesis, prolonged starvation causes protein degradation, especially in skeletal muscle.

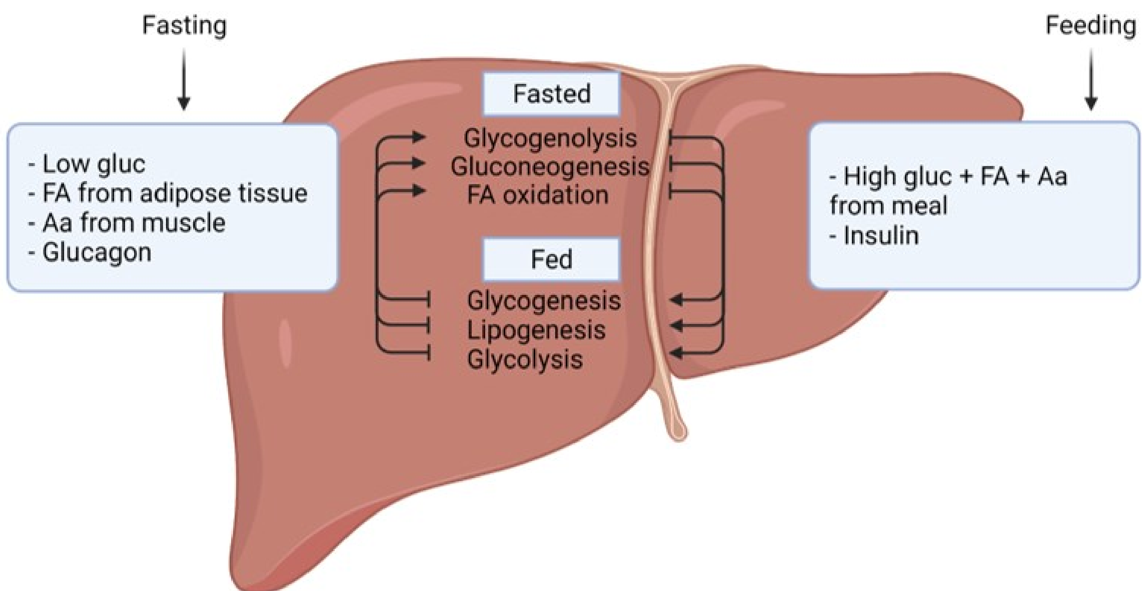

Figure 2. – Hepatic energy metabolism.

Energy metabolism in the liver in fed and fasted states. FA, fatty acids; Aa, amino acids; Gluc, glucose.

Ammonia detoxification

The detoxification of potentially harmful substances has significant importance for homeostasis. The liver detoxifies several endogenous and exogenous substances such as alcohol, drugs, and by-products of nitrogen metabolism. Among them, ammonia, produced by the gut via protein

metabolism and urease-containing bacteria, presents the most important endogenous toxin cleared by the liver.

The urea cycle

The urea cycle as a family of enzymes are exclusively found in the liver, as some individual enzymes might be found in extra-hepatic tissues (Lucas *et al.*, 2014). The urea cycle is primarily responsible for converting ammonia into the non-toxic product urea, which is then excreted by the kidneys. Ammonia produced in the intestines enters the liver via the portal vein and reaches the periportal hepatocytes equipped with all the enzymes needed for ureagenesis. First, the enzyme carbamyl phosphate synthetase (CPS I), found in mitochondria of periportal hepatocytes, mediates the synthesis of carbamyl phosphate from ammonia, bicarbonate and ATP. Subsequently, the enzyme N-acetyl-glutamate synthetase converts glutamate and acetyl-CoA into N-acetyl-glutamate. Next, the carbamyl phosphate can enter an alternative pathway and form orotic acid or, be condensed with ornithine by the enzyme ornithine transcarbamylase, forming citrulline. Citrulline is released into the cytosol and condensed with aspartate to form argininosuccinate by argininosuccinate synthetase. Then, fumarate and arginine are produced from argininosuccinate by argininosuccinate lyase. While fumarate is oxidized in the tricarboxylic acid (TCA) cycle, arginine is transformed into urea and ornithine by the hepatic arginase. Urea is a non-toxic product and will be excreted in urine by the kidneys. Glutamate dehydrogenase (GDH) is an enzyme found in both periportal and pericentral hepatocytes and catalyzes the conversion of glutamate in alpha-ketoglutarate + ammonia (and vice versa) to provide substrates for ammonia detoxification and the TCA cycle. The urea cycle is responsible for the majority of the ammonia removal in the liver, being a high-capacity but low-affinity system. However, ammonia detoxification also involves other enzymes such as glutamine synthetase (GS) and ornithine aminotransferase (OAT) present in perivenous hepatocytes (Zhou *et al.*, 2020).

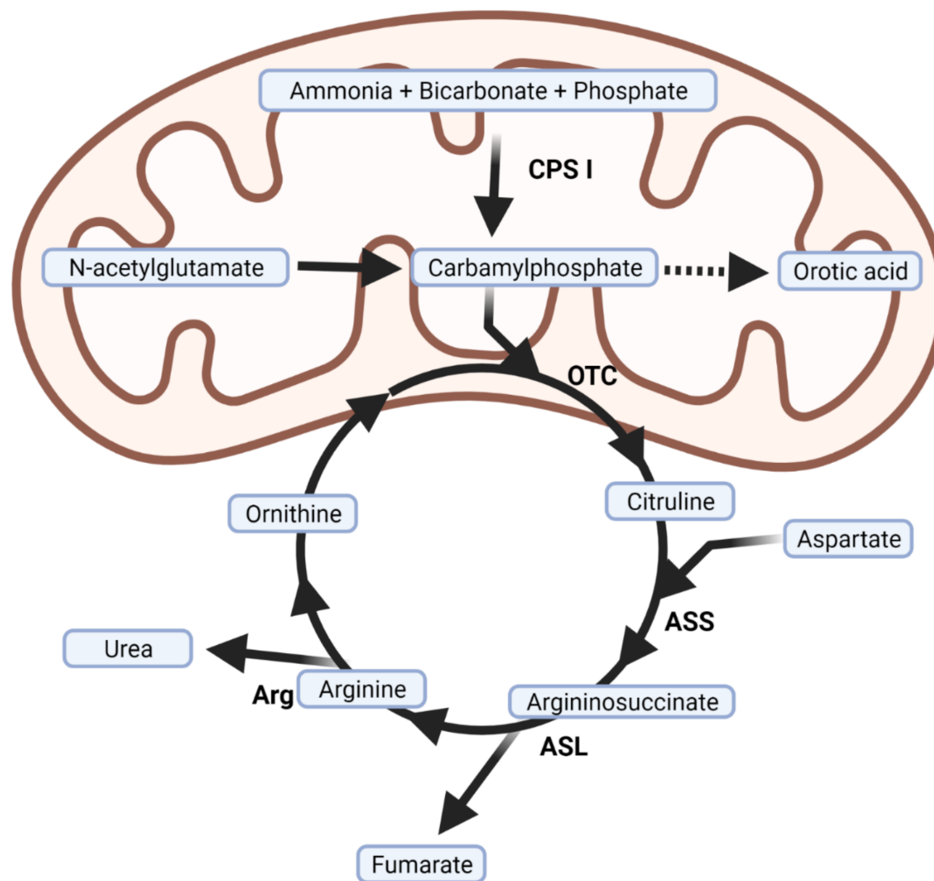

Figure 3. – Urea cycle

The urea cycle. CPSI, carbamyl phosphate synthetase; OTC, ornithine transcarbamylase; ASS, argininosuccinate synthetase; AS, argininosuccinate lyase; Arg, arginase.

Synthetic properties

The liver is responsible for the synthesis of most plasmatic proteins. Albumin is the most abundant protein in circulation, accounting for approximately 50% of all proteins in the blood and being a significant modulation of oncotic pressure. In addition, albumin is essential for its binding properties, transporting several ligands (such as fatty acids, ions, toxins) and acting as an antioxidant (Moman, Gupta and Varacallo, 2021). The liver also produces clotting factors, such as fibrinogen, prothrombin, and factors V, VII, IX, X, XI, XII among others in its hepatocytes, and factor VIII and von Willebrand factor in the liver sinusoidal endothelial cells (Heinz and

Braspenning, 2015). Furthermore, the liver contributes to the production of complement proteins, having an essential role in innate immunity (Thorgersen *et al.*, 2019).

Besides the production of circulating factors, the liver synthesizes bile, which is essential for normal digestive function. Bile is composed of fats, bile salts, and bilirubin, and the liver and intestine regulate the amounts of bile acids tightly to prevent cytotoxic accumulation. Bile salts are produced in the hepatocyte and modified by the cholangiocytes in the bile ductules and gallbladder (in species in which the gallbladder is present). The bile is released into the duodenum during a meal, exerting fat emulsification, which is essential for lipid absorption in the small intestine. In the ileum, most of the bile salts are reabsorbed and recycled by the liver.

Antioxidant system

Detoxification of xenobiotics produces high levels of reactive oxygen species, causing cell damage (Ghosh *et al.*, 2016). Reactive oxygen species (ROS) production is linked with the activity of cytochrome P450 enzymes (CYP), which is mediated by several receptors such as the aryl hydrocarbon receptor, pregnane X receptor, and the constitutive androstane receptor (He *et al.*, 2017). Since the liver is the main organ for detoxifying such molecules, it becomes the target of many insults resulting in oxidative stress. Because of that, the liver is equipped with a powerful antioxidant system.

Antioxidants are high-affinity scavengers of free radicals, preventing or delaying the oxidation of a substrate. Antioxidants donate electrons to stabilize free radicals and reduce their reactivity, balancing the body’s redox state (Casas-Grajales and Muriel, 2015). The liver has both enzymatic and non-enzymatic antioxidants. Enzymatic antioxidants effectively protect against oxidative attacks, facilitating antioxidant reactions and decomposing ROS (Christofidou-Solomidou and Muzykantov, 2006). Among the enzymatic antioxidants in the liver are superoxide dismutase (SOD) and catalase (CAT) (Jurczuk *et al.*, 2004). Amongst the non-enzymatic antioxidants produced by the liver, glutathione and albumin are the most important due to their high abundance (Roche *et al.*, 2008; Lu, 2020).

The central nervous system

The central nervous system (CNS) is composed of the brain and the spinal cord, and it exerts control upon the rest of the body. The CNS is a complex entity that coordinates all voluntary movements (e.g., speech and locomotion) and part of the involuntary movements (e.g., breathing and eye blinking). The CNS also regulates cognition, behavior, and growth, and it receives and responds to sensory information from the environment. The brain is a complex structure divided into the cerebrum, cerebellum, and brain stem.

The cerebrum is the largest part of the brain, and it is divided into two (right and left) hemispheres, which can also be divided into four lobes: frontal, parietal, temporal, and occipital. The frontal lobe controls complex brain functions such as attention, problem-solving, movement coordination, and personality. More specifically, the anterior part of the frontal cortex, the prefrontal cortex (PFC) receives highly processed inputs from other parts of the cerebrum. The PFC is responsible for modulation of social behavior, ability to concentrate, expression of personality, learning, and decision making.

The limbic system is part of the cerebrum composed of the limbic lobe (cingulate gyrus + parahippocampal gyrus) and structures from the deep brain such as the amygdala, mammillary bodies, and the hippocampus. The hippocampus has a significant role in learning, memory formation and retrieval, and it is a site for adult neurogenesis (Eriksson *et al.*, 1998). The hippocampus is essential for both long and short-term memory. Long-term memory can be divided into non-declarative (implicit) and declarative (explicit) memory, and declarative memory can be further divided into episodic (episodes) and semantic (facts) memory. The limbic system, especially the hippocampus, also connects with the brain cortex to link emotion with memory, giving a top-down cortical control of emotional responses.

The basal ganglia are also formed by deep brain structures such as the caudate nucleus, the globus pallidus, and the putamen. The basal ganglia are mainly responsible for motor control, in addition to motor learning, emotions, and executive functions.

The cerebellum is found below the occipital lobe, and it is divided into anterior and posterior lobes and a medial structure called the vermis. The primary function of the cerebellum is to regulate fine motor control, influencing balance, posture, motor coordination, and learning, allowing proper body movement.

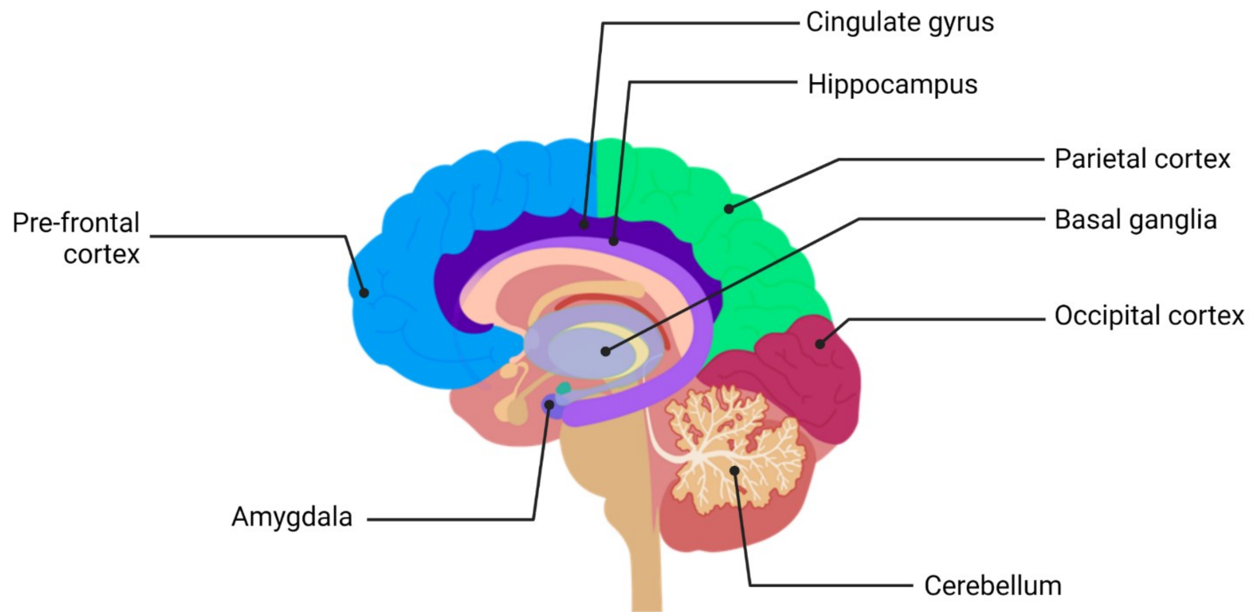

Figure 4. – Brain anatomy

The components of the CNS

Several types of cells populate the brain. Neurons are equipped with several transporters, pumps, and ion channels. Neurons generate and propagate electrical signals, which are transformed into chemical signals by releasing neurotransmitters. They act on the reception, transmission, and processing of information from both excitatory and inhibitory stimuli.

The glial cells are the most abundant cell type in the brain (up to 90% of the cells in the CNS), providing support to the neurons by regulating the brain microenvironment. Glial cells are diverse and have a variety of functions, such as producing myelin sheath for the neurons in the CNS

(oligodendrocytes), producing cerebrospinal fluid (ependymal cells), being part of the immune system (microglia), and supporting the neurons directly (astrocytes).

Astrocytes are star-shaped cells responsible for supporting neurons as well as communicating with neurons and blood capillaries. Astrocytes also regulate the ionic and molecular extracellular environment of the brain and have an important role regulating neurotransmission via the tripartite synapse (the functional association of pre- and post-synaptic neurons and adjacent glia). The astrocytic foot processes wrap around the blood vessels and transfer molecules and ions from the blood to the neurons. In addition, astrocytes have an essential role in brain energy metabolism by synthesizing lactate from glucose and transporting it to be used as an energy source by neurons (Pellerin *et al.*, 1998). Finally, the astrocytes play a role in protecting the brain, metabolizing toxins and shielding the neurons and other central cells from potential harmful systemic molecules.

The blood-brain barrier

The blood-brain barrier (BBB) is a vital biological barrier that protects the brain, a highly complex, dynamic organ sensitive to external insults. Highly regulated, the BBB physically and biochemically limits the crossing of certain blood-derived factors into the brain tissue, restricting the transcellular passage of charged, non-lipophilic, and large molecules. In addition, the cells from the BBB are metabolically active, containing enzymes, receptors, and transporters. Therefore, even though potentially harmful molecules can enter the cellular components of the BBB, they can be metabolized or restricted from entering into the brain parenchyma. The BBB is composed of astrocyte foot processes wrapped around a monolayer of endothelial cells with the adjacent pericytes.

Components of the BBB

Endothelial cells

The endothelial cells play a central role in the composition of the BBB. While the luminal side (blood facing) of the endothelial cells is in direct contact with the systemic circulation, the abluminal side (brain) is in contact with astrocyte foot processes and perivascular cells (pericytes).

Therefore, endothelial cells play a significant role in regulating the interaction between blood and the brain.

Endothelial cells restrict harmful substances from passing into the brain through two different mechanisms. First, in the paracellular barrier, tight junction proteins expressed between endothelial cells such as claudin, occludin, and zona occludens (ZO) restrict the paracellular passage (between cells) of molecules. Secondly, in the transcellular barrier, unwanted molecules that manage to get into the intracellular space of endothelial cells are either flushed by luminal efflux transporters or metabolized by enzymes. The transcellular barrier prevents harmful molecules such as drugs and other foreign substances from exiting endothelial cells and crossing into the brain.

Besides protecting the brain against the free passage of potentially harmful molecules, the endothelial cells also regulate the brain microenvironment tightly. The endothelial cells allow only selected molecules to cross into the brain via the expression of specific transporters, pumps, and receptors that perform receptor-mediated transcytosis (RMT) (Wang, Lui and Li, 2009).

Astrocytes

As part of the BBB, the astrocytes interact with the other cellular components, with the astrocytic foot processes providing structural stability to the BBB and regulating the barrier phenotype (reviewed by Cheslow and Alvarez, 2016). *In vitro* experiments have shown that cultured astrocytes can induce the tightening of the endothelial barrier (Janzer and Raff, 1987), likely by regulating the expression of tight junction proteins (Tao-Cheng, Nagy and Brightman, 1987). Moreover, astrocytes help control the brain's blood flow and the extracellular ionic and molecular environment within the synaptic cleft, regulating the blood-derived molecules that reach the neurons (Marina *et al.*, 2020).

Pericytes

Pericytes are perivascular cells derived from smooth muscle cells, which like astrocytes, interact with endothelial cells and contribute to BBB's stability. Although the importance of pericytes is often overlooked, there is evidence that these cells are essential for proper barrier function. Pericytes are necessary for proper tight junction protein organization, which dictates the tightness of the BBB, and influence endothelial transcytosis, regulating the passage of molecules through the BBB (Daneman *et al.*, 2010). Moreover, pericytes prevent apoptosis of the endothelial cells, stimulate basement membrane proteins' expression and, contribute to the normal capillary formation (Ramsauer, Krause and Dermietzel, 2002; Stratman and Davis, 2012).

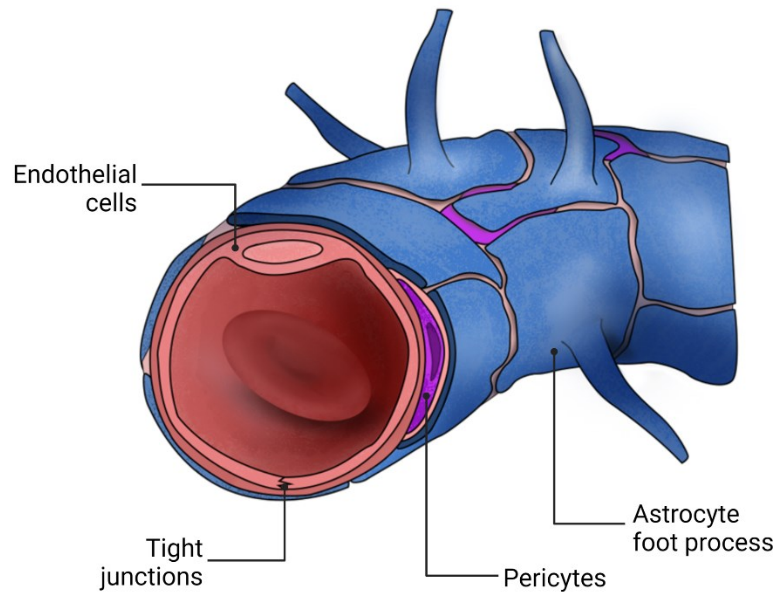

Figure 5. – The structure of the blood-brain barrier

The blood-brain barrier is composed by endothelial cells held together by tight junctions and wrapped by pericytes and astrocytic foot processes.

Liver disease

Liver disease is responsible for 2 million deaths every year globally, with cirrhosis and liver cancer accounting for 3.5% of all deaths worldwide (Asrani *et al.*, 2019). In Canada, cirrhosis is the 11th most common cause of death, responsible for 3662 deaths in 2019. Patients with liver disease might die from acute decompensations and complications such as sepsis (Biecker, 2013; Singal *et al.*, 2017; Philips *et al.*, 2020). Moreover, liver transplantation is the only curative treatment for cirrhosis. However, mortality also arises from the insufficient number of liver transplants, even though liver transplant is the second most common solid organ transplant (Asrani *et al.*, 2019). In addition, the increased incidence of liver disease, especially non-alcoholic steatohepatitis (NASH), also contributes to the increase in mortality over the years (Asrani *et al.*, 2019).

Depending on its progression, liver disease can be defined as either acute liver failure (ALF) or chronic liver disease (CLD). ALF is marked by a rapid progression of severe hepatocyte necrosis and liver damage without evidence of pre-existing liver disease (Lee, 2012). The leading causes of ALF are overdose of drugs such as acetaminophen in western countries and viral hepatitis in eastern countries (Bernal and Wendon, 2013). Patients with ALF present jaundice, coagulopathy, HE, and multi-organ failure, leading to death (Dong, Nanchal and Karvellas, 2020).

CLD is the most common type of liver disease, having a slow progression (from 5 to 50 years). The progression of hepatic injury regularly leads to fatty liver, fibrosis, and then cirrhosis, with replacement of liver parenchyma by scar tissue and distortion of morphological architecture. Fibrosis is the first stage of liver scarring, and it occurs upon stimuli such as the presence of cytokines, resulting in the activation of stellate cells of the liver and subsequent collagen secretion (Friedman, 2008). The pattern of fibrosis depends on the etiology of the liver disease (Lo and Kim, 2017), and with continuous injury, the typical liver architecture is replaced by nodules, characterizing cirrhosis. The hepatic injury from CLD promotes carcinogenesis, accounting for 80% of hepatic cancers, with hepatocellular carcinoma (HCC) being the most frequent. The severity of CLD is frequently measured by the Model for End-stage Liver Disease (MELD) score, primarily used to estimate post-transplant survival. The MELD score is based on levels of bilirubin, creatinine, and the International Normalized Ratio (INR), which measures blood coagulation time. In

addition, the severity of CLD can also be evaluated by the Child-Pugh score, which evaluates levels of albumin, bilirubin, and INR and the presence and severity of HE and ascites.

There are several causes of CLD, which may vary with age, sex, and continent of residence. While alcoholic liver disease and NASH are the leading causes of CLD in western countries, viral hepatitis is still a primary cause in Asian countries (Sepanlou *et al.*, 2020). In addition, biliary cirrhosis, such as primary biliary cholangitis (PBC) and primary sclerosing cholangitis (PSC), are also present, especially in developed countries (Asrani *et al.* 2019).

<u>Alcoholic liver disease</u>

Alcohol is the most commonly used recreational drug in the world, and as a result, alcohol-associated liver disease is one of most common cause of liver cirrhosis in the United States. According to the World Health Organization, alcohol abuse is responsible for more than 60% of the cases of cirrhosis and almost 50% of cirrhosis deaths (Yoon and Chen, 2016) In Canada, 19% of the population above 12 years old is defined as heavy drinkers (consumption of 4 or more drinks and five or more drinks per occasion, at least once per month for females and males, respectively) (Government of Canada, 2017). According to the Canadian Center on Substance Use and Addiction, alcohol abuse in Canada leads to a mortality rate of 8.4 per 100000 habitants.

<u>Non-alcoholic fatty liver disease</u>

According to the World Health Organization, with the increased prevalence of obesity, 52% of the world population was considered overweight or obese in 2016. Therefore, NASH is on the rise to become the primary cause of CLD in developed countries (Asrani *et al.*, 2019). Non-alcoholic fatty liver disease (NAFLD) affects as much as 30% of the general population, leading to NASH, which is the second cause of liver transplantation in the US (reviewed by (Bellentani, 2017).

<u>Viral hepatitis</u>

Hepatitis C virus (HCV) and hepatitis B virus (HBV) infections are systemic disorders marked by primary hepatic impairment that causes viral hepatitis, fibrosis, and cirrhosis. Globally, viral hepatitis is still one of the first causes of CLD and it is estimated that around 71 million people live with chronic hepatitis C infection. According to the World Health Organization report from 2016,

antiviral medicines are the treatment of choice and can cure 95% of the cases of hepatitis C, reducing the risk of progression into cirrhosis and liver cancer (Berenguer *et al.*, 2017). HBV infection is a significant health problem worldwide, even with HBV transmission being prevented by vaccination. Even so, there is still a high incidence of HBV worldwide (El-Serag, 2012).

<u>Cholestasis</u>

PBC and PSC are the most common forms of cholestasis, with the incidence of PBC increasing over the years (Boonstra *et al.*, 2014). During cholestasis, the bile flow is reduced or interrupted in the biliary tract. Therefore, the bile cannot reach the intestines, resulting in liver damage and increased level of systemic bile acids (Bremmelgaard and Sjövall, 1979; Bremmelgaard and Almé, 1980).

Complications of liver disease

Regardless of its etiology, CLD can lead to complications. Compensated cirrhosis is defined as patients living with cirrhosis without any overt complications whereas decompensated cirrhosis is defined when complications related to cirrhosis develop.

Due to stiffening of hepatic structures, CLD patients develop portal hypertension, which could cause ascites and variceal bleeding. In addition, these patients often present sarcopenia, jaundice (yellowing of the skin), and HE, among other complications.

<table>
<tr><th>Primary complications</th><th>Secondary complications</th><th>Associated complications</th></tr>
<tr><td rowspan="5">Portal hypertension</td><td>Gastroesophageal varices</td><td>Gastrointestinal bleeding</td></tr>
<tr><td>Portal hypertensive gastropathy</td><td>Gastrointestinal bleeding</td></tr>
<tr><td>Splenomegaly</td><td></td></tr>
<tr><td>Ascites</td><td>Spontaneous bacterial peritonitis</td></tr>
<tr><td>Portopulmonary hypertension</td><td></td></tr>
<tr><td>Hepatic encephalopathy</td><td rowspan="3"></td><td rowspan="3"></td></tr>
<tr><td>Hepatorenal syndrome</td></tr>
<tr><td>Malnutrition</td></tr>
</table>

Sarcopenia		
Coagulopathy		
Bone disease		
Anemia		
Jaundice		

Table 1. - Complications from liver disease

Ascites

Ascites is a common complication of CLD, frequently being the first decompensating event of cirrhosis (Planas *et al.*, 2004). It occurs in up to half of all patients with cirrhosis, being presented as mild to severe and, from uncomplicated to refractory (Ginés *et al.*, 1987). Moreover, ascites is associated with 32% three-year mortality in patients (Tonon *et al.*, 2021).

Ascites arises from portal hypertension and sodium and water retention. The endothelial cells of the liver sinusoids form an extremely porous membrane, highly permeable to molecules, including plasmatic proteins. Scarring of the liver with increased collagen production and deposition increases liver stiffness, restraining the blood flow and causing portal hypertension. Portal hypertension consists of elevated blood pressure in the portal venous system, which can be caused by increased hepatic venous flow or by increased flow resistance due hepatic stiffening. Portal hypertension increases the hydrostatic pressure from the hepatic sinusoids and causes fluid migration from the vessels into the peritoneal cavity. Fluid builds up in the abdominal cavity, resulting in ascites. While normal portal pressure is around 5 mmHg, clinical complications from portal hypertension arise with a portal pressure above 10 mmHg (Groszmann *et al.*, 2005). Portal hypertension is critical to the development of ascites, which rarely develops in patients with normal portal pressure (Moore and Aithal, 2006). In agreement, the insertion of a transjugular intrahepatic portosystemic shunt (TIPS), decreases portal pressure and can be used to treat refractory ascites (Mah *et al.*, 2020).

Sodium and water retention occur due to renal dysfunction and systemic vasodilation, which impairs systemic hemodynamics with decreased arterial blood volume. Systemic vasodilation might arise from lower sensitivity to vasoconstrictors, an increase in circulating vasodilators such as nitric oxide and prostacyclin or inflammation (Ginès *et al.*, 1997; Bernardi *et al.*, 2015). CLD is

associated with decreased renal sodium excretion, with increased sodium reabsorption at the proximal and distal tubule (Ginès *et al.*, 1997; Bernardi and Zaccherini, 2018).

Gastrointestinal bleeding

After ascites, gastrointestinal (GI) bleeding is the most common complication of CLD (Planas et al., 2004). It affects up to 40% of patients without other complications and 60% of the patients with ascites (Biecker, 2013). GI bleeding, like ascites, is a consequence of increased portal hypertension, which results in esophageal varices. Once the varices occur, they linearly increase in size over time, resulting in rupture and bleeding (de Franchis and Primignani, 2001). The risk of bleeding increases in patients with severe liver disease (Child-Pugh C score), medium to large varices, or small varices with a red wale sign (Biecker, 2013). In this case, prophylactic treatment should be given, reducing the risk of bleeding by up to 50% (Biecker 2013). Management of patients with a high risk of GI bleeding targets reduction of portal hypertension with nonselective beta-blockers, TIPS and endoscopic variceal ligation.

Jaundice

Marked by the yellowing of the skin and sclera and bilirubin levels greater than 2.5 to 3 mg per dL, jaundice is a far less severe complication of CLD (Roche and Kobos, 2004). However, jaundice is indicative of decompensation in cirrhosis and should be evaluated. Being the third most common first decompensation (Sangiovanni *et al.*, 2006), jaundice has a prevalence of 4.1% of all patients with CLD, and 17% of the patients with liver disease will eventually develop jaundice over the course of their disease. Jaundice can arise from either unconjugated or conjugated hyperbilirubinemia. Unconjugated hyperbilirubinemia is caused by hemolysis and disorders that involve bilirubin conjugation. Meanwhile, conjugated hyperbilirubinemia is present in conditions related to hepatocyte damage, such as CLD from viral hepatitis, alcohol intake, and biliary cirrhosis (Fargo, Grogan and Saguil, 2017).

Other complications

Besides the complications already mentioned, the impact of liver disease on the body is profound, resulting in several complications, as shown in Table 1.

Sarcopenia

Skeletal muscle is one of the most dynamic tissues, accounting for around 40% of the body weight and 50-75% of all body proteins (Frontera and Ochala, 2015). Skeletal muscle tissue is organized into muscle fibers (myofibers) and associated connective tissue. Muscle fibers are multinucleated and therefore do not divide. Therefore, cell division is carried by the satellite cells, which are muscle stem cells that proliferate and differentiate, forming myofibers (Macaluso and Myburgh, 2012).

The main functions of the skeletal muscle are to generate force to produce movement, maintain posture, and contribute to basal energy metabolism, especially as storage of amino acids (Wolfe 2006). Muscle is mainly made from water and proteins, with actin and myosin, the main contractile proteins, accounting for 70-80% of the protein content in a muscle fiber (Frontera and Ochala 2015). The muscle mass depends on the balance between protein synthesis and degradation, which are processes susceptible to nutritional status, hormonal balance, exercise, and diseases.

Sarcopenia is defined as the loss of muscle mass and function, which leads to physical disability and poor quality of life. Sarcopenia develops from multiple causes, including aging, nutritional deficiencies, and chronic diseases (Fielding *et al.*, 2011). Since age is a risk factor for muscle loss, sarcopenia is commonly present in the elderly population. Sarcopenia is an essential factor considering the overall health and financial burden in older adults (Landi *et al.*, 2010), and it is associated with an increased risk of poor cognition (Cooper *et al.* 2011). Muscle mass and function are shown to predict disability and mortality over the years, increasing the incidence of falls, hospitalization time, and healthcare costs (Rantanen, 2003). In liver disease, sarcopenia has a high incidence, worsening the patient's quality of life. Since the muscle is an important site for extra-hepatic ammonia detoxification, the presence of sarcopenia is associated with increased risk of HE (Bhanji *et al.*, 2018). Also, it increases the incidence of other complications of CLD such as ascites and portal hypertension and the mortality risk before and after liver transplantation (Huisman *et al.*, 2011).

The diagnosis of sarcopenia is performed by measurements of muscle mass and quality by scans (CT-scans or MRIs) generally at the level of the third lumbar vertebrae, muscle strength by grip strength or chair stands, and by the assessment of muscle performance by gait speed test among others (Cruz-Jentoft *et al.*, 2019). A loss of fat mass can accompany the loss of muscle mass, and when fat mass loss is absent, sarcopenia might be difficult to perceive in clinic settings. In addition, sarcopenia might also be associated with high-fat mass content in the skeletal muscle, known as myosteatosis. Myosteatosis presents as the loss of muscle quality rather than muscle mass alone and is also associated with health complications and mortality in patients with CLD (Montano-Loza *et al.*, 2016, p. 20016; Bhanji *et al.*, 2018).

Types of HE

HE is a serious CNS dysfunction resulting from either acute or chronic liver failure. HE leads to a wide range of neuropsychiatric symptoms. HE can be divided into types A, B, and C according to the underlying liver disease.

Type A HE

Type A HE arises from **a**cute liver failure, and it is generally a critical condition. It presents a quick progression of cerebral edema, which evolves to life-threatening intracranial hypertension in up to 25% of the cases and can culminate in brain stem herniation and death (Bernal et al., 2007). Patients with type A HE present with fast deterioration of neurological symptoms, including severe lethargy, progressing to coma and death.

Type B HE

Type B HE is a rare condition caused by insufficient blood detoxification by the liver due to a congenital malformation, which shunts (**b**ypass) the blood from the intestines away from the liver and into the circulation (Franchi-Abella *et al.*, 2018). Patients and animal models of type B HE present typical neurocognitive impairments from HE such as lethargy, confusion, changes in behavior, and attention disorders (Bernard *et al.*, 2012), but without the underlying liver disease.

Type C HE

Type C HE greatly affects patients with **c**irrhosis. HE develops after the slow progression of the liver disease. Therefore, toxins build-up and reach the brain, causing type C HE to develop, with symptoms that arise from mild lack of attention, memory impairments, and impaired motor coordination to lethargy and coma.

Covert HE

Covert HE (CHE) converges HE grade 1 based on the West Haven criteria (mild lack of awareness, euphoria or anxiety, shortened attention span, altered sleep rhythm) and minimal HE (minimal impairments only observed by psychometric or neuropsychological tests, without clinical evidence of neurocognitive changes). Being present in the majority of the patients with CLD (Bajaj 2008) and often undiagnosed, CHE is the subclinical form of HE. Even so, CHE affects patients impairing performance in attention-demanding tasks such as working and driving (Wein *et al.*, 2004). In addition, CHE patients have a higher risk (4-fold higher) of developing OHE (Hartmann *et al.*, 2000) and a higher risk of persistent neurological complications after liver transplantation (Chavarria and Cordoba, 2013).

Since CHE is subtle, its diagnosis is made using careful and extensive cognitive testing. Although there is no definitive test for its diagnosis, two different cognitive tests can be used to diagnose CHE (Duarte-Rojo *et al.*, 2018). The Psychometric Hepatic Encephalopathy Score (PHES) test is considered the gold standard for CHE diagnosis. PHES comprises five tests, which assess motor coordination, psychomotor speed, processing speed, working memory, visuospatial processing, and attention. However, the PHES test is time-consuming and challenging to analyze. So, other psychometric tests present a potential improvement for CHE assessment. The EncephalApp Stroop test is an electronic test that measures selective attention, psychometric speed, and cognitive flexibility. Validated in several countries and easy to use, it presents a good alternative for patients with CHE (Bajaj *et al.*, 2015; Zeng *et al.*, 2019; Cunha-Silva *et al.*, 2022). However, both PHES and Stroop tests are subjected to a learning effect. Because of that, tests that are quantitative and which do not involve cognition (and therefore have no learning effect) are wanted. Tests such as the critical flicker frequency (CFF), which evaluates visual temporal

resolution, and electro-encephalogram (EEG), which evaluates brain activity, are potential tools for CHE diagnosis (Kircheis *et al.*, 2002; Amodio and Montagnese, 2015).

However, there is not a perfect test since HE has a broad range of symptoms and the development of specific cognitive impairments is not uniform in patients. In agreement, patients diagnosed as non-HE by the PHES test failed other psychometric tests evaluating different aspects of cognitive and motor function (Giménez-Garzó *et al.*, 2017). For now, a combination of tests is the best way to ensure CHE diagnosis. More importantly, research is still needed to understand which factors drive the different HE domains, so we can target CHE testing accordingly.

Overt HE

OHE is the clinically evident form of HE, defined by the West-Haven criteria as grades II (lethargy or apathy, time disorientation, obvious personality change, inappropriate behavior, motor incoordination, and asterixis), grade III (somnolence to stupor, confusion, gross disorientation, bizarre behavior with response to stimuli) and grade IV (coma) (Vilstrup *et al.*, 2014, p. 2014).

OHE usually arises due to a precipitant factor such as constipation, electrolyte imbalance, infections, or GI bleeding, among others (Ferenci *et al.*, 2002). When no precipitant factors can be found, OHE is considered spontaneous. However, the precipitant factor may be simply missed. Moreover, OHE can be further divided into episodic when it is sporadic, recurrent when episodes present more than once over 6 months, or persistent if the episode is not reversed.

Pathogenesis of HE

During liver disease, the systemic factors that arise from the ailing liver might accumulate in the systemic circulation and impact brain function. The pathogenesis of HE is multifactorial, with the precise underlying mechanisms still not fully understood. However, there is evidence that systemic pathogenic factors such as ammonia, oxidative stress, and inflammation play an important role.

Ammonia

In liver disease, hyperammonemia develops due to a decrease in ammonia detoxification and an increase in ammonia production. Indeed, the loss of hepatic function results in a decrease in

ammonia's removal capacity. Also, an increase in glutaminase activity (deamination of glutamine) in the gut and the changes in the gut microbiota favoring ammonia-producing bacteria further increase blood ammonia (Bajaj *et al.*, 2012; Chen *et al.*, 2016).

Ammonia exists as both a weak acid (NH_4^+) and a weak base (NH_3). At physiologic pH (7.4), around 2% of the ammonia is a gas (NH_3), while the remaining 98% is found as an ion (NH_4^+). NH_3 can freely cross all biological membranes via diffusion, including the BBB, while the ionic form, NH_4^+, can cross only via channels and transporters. Since NH_4^+ has similar ionic properties as potassium, it can be transported by potassium carriers such as the K^+, $2Cl^-$ transporter (KCC) (Bergeron *et al.*, 2003). Ammonia can also cross membranes through specific ammonia transporters. The Rhesus-associated glycoprotein (RhCG and RhBG) are found in several cell types, including the endothelial cells of the BBB (Huang and Liu, 2001; Nakhoul and Hamm, 2013), and allow ammonia to reach the brain easily.

Amongst the multiple factors involved in the pathogenesis of HE, ammonia toxicity is central to the development of this syndrome. Although the relationship between blood ammonia levels and cognitive decline in patients with liver disease is not always linear, different studies demonstrate the association between ammonia and HE. It has been shown that the presence of HE is associated with increased ammonia (Xie *et al.*, 2018) and that patients with higher baseline ammonia have increased incidence and frequency of HE episodes (Vierling *et al.*, 2016). In agreement, ammonia-lowering strategies remain at the forefront of treatment (Ong *et al.*, 2003; Rose, 2012; Qureshi, Khokhar and Shafqat, 2014). Ammonia lowering strategies improve cognitive functions, such as health-related quality of life (Prasad *et al.*, 2007) and prevent new episodes of overt HE (Sharma *et al.*, 2009). Also, ammonia levels correlate, at least in part, with the severity of HE, and different studies found that the higher the blood ammonia levels, the worse is the neurocognitive deficits associated with the HE (Kramer *et al.*, 2000; Ong *et al.*, 2003; Qureshi, Khokhar and Shafqat, 2014).

Oxidative stress

Oxidative stress arises when reactive oxygen species (ROS) production is higher than the ability to remove such molecules, causing an imbalance between antioxidants and pro-oxidants. Natural

products of cell metabolism, ROS are highly reactive molecules containing oxygen such as nitric oxide (NO), hydrogen peroxide (H_2O_2), and the anions superoxide (O_2^-) and hydroxyl (OH^-). However, excess ROS exert deleterious effects by damaging lipids, proteins, DNA, and RNA. In cirrhosis, oxidative stress is a systemic event induced by decreased expression of antioxidants and increased systemic pro-oxidants. Albumin, the most abundant circulating protein and a vital antioxidant produced by the liver, is decreased during liver disease (Oettl *et al.*, 2008). Another hepatic antioxidant, the peptide glutathione, is also decreased during liver disease, adding to the oxidative stress imbalance (Lu, 2020). Several other factors are responsible for ROS and oxidative stress generation, which arise from the ailing liver, including inflammation.

There is an ample amount of evidence demonstrating that ROS is instrumental in the pathogenesis of HE. In liver disease patients, 3-nitrotyrosine, a blood marker of systemic oxidative and nitrosative stress, differentiated patients with and without minimal HE (Montoliu *et al.*, 2011; Giménez-Garzó *et al.*, 2018). In addition to increased pro-oxidants, impairment of antioxidant capacity is a feature of oxidative stress found in patients with HE. In agreement, blood levels of glutathione peroxidase, among others, correlate with the presence of minimal HE (Montoliu *et al.*, 2011; Irimia *et al.*, 2013; Sangeetha *et al.*, 2016). Systemic ROS can differentiate patients with minimal HE from patients without HE, even without evidence of ROS in the brain. However, ROS in the CNS might have a role in the severity of HE. One of the few studies using brains of patients with HE showed increased tyrosine-nitrated proteins and RNA oxidation compared to patients with liver disease without HE (Görg *et al.*, 2010). In addition, systemic (but not central) oxidative stress also has a vital role in the pathogenesis of brain edema, a central feature of HE. In animal models of HE, high systemic oxidative stress causes brain edema, which is reduced by antioxidant treatment (Bosoi *et al.*, 2012).

Inflammation

Inflammation is part of the immune response towards injury caused by infection, physical damage, or toxic cellular components. This response involves cells of the immune system (including macrophages, lymphocytes, and dendritic cells, among others) and their pro-inflammatory mediators, such as cytokines and chemokines. Inflammation is a physiological and

beneficial process used to protect the body against potentially harmful stimuli. However, once it becomes chronic and unregulated, it can be a source of detrimental processes.

Systemic inflammation is a common finding in liver disease patients (Haukeland *et al.*, 2006; Tilg and Moschen, 2010), and it is associated with hospital complications and worse prognosis (Cazzaniga *et al.*, 2009). Higher levels of inflammation are found in patients with both covert and overt HE. Systemic inflammation markers such as TNF-α correlate with both the presence and the severity of OHE assessed by the West Haven criteria (Odeh *et al.*, 2004). Also, the presence of systemic inflammatory response syndrome (SIRS), defined by increased temperature, heart and respiratory rate, white cell count, and blood glucose, correlates with the presence of overt HE in cirrhotic patients (Shawcross *et al.*, 2011). Besides SIRS, other inflammation markers such as systemic IL-6, neutrophil count, white cell count, and systemic C-reactive protein also correlate with the presence of CHE (Shawcross *et al.*, 2007).

All the information was on systemic inflammation. I have modified the text for clarification. In addition, a paragraph on neuroinflammation was added: "In addition, HE is associated with neuroinflammation. Although not present in all animal models of HE, neuroinflammation in CLD is marked by increased cytokine levels such as IL-6 and microglial activation (Dadsetan et al. 2016). In acute liver failure models of HE, neuroinflammation arises from activation of neuronal TGFβR2 signaling, and although protective mechanisms such as the activation of the Takeda G protein-coupled receptor 5 (TGR5) signaling lessens neuroinflammation, it still persists driving the neurological decline (McMillin et al. 2015; 2019).

Other factors are also involved in the pathogenesis of HE. Increased bile acids due to primary or secondary cholestasis, impaired lactate metabolism and altered neurotransmission are among those factors (Liere, Sandhu and DeMorrow, 2017).

Brain edema

An increase in brain water is often a part of HE's syndrome. Elevated BBB permeability (with or without physical BBB breakdown) can cause an osmotic shift and consequently lead to an influx

of water, resulting in brain edema. The types of brain edema are defined as vasogenic or cytotoxic.

Vasogenic edema

In vasogenic edema, the physical disruption of the BBB allows molecules that generally do not enter the brain to enter freely, changing the osmolarity within the brain and subsequently resulting in an accumulation of water. Vasogenic edema might occur in patients with CLD or ALF, resulting in large increases of brain volume when associated with type A HE. Since the skull is a rigid structure, it cannot accommodate a swollen brain. Therefore, intracranial pressure rises in 20% of the patients with ALF, from which 55% die due to brain stem herniation (Bernal et al., 2013).

Cytotoxic edema

Cytotoxic edema is present in patients with CLD. In minimal HE, brain edema has been considered "low grade," likely being cytotoxic since there is no increase in intracranial pressure (Häussinger, 2006; Kale *et al.*, 2006; Rai *et al.*, 2015). However, it is difficult to assess the BBB status in patients, and because of that, the presence of BBB breakdown in those patients is unknown. Moreover, cytotoxic edema is present in type C HE animal models of minimal HE, like the bile-duct ligated (BDL) rat, when increased brain water is present with an intact BBB (Bosoi *et al.*, 2012).

Cytotoxic edema is characterized by intracellular or interstitial swelling in response to osmolarity changes when the BBB is not physically impaired (no breakdown). Here, an increase in the passage of osmolytes such as ions, lactate, and glutamine, when crossing the BBB will change the osmotic pressure inside the brain (cells or interstitial fluid). This process will culminate in the water entrance and an increase in volume, characterizing cytotoxic edema.

Brain osmolytes

Glutamine is the most abundant plasmatic amino acid, and high glutamine is associated with cytotoxic brain edema. Glutamine metabolism is strongly related to ammonia clearance. Ammonia detoxification by the enzyme GS in astrocytes produces glutamine, potentially contributing to brain edema. Glutamine represents up to 15% of the pool of all brain osmolytes

in health (Pasantes-Morales and Cruz-Rangel, 2010). During liver disease, increased systemic and brain glutamine are found in both patients and animal models with ALF and CLD (Lavoie *et al.*, 1987; Laubenberger *et al.*, 1997; Desjardins *et al.*, 1999; Chatauret *et al.*, 2006; Fries *et al.*, 2014), contributing to the pathogenesis of brain edema.

Lactate is a product of anaerobic metabolism and a substrate used in multiple cell-to-cell shuttles (Gladden, 2004). In the brain, lactate participates in the astrocyte-neuron lactate shuttle (ANLS), by which astrocytes convert pyruvate into lactate that is sent to the neurons and used as a source of energy. In addition, lactate modulates neuronal activity by activating lactate receptors (Bergersen, 2015) and interaction with GABA and α-adrenergic receptors (de Castro Abrantes *et al.*, 2019). Lactate also acts as an osmolyte, and therefore, it plays a role in brain edema during CLD. In BDL rats, increased brain (but not systemic) lactate was associated with brain edema. Furthermore, when lactate production was blocked by treatment with dichloroacetate, a lactate lowering drug, brain water was normalized (Bosoi *et al.*, 2014). The liver metabolizes lactate from the muscle into glucose by the Cori's cycle, and patients with advanced cirrhosis have increased systemic (Sun *et al.*, 2018; Drolz *et al.*, 2019; Mahmud *et al.*, 2021) and cerebrospinal fluid (CSF) lactate (Yao *et al.*, 1989). Hyperlactatemia during liver disease arises from the loss of hepatic lactate metabolism caused by hepatic loss of function (Almenoff *et al.*, 1989) and increased lactate production by the hypoxic liver tissue (Rao *et al.*, 2008), problems that are known to affect CLD patients (Purnak and Yilmaz, 2013; Lee *et al.*, 2015).

Glutamate, myoinositol, and taurine are other osmolytes involved in brain edema. Glutamate, like glutamine, is increased during CLD (Bosoi *et al.*, 2014), providing extra substrate for glutamine formation through GS. However, other osmolytes such as myoinositol and taurine are decreased in the brain of animal models of CLD (Cordoba, Gottstein and Blei, 1996). Although the brain tries to balance the osmotic gradient to prevent or reduce brain edema by decreasing some osmolytes, this compensatory mechanism is not enough to maintain homeostasis and brain edema still occurs.

Glutamine synthetase

During liver disease, hepatocytes are replaced by scar tissue and the liver's detoxifying potential decreases. Hyperammonemia installs, and ammonia reaches the brain, provoking neurological complications. In the setting of liver disease, the enzyme GS arises as an important compensatory mechanism for ammonia detoxification, reaching up to 50% of the hepatic and all extra-hepatic detoxifying capacity (Hakvoort *et al.*, 2016).

Characteristics

GS is a glutamate-ammonia ligase present in animals, plants, and bacteria. The GS structure varies amongst eukaryotes and prokaryotes, and three isoforms are known to date (GS I, GS II, and GS III) (Llorca *et al.*, 2006; van Rooyen *et al.*, 2011). In mammals, GS II has a molecular weight of around 44 kDa and is the product of the GLUL gene (Wang *et al.*, 1996). GS is found in most mammalian organs, including the brain (Matthews *et al.*, 2010). The 373 amino acid protein has three domains, an N-terminal, a β-grasp domain, and a catalytic domain. Each subunit is linked and then stacked into a double ring, forming the active enzyme, octameric or decametric, in mammals (Boksha *et al.*, 2002; Krajewski *et al.*, 2008). The active sites that form the bifunnel have openings on either side to bind to GS's substrates, glutamate, and ammonia (Berlicki 2008).

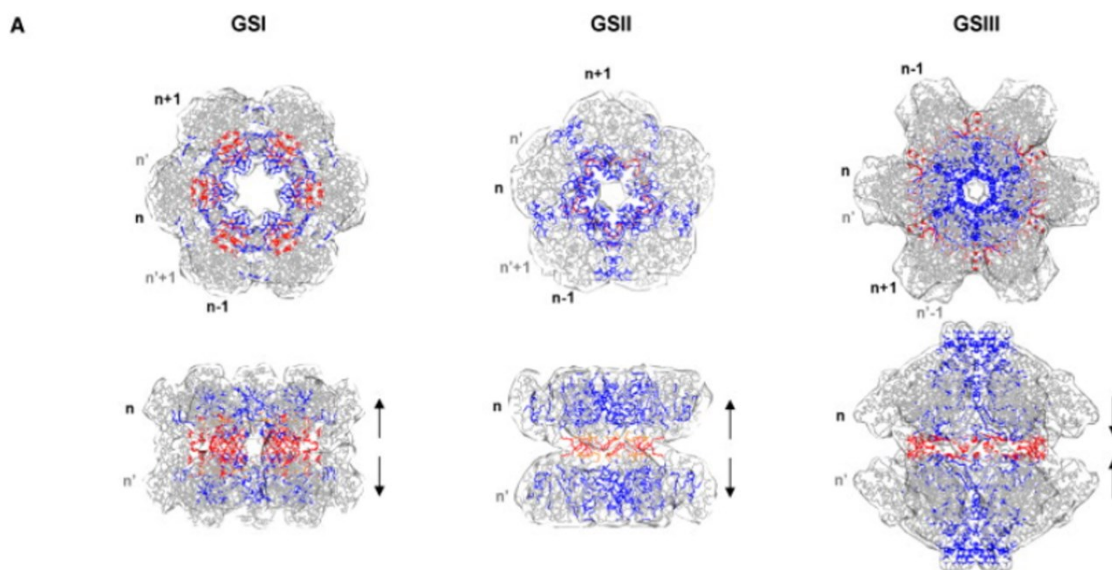

Figure 6. – Structure of glutamine synthetase type I (GSI), II (GSII), and III (GSIII)

Quaternary structures of GSI, GSII and GSIII. “n” labels the subunits to stress the contact points of the inner-ring (blue) and the outer-ring (red) of the GS structure. Black arrows indicate the orientation of the component rings (van Rooyen *et al.*, 2011)

Localization and function

GS’s primary role in health is the hepatic and extra-hepatic detoxification of metabolic nitrogen waste and glutamine formation. The detoxification of systemic ammonia occurs by a two-step reaction that is ATP dependent and uses divalent ions (Mg_2^+ or Mn_2^+) as co-factors, with Mg_2^+ being more efficient for glutamine biosynthesis (Wu, 1977). Besides its biosynthetic activity amidating glutamate into glutamine, GS can catalyze the conversion of glutamate + hydroxylamine into γ-glutamyl hydroxamate. Although the glutamyl transferase activity of GS does not occur biologically, it is the basis for several GS activity assays (Wellner and Meister, 1966; Calas *et al.*, 2008).

Besides GS expression in perivenous hepatocytes, GS expression has been found in kidney, in muscles (cardiac and skeletal), and CNS (exclusively in astrocytes) (Iqbal and Ottaway, 1970; Anlauf and Derouiche, 2013) and recently in endothelial cells of the retina (Eelen *et al.*, 2018). Selective knock-out (KO) of GS in healthy mice’s muscle impacts the whole body's ability to detoxify ammonia, suggesting that skeletal muscle can detoxify up to 10% of circulating ammonia levels (He, Theodorus B. M. Hakvoort, *et al.*, 2010). However, GS might have a more prominent role in ammonia detoxification during liver disease since it GS in muscle tissue is upregulated during liver failure ((Zhou *et al.*, 2020).

In the brain, GS is expressed in astrocytes, regulating the production of neurotransmitters via the glutamine-glutamate cycle (Anlauf and Derouiche, 2013) by limiting glutamate availability and inhibiting neurotransmission. During neurotransmission, glutamate is released on the synaptic cleft and is subsequently up-taken by astrocytic transporters. Glutamate is then converted into α-ketoglutarate to enter the TCA cycle, converted into glutamine by GS, or becomes a substrate

for synthesizing the inhibitory neurotransmitter γ-aminobutyric acid (GABA) (Schousboe, Bak and Waagepetersen, 2013). From the glutamine produced by the astrocytes, a portion is then transported to the neurons and converted back into glutamate by the enzyme glutaminase completing the glutamine-glutamate cycle (Cooper and Jeitner, 2016).

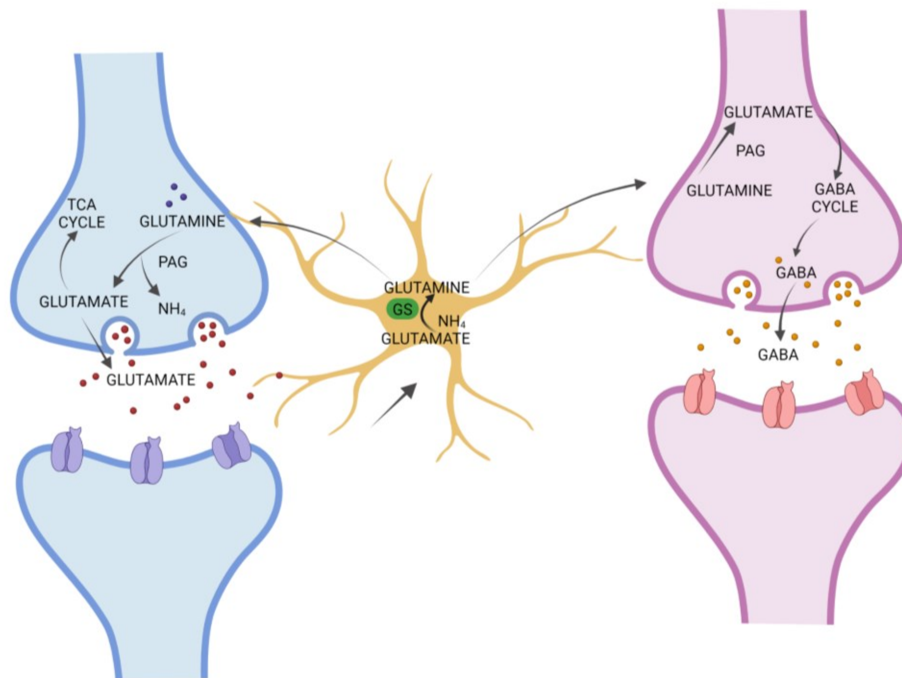

Figure 7. – The glutamine glutamate cycle

Animal models of HE

Type A HE

Animal models of type A HE are achieved by two processes: the administration of hepatotoxic substances or surgical manipulation.

Hepatotoxic models

Among the hepatotoxic models, rodents injected with galactosamine, thioacetamide, or azoxymethane are the most common. Although each toxin has a specific target, all of them in

high doses cause severe hepatic necrosis with subsequent brain impairments, including progressive loss of reflexes, brain edema, and hepatic coma (DeMorrow *et al.*, 2021). In addition, the administration of acetaminophen, a drug responsible for most ALF cases in humans, also causes type A HE in rodents with hepatic necrosis, increased systemic ammonia, oxidative stress, inflammation, and brain edema (DeMorrow *et al.*, 2021). However, this model is not frequently used due to its poor reproducibility.

Surgical models

Surgery excluding the liver from circulation by a combination of portal vein shunting and ligation of hepatic artery in rodents or pigs also leads to type A HE, culminating in a progressive rise in systemic ammonia levels, brain edema, and hepatic coma (DeMorrow *et al.*, 2021). Moreover, partial or total hepatectomy also causes type A HE, with increased ammonia and hepatic coma (Rahman and Hodgson, 2000).

Type B HE

Animals with partial or total portal-systemic shunting develop type B HE. The shunt might be created surgically, such as in the portocaval anastomosis (PCA) rat or a congenital malformation in dogs and mice (Lamb and White, 1998). In both cases, the shunt diverts the portal blood from the liver into the systemic circulation resulting in hyperammonemia and psychomotor dysfunctions without the underlying liver disease (DeMorrow *et al.*, 2021).

Type C HE

Models of CHE

Hepatotoxic models

When chronically administered in lower doses, thioacetamide constitutes a model of type C HE, presenting milder symptoms than its respective type A model, like decreased exploratory behavior, memory impairments and lack of attention (Hajipour *et al.*, 2021). Similarly, long-term administration of the hepatotoxin carbon tetrachloride causes liver injury, hyperammonemia,

neuroinflammation, and BBB breakdown, with impaired memory assessed by the Morris water maze test (Yang *et al.*, 2015).

Surgical models

The most used model of type C HE is the BDL rat, in which the ligature of the common bile duct produces progressive liver impairment with severe fibrosis after three weeks (Tarcin *et al.*, 2011). After 4-6 weeks, hyperammonemia is present with cognitive impairments assessed by behavioral tests. BDL rats have minimal HE characterized by impaired activity, motor coordination, and memory, associated with increased blood ammonia, systemic oxidative stress, and pro-inflammatory cytokines (Bosoi *et al.*, 2012; Dhanda *et al.*, 2018; Ochoa-Sanchez *et al.*, 2021). In addition, the BDL rat develops other complications of liver disease similar to humans, such as portal hypertension, ascites, jaundice, sarcopenia, and GI bleeding (DeMorrow *et al.*, 2021).

Models of OHE

Acute insults are administered to models of type B or C HE to produce an OHE episode and elucidate the pathogenic factors involved in HE. As the driver of many precipitating HE factors, ammonia is the most common toxin used (by injection or oral administration in the diet) to induce an OHE episode (DeMorrow *et al.*, 2021).

Ammonia-lowering strategies

Lactulose

Lactulose is a non-absorbable disaccharide and the standard of care treatment for HE. Lactulose acts by inhibiting ammonia production and absorption in the intestines. Upon oral intake, lactulose reaches the intestines and act as an osmotic laxative, increasing fecal mass movement and improving constipation. Because slow-moving fecal matter increases intestinal ammonia absorption, lactulose decreases ammonia levels. Lactulose can also prevent glutamine absorption from the fecal matter, decreasing its intestinal deamination by glutaminase from the intestines and reducing ammonia production and absorption. In addition to the laxative effect, once in the colon, lactulose is metabolized by colonic bacteria into lactic acid and acetic acid. In turn, these two acids will reduce the colonic pH and might suppress the growth of urease bacteria in the

intestines (Rose, 2012). Different studies support lactulose's role in reducing ammonia in clinical (Rahimi *et al.*, 2014; Naderian *et al.*, 2017) and pre-clinical (Kawai *et al.*, 2012) studies. However, compliance with treatment is a major drawback of lactulose therapy. The gastrointestinal effects (abdominal cramping, nausea, vomiting, flatulence, and abdominal distension), the sweet taste, and the need to titrate the dose (to achieve 2 or 3 semi-soft stools per day) are among the causes why patients do not stick to treatment with lactulose (Neff, 2010).

Rifaximin

Rifaximin is a broad-spectrum antibiotic that inhibits colonic bacteria, including urease bacteria, thereby reducing ammonia production. It acts by binding to bacterial RNA-polymerase and preventing bacterial RNA and protein synthesis. One of the significant advantages of rifaximin is that it is poorly absorbed in the gut, allowing for longer treatments and fewer side effects (Williams and Bass, 2005). Rifaximin modulates colonic bacteria (Bajaj *et al.*, 2013) and reduces hospitalizations duration and frequency. Moreover, many studies found rifaximin to be as good as lactulose in lowering ammonia (Bucci and Palmieri, 1993; Paik *et al.*, 2005; Bass *et al.*, 2010). Also, rifaximin is generally better tolerated than lactulose, although its cost still makes its prescription restrictive (Williams and Bass, 2005).

Ornithine Phenylacetate

Ornithine phenylacetate (OP) has a dual mechanism of action. First, ornithine is converted into glutamate by ornithine aminotransferase, supplying substrate for ammonia detoxification via glutamine formation by muscle GS. Secondly, phenylacetate binds to the produced glutamine forming phenylacetylglutamine which will be excreted by the kidneys, preventing the reconversion of glutamine into glutamate + ammonia by the enzyme glutaminase. In addition, phenylacetate also binds to glycine forming phenylacetylglycine, facilitating glycine's nitrogen excretion. OP is not clinically available in Canada since it has not been FDA approved. However, using the BDL rat as an animal model of cirrhosis with hyperammonemia, our laboratory demonstrated that OP reduces ammonia levels (Bosoi et al., 2017). OP lowering capacity was also demonstrated in other animal models of liver disease (Ytrebø et al., 2009) and patients (Ventura-Cots et al., 2016).

L-ornithine-L-aspartate

L-ornithine L-aspartate (LOLA) is an ammonia lowering strategy that aims to stimulate ammonia detoxification by GS and by the urea cycle in residual hepatocytes. LOLA provides glutamate as a substrate for muscle GS and aspartate for the urea cycle. Glutamate can be produced via ornithine aminotransferase and, the transamination of aspartate with α- ketoglutarate. Unfortunately, since glutamine can be reconverted into glutamate and release ammonia, there might be a rebound hyperammonemia after LOLA treatment (Rose, 2012). LOLA is also not licensed for use in Canada, but it has been studied in cirrhotic patients with TIPS and it effectively lowered ammonia levels (Bai *et al.*, 2014).

Sodium Benzoate

Sodium benzoate (SB) is an ammonia-lowering drug used primarily to treat hyperammonemia that arises from urea cycle disorders (UCD) (Batshaw, MacArthur and Tuchman, 2001). SB acts on a three-step process that first comprises the conjugation of benzoate with coenzyme A to form benzoyl CoA in the liver and kidney. Subsequently, the conjugation of benzoyl CoA with glycine forms hippuric acid in the liver and kidney. Finally, hippuric acid is excreted in the kidneys by glomerular filtration. The second step of the process is dependent on glycine concentration, and each molecule of hippuric acid removes one nitrogen from ammonia, which comes from glycine. There is evidence that SB lowers ammonia in CLD in pre-clinical (Campollo *et al.*, 1994) and clinical (Sushma *et al.*, 1992) studies. Even so, the use of SB in the context of CLD needs careful consideration since the sodium load is not advised for patients with fluid retention and kidney impairments, both common features present in CLD patients (Hartleb and Gutkowski, 2012).

Glycerol Phenylbutyrate

Glycerol phenylbutyrate (GP) is another drug used to decrease high ammonia levels from UCD, although not used in Canada. Once ingested, GP is digested by pancreatic lipases, and phenylbutyrate is released and converted into phenylacetate by β-oxidation in most organs. Like with OP, phenylacetate will bind to glutamine, forming phenylacetylglutamine, and will be excreted by the kidney. As it decreases glutamine levels, GP might also increase glutamine production by GS. Each GP molecule removes two molecules of ammonia, both from glutamine.

Treatments with GP reduce ammonia levels and the frequency of hyperammonemic episodes in children with UCD (Berry *et al.*, 2017). Phenylacetate also binds to glycine, a potential source of ammonia, leading to the formation and excretion of phenylacetylglycine and therefore preventing ammonia formation. GP was also tested in patients with CLD and was shown to reduce ammonia levels and protects against HE (Ghabril *et al.*, 2013; Rockey *et al.*, 2014).

Gender and sex bias in research

From the evolutionary point of view, sex differences exist because selection acts on the two sexes differently (Darwin, 1871). However, those differences are often disregarded in biomedical research, and most research so far has been conducted majorly with male subjects (Zajitschek *et al.*, 2020). Consequently, our knowledge becomes sex or gender-biased, creating a gap in knowledge that leads to misdiagnosis, overmedication, and adverse drug interactions in women (Zucker and Prendergast, 2020).

Clinical research

Ignoring the differences between males and females might come with severe consequences. Neurological conditions such as stroke and Attention-Deficit Hyperactivity Disorder (ADHD) are massively underdiagnosed in females since their symptoms are different from the standard male-based criteria (Hinshaw *et al.*, 2012). Disregarding sex differences results in public health problems that have serious consequences, such as permanent disability and deaths (Shansky and Murphy, 2021). Even when females are correctly diagnosed, they might be incorrectly treated since females are often underrepresented in drug development research (Anderson, 2008). A study showed that eight of the ten drugs removed from the market by the FDA from 1997 to 2000 held more significant risks for female patients. Lack of dose adjustments was one of the critical reasons for treatment removal. The slower metabolism in females for the drug zolpidem, used for insomnia, resulted in several motor vehicle crashes until the dose was adjusted by 50% (Carey *et al.*, 2017). When drugs based on single-sex studies are given to both sexes, there is a high risk of unseen adverse effects. That is caused by sex-specific differences in basal and drug metabolism and generally results in a higher incidence of side effects from treatments in women compared to men.

Pre-clinical research

Male rodents have been the standard model in pre-clinical research, an issue that likely contributed to the clinical problems women face with misdiagnosis and high adverse effects from medication. The knowledge gap on the pathogenesis of many diseases, including neurological diseases in women, is partly due to the lack of studies with female animals in preclinical research. The use of male mammals in research is up to 5x the use of females (Beery and Zucker, 2011). Although the inclusion of female patients in clinical research increased over the past 50 years, the same is not valid for female animals in pre-clinical studies (Beery and Zucker, 2011). Therefore, funding agencies like the Canadian Institutes of Health Research and the US National Institutes of Health (NIH) stipulated rules for including sex as a biological variable in 2016, aiming for the inclusion of males and females in research (Shansky and Murphy, 2021).

Hypothesis and aims

Hyperammonemia is central in the pathogenesis of HE as ammonia easily crosses the BBB causing neurotoxicity. Since the endothelial cells of the BBB are the interface between the blood and the brain, it is plausible that ammonia is metabolized in endothelial cells to help protect the brain against ammonia-induced neurotoxicity.

In addition, response to HE treatments is heterogenous and sex is among the factors that might dictate incidence and developments of CLD and HE. However, the impact of sex has not been explored in animal models of CLD and HE.

Therefore, our aims were:

1- To explore ammonia metabolism in endothelial cells of the BBB through the presence of GS.

2 - To assess the impact of sex on complications from CLD including sarcopenia, CHE and ammonia-induced OHE.

Article presentation

First article: Glutamine synthetase in endothelial cells of the blood-brain barrier

Glutamine synthetase in endothelial cells of the blood-brain barrier

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Authors contributions

MMO, MT and CFR contributed for the study's concept and design. MMO and OMF carried out the experiments. MMO, OMF, MT and CFR analysed the data. MMO, OMF, MT and CFR wrote the paper. CFR funded and supervised the study. All authors participated on the paper's revision and approval.

Abstract

Background: The catalytic reaction of glutamine synthetase (GS), the amidation of glutamate into glutamine, involves the removal of ammonia. Liver disease leads to hyperammonemia and causes deleterious effects to the brain. When hepatic ammonia detoxification is impaired, extra-hepatic GS plays an important compensatory role in detoxifying ammonia. The blood-brain barrier (BBB) is the interface between the blood and the brain, responsible for protecting the brain against harmful insults. With the endothelial cells of the BBB being the first line of defense for the brain against ammonia toxicity, our aim was to investigate and characterize GS in endothelial cells of the BBB.

Material and methods: We assessed GS protein expression (western blot and confocal immunofluorescence) and activity *in vitro* (primary rat brain microvascular endothelial cells), including cells treated with the GS inhibitor methionine sulfoximine (MSO) and *ex-vivo* (in isolated cerebral microvessels and brain slices of naïve rats). We also investigated the roles of different substrates in the stimulation of GS using 5-^{13}C ornithine, glutamate

and α-ketoglutarate, with 0, 0.5 and 1 mM of ammonium chloride for up to 24 hours and measuring 5-^{13}C glutamine production.

Results: The expression and activity of GS was found in endothelial cells from both *in vivo* and *in ex-vivo* systems, and was blocked by the MSO *in vitro*. Ornithine (compared to α-ketoglutarate and glutamate) was found to generate the highest amount of glutamine.

Conclusions: Our results demonstrate that GS is expressed in endothelial cells of the BBB possibly playing a role in attenuating or delaying ammonia entry into the brain. Our results also demonstrate that GS in the BBB can be a target to increase ammonia removal using ornithine supplementation.

Introduction

Ammonia metabolism is essential to maintain proper body function, with ammonia being generated or required in several biochemical reactions (Felipo and Butterworth 2002). Ammonia is primarily produced in the gut with the majority detoxified by the liver via the urea cycle in periportal hepatocytes and the enzyme glutamine synthetase (GS) in perivenous hepatocytes. GS removes ammonia through the amidation of glutamate into glutamine. GS is also expressed in extra hepatic tissues, and this high-affinity (but low-capacity) ammonia detoxifying reaction accounts for half of the hepatic and the majority of the extra hepatic ammonia detoxification during hyperammonemia (Hakvoort *et al.* 2016).

In conditions where the capacity to remove ammonia is significantly decreased such as liver disease or urea cycle disorders, GS from muscle plays important compensatory roles in detoxifying ammonia. However, hyperammonemia persists,

reaching the brain and leading to neurotoxicity and neurocognitive impairments (Rose *et al.* 2020). To treat hyperammonemia, ammonia scavenger therapies like ornithine phenylacetate (OP) or L-ornithine L-aspartate (LOLA) are used to stimulate GS-mediated ammonia detoxification.

In the brain, GS is exclusively present in astrocytes (Anlauf and Derouiche 2013) and plays a vital role in the neuron-astrocyte glutamate-glutamine cycle responsible for preventing excitotoxicity and replenishing the neuronal glutamate neurotransmitter pools (Schousboe *et al.* 2014). Upon blood-derived ammonia entering the brain, the astrocytes bear the brunt in helping clear ammonia in the brain. Although high brain ammonia increases GS activity (Cudalbu *et al.* 2012), central ammonia levels remain high, being accountable for brain edema (Albrecht and Norenberg 2006), mitochondrial dysfunction and alterations in energy metabolism in astrocytes (Drews *et al.* 2020; Jayakumar *et al.* 2012). Furthermore, removal of ammonia via GS in astrocytes concomitantly creates an ammonia gradient across the blood-brain barrier (BBB) and therefore does not sufficiently reduce or prevent high brain ammonia levels (Bosman *et al.* 1990).

The BBB is essential in controlling the brain microenvironment by physically, biochemically and enzymatically regulating the transport of molecules into the brain (Brownson *et al.* 1994; Ballabh *et al.* 2004). The BBB is composed of endothelial cells and adjacent pericytes, wrapped by astrocytic foot processes. Endothelial cells have a central role in the BBB by being the interface between the blood and the brain and therefore regulating the entrance of ions and molecules by their tight junctions and transporters (Wang *et al.* 2009; Liu *et al.* 2012).

Ammonia both as an ion (NH_4^+) and gas (NH_3), can cross cellular barriers via ammonia or potassium transporters (due its similar ionic properties) or by diffusion as a

gas. Knowing the sensitivity of the brain to ammonia's toxicity and how the BBB shields the brain from potentially harmful molecules, the role of the BBB in ammonia metabolism and subsequently protection against ammonia's neurotoxicity remains unknown.

Since the endothelial cells of the BBB are the first line of defense against ammonia neurotoxicity, our aim was to evaluate the metabolism of ammonia via the expression of GS in endothelial cells of the BBB.

Material and methods

Cerebral microvessels isolation

Cerebral microvessels (CMV) isolation of frontal cortex and cerebellum of 225-250g naïve Sprague-Dawley rats (Charles River, Montreal, CA) was done as previously described (Faropoulos *et al.* 2010). Briefly, after dissecting the brain regions, brain tissue was homogenized at 1300 rpm (Heidolph Instruments GmbH & Co., USA) in sucrose buffer (0.32 M sucrose, 3 mM HEPES, 1:500 protease inhibitor cocktail (PIC)) and centrifuged 4˚C twice at 1000 g for 10 min, 100 g for 30 sec, 100 g for 15 sec, thrice 200 g for 1 min, removing the supernatant and resuspending the pellet with sucrose buffer after each centrifugation. Microvessel isolation was confirmed by light microscopy after placing one drop of the final sample on a slide. Whole tissue from brain frontal cortex of naïve rats was used as a positive control for GS expression. All studies were approved by the Institutional Animal Care and Use Committee at the CRCHUM.

Cell culture

Rat primary microvascular endothelial cells

Rat primary brain microvessel endothelial cells from neonatal Sprague-Dawley rats were purchased from Cell Biologics (Cell Biologics, USA). Cells were kept at 37 °C in humidified 5% CO_2 atmosphere, in basal endothelial cell medium (Cell Biologics, USA) supplemented with 5% fetal bovine serum (FBS), endothelial growth factor, vascular endothelial growth factor, L-glutamine and antibiotics/antimycotic (Cell Biologics, USA). For protein extraction, cells were plated at 40000 cells per cm^2 to reach around 80 % confluence and allowed to attach to Petri dishes overnight. Then, cells were washed twice with warm PBS and lysed for further experiments.

Rat primary astrocytes

Rat primary astrocytes from pups were used as a positive control for GS expression. Briefly, after sterilizing the skin with 70 % ethanol, the cranium of 1-day old rat pups was opened with scissors and the brains exposed. The brain cortex was removed with a spatula and minced with scissors for two min. *Dulbecco's Modified Eagle Medium* ((DMEM), Sigma, USA) was added, and the mixture was then homogenized by passing through a 22 G needle in a 10 ml syringe, adding 2 ml of DMEM after each homogenization until 8 ml. The mixture was then centrifuged, vortexed and filtered using first an 80 µm mesh and subsequently a 10 µm mesh. Culture media was added (DMEM supplemented with 10 % horse serum + penicillin (5 mg/ml), streptomycin (5 mg/ml) and neomycin (10 mg/ml)) and astrocytes were plated in petri dishes. The cells from 11 rat pups were pooled and cultured together.

<u>GS protein expression by western blot</u>

Western blot assays were performed with rat brain tissue, isolated CMV, astrocytes and brain microvascular endothelial cells. Tissues and cells were lysed with RIPA buffer (trisaminomethane (Tris) 50 mM, sodium chloride 150 mM, ethylenediaminetetraacetic acid (EDTA) 1 mM, sodium dodecyl sulfate (SDS) 0.1% and PIC 1/500) and protein concentration was determined using the Bio-Rad DC protein assay kit (Bio-Rad Laboratories, USA). Twenty µg of protein lysates were mixed with Laemmli buffer, heated for 5 min at 100 ˚C and loaded into 9 % stain-free SDS gels (SDS polyacrylamide gels (resolving gel: 0.375 M Tris pH 8.8; 0.1 % SDS; 9 % acrylamide and 0.5 % trichloroethanol). Samples were separated by electrophoresis for 1 hour at 60 V and after for more 1.5 hours at 150 V. At the end of the electrophoresis, the gel was activated with UV light for 45 sec using the ChemiDoc imaging system (Bio-Rad Laboratories, USA) and proteins were transferred into nitrocellulose membranes by wet transfer overnight at 30 V. Following transfer, the membranes were imaged using ChemiDoc for total protein quantification and subsequently blocked with TBST-Milk (1 mM Tris; 10 mM NaCl; 0.5 % Tween-20; 5 % skimmed milk) for one hour at room temperature. Then, membranes were probed with 1/2000 GS primary antibody reference number 610518 (mouse anti-GS, BD Biosciences, USA), washed with TBST solution 5 times, probed with the secondary antibody reference number AB_10015289 (goat anti-mouse IgG coupled to horseradish peroxidase, Jackson ImmunoResearch, USA) for one hour at room temperature, washed with TBST solution for 5 times and exposed to an enhanced chemiluminescence substrate (Clarity Western ECL substrate, Bio-Rad Laboratories, USA) and imaged by ChemiDoc. Images were quantified with Image Lab 6.0.1 software (Bio-Rad Laboratories, USA) and results are expressed as a ratio of the GS band by the total protein lane.

In vitro GS immunofluorescence

Endothelial cells were plated on coverslips (10000 cells/cm^2) and allowed to attach overnight. Cells were than washed with ice-cold PBS followed by fixation with ice-cold methanol for 15 min. Cells were then washed three times with PBS, permeabilized for 15 min with PBS-0.5 %Triton and washed again three times with PBS. Samples were blocked with PBS-0.5 %Triton + 10 % donkey serum for 30 min at room temperature, washed 3 times in PBS and exposed to 1/200 rabbit anti-GS, reference number G2781 (Sigma-Aldrich) overnight at 4 ˚C. Cells were washed three times with PBS, probed with 1/200 rabbit anti-IgG coupled to Alexa 694 fluorophore, reference number 711-585-152 (Jackson ImmunoResearch) for 30 min in the dark at room temperature. After washes, cells were incubated with 4',6-diamidino-2- phenylindole (DAPI, 1 µg/ml) for 5 min, mounted with mounting medium (0.1 M Tris; 2.5 % 1,4-Diazabicyclo [2.2.2] octane; 9.6 % Polyvinyl alcohol; 24 % glycerol). Images were acquired with an Axio Imager M2 microscope (Carl Zeiss, Germany) using a 20 x objective. Pictures were taken using the software ZEN (Carl Zeiss, Germany), and analyzed using Fiji software (Schindelin et al., 2012).

Ex vivo GS confocal immunofluorescence

Immunostaining was carried out similarly to the procedure for *in vitro* immunofluorescence, with some modifications. First, formalin-perfused brains from two naïve Sprague-Dawley rats of ~200 g (Charles River, Canada) were enclosed in optimal cutting temperature (OCT) compound and frozen. Ten µm slices from the frontal cortex were cut and placed in glass slides. To prevent autofluorescence, brain slices were incubated with Sudan black stain (0.1 % in 70 % ethanol) for 20 min and washed 3 times

for 5 min in PBS-0.02 % Tween. After, slices were blocked with PBS-0.5 %Triton + 10 % donkey serum for 30 min at room temperature and probed with the following antibodies: 1/200 mouse anti-GS, reference number 610518 (BD Biosciences, USA) overnight at 4˚C, 1/300 rabbit anti-caveolin-1 (endothelial cell marker), reference number 3238 (Cell Signaling, USA) for 1 hour at room temperature and 1/500 chicken anti-GFAP, reference number AB4674 (Abcam, USA). After, washes, cells were incubated with 1/200 mouse anti-IgG alexa 488 fluorophore, reference number 715-545-150 (Jackson ImmunoResearch, USA), rabbit anti-IgG coupled to Cy3 fluorophore, reference number 711-165-152 (Jackson ImmunoResearch, USA), and chicken anti-IgG coupled to 647 fluorophore, reference number 703-605-155 (Jackson ImmunoResearch, USA) for 30 min in the dark at room temperature. After washes, cells were incubated with DAPI 1 µg/ml for 5 min, washed and mounted with ProLong Gold antifade mounting medium (Thermo Fisher Scientific, USA) and kept in -20°C until analyzed.

Confocal microscopy

Confocal images were acquired with a Leica TCS-SP5 inverted microscope using a HCX PL APO CS 63x/1.4 Oil UV objective with the Las-AF software. Excitation was performed using a 405nm diode laser for DAPI, a 488nm line of an Argon laser for Alexa-Fluor 488, a 561nm DPSS laser for Cy3, and a 633nm HeNe laser for Alexa-Fluor 647. Detection bandwidth was 415-478nm for DAPI using a PMT, 498-551nm for Alexa-Fluor 488 using a HyD under the Standard mode, 571-623nm for Cy3 using a HyD under the Standard mode, and 643-740nm for Alexa-Fluor 647 using a PMT. A sequential acquisition consisting of Cy3 in sequence 1, Alexa-Fluor 488 and Alexa-Fluor 647 in

sequence 2 and DAPI in sequence 3 was performed to avoid cross-excitations and cross-emissions between the different dyes. Images were acquired at 400Hz scan speed with a zoom factor 4 and final images are 12bits, 1024x1024 (axial pixel size of 60nm). Z-stacks were performed with a 0.29 µm step size for volume rendering.

Image analysis

Confocal images were analyzed using the Imaris software (Oxford Instrument, Full Spectrum, V9.5). A subtract background filter of 30 µm radius was applied on each channel before segmentation. For capillary detection, the Surface module was used on the Cy3 channel with a manual thresholding and a 0.7 µm grain size smoothing.

For GFAP$^+$ cells detection, the surface module was used on the channel with a manual thresholding and a 0.12 µm grain size smoothing. For GS detection, the Spot module was used on the Alexa-Fluor 488 channel with a manual thresholding, and an estimated diameter of 0.3 µm. Then, to obtain GS statistics associated with capillaries, we used the "Shorted Distance to Surface" filter in the spot module of Imaris to select GS$^+$ spots associated to capillary surfaces. The same technique was used for GS statistics associated with GFAP$^+$ surfaces. We finally filtered GS$^+$ spots associated to capillaries only with exclusion of GS$^+$ spots associated to GFAP$^+$ surfaces (giving the GS+Cav-GFAP statistics). Finally, we used the Cell module to generate statistics of GS localization importing the capillary surfaces as membrane, GS spots as vesicles and using the DAPI channel for nucleus detection.

Assessment of GS activity by labeled substrates

Cell treatment

Endothelial cells were plated on 6-well plates at 30000 cells/cm^2 and allowed to attach overnight. Cells were then exposed for 24, 48 and 72 hours to labeled substrates (Cambridge Isotope Laboratories, USA) glutamate ($^{13}C5$, ^{15}N; 22.5 μM), glutamine ($^{13}C5$ and 1,2 $^{13}C2$; 70 μM), α-ketoglutarate ($^{13}C5$; 50 μM) and ornithine ($^{13}C5$, $^{15}N2$; 0.17 μM). The concentration of labeled substrates was chosen to reach 5 % enrichment based on the basal concentrations. All groups had similar concentrations of total substrates (labeled + unlabeled). Cells were also exposed to ammonium chloride (0.5 and 1 mM) and, methionine sulfoximine ((MSO); 5 mM) added to the culture media. At the end of the incubation time, a sample of the cell media was collected and snap frozen and cells were washed twice with warm PBS and lysed with Tris-SDS buffer (Tris 62.5 mM pH7.5, SDS 0.3 %).

GS substrates assessment – tracers

Preparation of calibrators

Primary stocks of the labeled amino acids were prepared in the range from 677 to 3264 μM in ultrapure water:methanol (1:1). An 8 point calibration curve was prepared by dilution of the stocks as follows: L-glutamic acid-$^{13}C5$, ^{15}N, l-glutamine-$^{13}C5$, l-glutamine-$^{13}C2$ and alpha-ketoglutaric acid-$^{13}C5$ were prepared in the range: 100- 0.78 μM. For l-ornithine-$^{13}C5$,$^{15}N2$ the range was 1000-7.8 nM. The calibrator solutions were stored at -70 °C before analysis.

Sample preparation

An Eppendorf Reference 2 (1-20 µl) pipette (Germany) was used for precision pipetting. To 1.5 ml PP centrifuge tubes (Sarstedt, Germany) 20.0 µL standards, cell

lysates or culture media samples were added 20.0 µL of aqueous internal standard (l-ornithine-$^{13}C5$, 0.5 µM; alpha-ketoglutaric acid-D6, 20 µM; l-glutamine-^{15}N, 20 µM and l-glutamic acid-^{13}C, 20 µM) and 500 µl ice-cold acetonitrile:methanol (1:1). Samples were mixed at 3000 rpm for 10 seconds (VWR mixer mini vortex, VWR International) and centrifuged at 21380 × g for 5 min (Hettich Micro 200 centrifuge, Germany). A 100 µL aliquot of supernatant from the mixtures were transferred to LC-vials.

Quantification of labeled amino acids

Samples were analysed by LC-MS/MS using a Waters Acquity UPLC I-Class FTN system with an autosampler and a binary solvent delivery system (Waters, Milford, MA) interfaced to Waters Xevo TQ-XS benchtop tandem quadrupole mass spectrometer (Waters, Manchester, UK). The mass spectrometer was operated in positive electrospray ion mode (ES+) and spray voltage was set to 0.80 kV. The system was controlled by MassLynx version 4.2 software. Desolvation gas temperature was 550 °C; source temperature was 150 °C; desolvation gas flow was 1000 L/h; cone gas flow was 150 L/h; collision gas pressure was 4 x 10-3 mBar (argon) and the ion energies were 0.4 V for both quadrupoles. Chromatographic separations were performed on a 2.1 x 100 mm Waters Acquity Premier BEH Amide column, 1.7 µm 2.1 mm x 100 mm maintained at 20.0 °C. The injection volume was set to 1.0 µL. Eluent A consisted of 0.05% formic acid in water; eluent B consisted of 0.05% acetic acid in acetonitrile. Gradient elution was performed with 1% A at start with hold for 0.5 min followed by a linear increase to 39% A until 5 min, a linear increase to 50% A until 5.5 min, hold at 50% A until 5.8 min, and re-equilibration from 5.81min to 7 min. The flow rate was 0.44 mL/min at start of run and 0.3 ml at end.

MRM transitions

For quantitative analysis the following MRM transitions were used (bold transitions are qualifiers): L-glutamic acid-$^{13}C5$,^{15}N: m/z 154 > 136/89/107, L-glutamine-$^{13}C5$: m/z 152 > 135/88, L-glutamine-$^{13}C2$: m/z 149 > 132/85, alpha-ketoglutaric acid-$^{13}C5$: m/z 150 > 60/105, L-ornithine-$^{13}C5$,$^{15}N2$: m/z 140 > 75/122 were monitored respectively

L-Glutamic acid and L-glutamine were chromatographically separated and method for all analytes were found to be linear (r2 > 0.99) in the range of the calibrators. Between-day coefficient of variation for labeled amino acids were < 12 % on three consecutive days.

Statistics

Data are expressed as mean ± standard deviation (SD) or percentages, and *p*-values < 0.05 were considered statistically significant. Statistical significance was tested using student t-test for one continuous variable comparing two groups, one-way ANOVA with Dunnett's multiple comparisons test for one continuous variable comparing three groups and the two-way ANOVA with Tukey's multiple comparisons test post-hoc for comparison between 2 variables. Statistical analysis was done using GraphPad Prism 8 (La Jolla, USA).

Results

GS expression in vitro

In primary cultured brain microvascular endothelial cells, the expression of GS protein was detected as shown by western blot (Fig. 8A). Compared to primary cultured

astrocytes, expression of GS in rat brain microvascular endothelial cells was significantly lower (0.22 ± 0.04 band intensity vs. 1.08 ± 0.33 band intensity respectively; $p < 0.05$). Activity of GS was found in cultured endothelial cells (Fig. 8B) which was significantly lower compared to cultured astrocytes (0.7 ± 0.2 mM of γ-glutamylhydroxamate by μg of protein vs. 2.0 ± 0.2 mM of γ-glutamylhydroxamate by μg of protein respectively; $p < 0.05$). In order to confirm the activity, we inhibited GS activity with MSO which resulted in a significant decrease of glutamine vs controls both intracellularly (2.18 ± 0.13 mM of glutamine for control vs. 0.86 ± 0.02 mM of glutamine for MSO treated, respectively; $p < 0.001$) (Fig. 8C) and extracellularly (65.10 ± 5.95 mM of glutamine for control vs. 49.84 ± 3.79 mM of glutamine for MSO treated, respectively; $p < 0.05$) (Fig. 8D). In addition, we demonstrated the expression of GS with immunofluorescence in cultured endothelial cells of the BBB (Fig. 8E).

GS expression ex vivo

GS protein expression was found in isolated CMV from the frontal cortex of naïve rats. Similar levels of GS protein expression were found in the frontal cortex tissue (Fig. 9A). Similarly, GS activity was not different in CMV's vs whole tissue from frontal cortex (Fig. 9B). Comparing isolated CMV's from cerebral cortex and cerebellum, GS expression and activity were not significantly different (Fig. 9C-D).

Confocal microscopy of brain slices from naïve rats showed GS expression in both endothelial cells (Caveolin-1) and astrocytes (GFAP) (Fig. 10A). 3D reconstruction revealed GS colocalization with the endothelial cell marker caveolin-1 (green spots) after exclusion of astrocytic GS (purple spots) (Fig. 10B). Quantification of GS fluorescence indicated that endothelial cells show lower GS intensity (862 ± 287) compared to

astrocytes (998 ± 388) ($p < 0.001$) (Fig. 10C). Furthermore, the cellular compartmentalization of GS in endothelial cells revealed GS to be localized primarily in the cytoplasm (90%) with a small fraction of GS (10%) being membrane bound ($p<0.001$)(Fig. 10D).

Assessment of GS's substrates efficiency

We assessed the total, intracellular and extracellular concentration amount of 5-^{13}C glutamine formed by each of GS's substrates.

Total labeled glutamine

Cells treated with 1mM ammonium chloride

Total labeled glutamine levels in cultured primary brain endothelial cells treated with 1 mM ammonium chloride were higher at all time points (1, 8, 16, 24 hours) following incubation with labeled ornithine and labeled glutamate compared to labeled α-ketoglutarate: $p<0.05$ for 1 hour and $p<0.001$ for 8, 16 and 24 hours (Table 2).

Cells treated with 0.5 mM ammonium chloride

Ornithine and glutamate lead to a higher production of glutamine in primary endothelial cells treated with 0.5 mM ammonium chloride compared to α-ketoglutarate at all time points: $p<0.05$ for ornithine and $p<0.01$ for glutamate for 1 hour and $p<0.001$ for both at 8, 16 and 24 hours (Table 2).

Untreated cells

Ornithine and glutamate generated higher glutamine levels in untreated cells compared to α-ketoglutarate: $p<0.05$ for ornithine and $p<0.01$ for glutamate for 1 hour and $p<0.001$ for both at 8, 16 and 24 hours, and higher labeled glutamine in cells incubated with ornithine vs. glutamate: $p<0.05$ at 24 hours (Table 2).

Intracellular labeled glutamine

Cells treated with 1 mM ammonium chloride

Intracellular labeled glutamine levels in cells treated with 1 mM ammonium chloride for 16 and 24 hours were higher in cells incubated with labeled ornithine compared to α-ketoglutarate: $p<0.01$. Also, labeled glutamine was higher in cells incubated with labeled ornithine vs. labeled glutamate at 16 hours: $p<0.05$ (Table 2).

Cells treated with 0.5 mM ammonium chloride

Intracellular labeled glutamine in cells treated with 0.5 mM ammonium chloride for 16 and 24 hours were higher in cells incubated with labeled ornithine compared to α-ketoglutarate: $p<0.05$ for 16 hours and $p<0.01$ for 24 hours (Table 2).

Untreated cells

In untreated cells, intracellular labeled glutamine levels at 16 and 24 hours were higher in cells incubated with labeled ornithine compared to α-ketoglutarate: $p<0.01$ for 16 hours and $p<0.001$ for 24 hours. Also, labeled glutamine was higher in cells incubated with labeled ornithine vs. labeled glutamate at 24 hours: $p<0.05$ (Table 2).

Extracellular labeled glutamine

Cells treated with 1 mM ammonium chloride

Extracellular labeled glutamine in cells treated with 1 mM ammonium chloride, after all time points was higher in cells incubated with labeled glutamate compared to α-ketoglutarate: $p<0.05$ for 1 hour and $p<0.001$ for 8, 16 and 24 hours, and were higher in cells incubated with labeled ornithine compared to α-ketoglutarate: $p<0.001$ for 8, 16 and 24 hours. In addition, cells incubated with labeled ornithine had higher labeled glutamine levels vs. cells incubated with labeled glutamate at 24 hours: $p<0.05$ (Table 2).

Cells treated with 0.5 mM ammonium chloride

Extracellular labeled glutamine levels in cells treated with 0.5 mM ammonium chloride, after all time points were higher in cells incubated with labeled ornithine or labeled glutamate compared to α-ketoglutarate: $p<0.05$ for 1 hour and $p<0.001$ for 8, 16 and 24 hours (Table 2).

Untreated cells

Untreated cells had higher total glutamine levels when incubated with labeled ornithine or labeled glutamate compared to α-ketoglutarate: $p<0.05$ for ornithine and $p<0.01$ for glutamate for 1 hour and $p<0.001$ for both at 8, 16 and 24 hours (Table 2).

Discussion

We demonstrated for the first time that GS is present in endothelial cells of the BBB, via protein expression, activity and cellular localization, using both *in vitro* and *ex vivo* preparations. In addition, GS activity was confirmed by inhibition of glutamine

production with MSO. From all potential GS substrates (ornithine, glutamate and α-ketoglutarate), ornithine demonstrated to be the most efficient in producing glutamine.

We characterized GS in endothelial cells of the BBB by evaluating GS protein expression and activity in cultured brain microvascular endothelial cells. GS in endothelial cells expressed protein levels and activity, although lower compared to astrocytes. The presence of GS in the BBB endothelial cells might not have been investigated so far due to its lower expression compared to astrocytes. In agreement, quantification of the 3D reconstruction in *ex vivo* confocal imaging confirmed lower expression of GS in endothelial cells of the BBB vs astrocytes in naïve rats. However, GS expression in isolated CMV was not different from whole brain tissue. Astrocytes represent only 20% of the brain, with other cells like microglia, neurons and oligodendrocytes accounting for the rest of the brain cells (Pelvig *et al.* 2008). Therefore, isolation of CMV concentrates GS expression per μg of protein while whole brain preparation dilutes GS by μg of protein, resulting in no differences between the two.

The BBB is the interface between the blood and the brain and the endothelial cells form the first barrier against blood-derived ammonia-induced CNS toxicity. The brain is susceptible to the deleterious effects of ammonia such as mitochondrial damage and astrocyte swelling. Ammonia both as an ion and gas, can cross cellular barriers via ammonia or potassium transporters or by diffusion as a gas. Ammonia is a natural endogenous toxin and therefore defence mechanisms should be in place to help protect the brain from its neurotoxicity.

To assess if GS was differentially regulated in CMVs in different brain areas, we compared GS protein expression and activity from cortical vs. cerebellar CMVs by western

blot, but we did not observe any differences. Studies found that GS is localized closely to glutamate transporters *in vitro* (Derouiche and Rauen 1995) and that GS expression by immunohistochemistry is upregulated in brain areas close to glutamatergic neurons (in the CA1 and CA3 areas of the hippocampus and molecular layer of the cerebellum) during hyperammonemia, protecting the nearby neurons against glutamate-induced neurotoxicity (Suárez *et al.* 1997). However, those studies evaluated GS expression in astrocytes and in hyperammonemic models, while we assessed GS in isolated CMV of naïve rats. Since CMV isolation yield a restricted amount of protein, it was not possible to assess the presence of GS in specific regions of the cerebellum nor in the hippocampus, due to its small size.

Although glutamate is the substrate for GS, other molecules such as α-ketoglutarate and ornithine have the potential to metabolize to glutamate. Alpha-ketoglutarate can deaminate to glutamate by glutamate dehydrogenase (GDH) while ornithine generates glutamate via ornithine aminotransferase (OAT) (Ventura *et al.* 2009).

We incubated endothelial cells for 1, 8, 16 and 24 hours with 5-^{13}C glutamate, ornithine and alpha-ketoglutarate. We chose these time points since incubation for shorter periods could restrict the potential reconversion of glutamine into other molecules, since the presence of glutaminase, which performs glutaminolysis, in endothelial cells of the BBB has been shown in bovine endothelial cells (Lee *et al.* 1998). Meanwhile, longer time-points such as 24 hours would allow for higher conversion of substrates into glutamine and possibly to upregulation of GS activity in cells treated with ammonia. We exposed endothelial cells to 0.5 and 1 mM of ammonia, similar concentrations to other studies evaluating the role of ammonia in endothelial cells (Bartolić *et al.* 2016). High ammonia levels have been shown to upregulate GS expression in astrocytes (Suárez *et al.* 1997).

However, we did not see an increase in the production of glutamine 5-^{13}C using labeled substrates in cultured endothelial cells.

Endothelial cells of the BBB regulate the entrance of glutamate via facilitative carriers and excitatory amino acid transporters (EAAT) (Sánchez del Pino *et al.* 1995). EAATs are expressed mainly on the abluminal side of the BBB while, facilitative carriers are expressed only on the luminal side (Sánchez del Pino *et al.* 1995). Considering that the concentration of glutamate is higher in the brain vs. plasma (10,000–12,000 μmol/L vs. 50–100 μmol/L respectively), and EAATs are exclusively expressed on the abluminal side of the BBB, glutamate rarely crosses the BBB from the blood into the brain (Hawkins 2009). Therefore, glutamate may not be the optimal substrate to stimulate GS in endothelial cells of the BBB.

Ornithine can enter endothelial cells of the BBB via cationic amino acid transporters (O'Kane *et al.* 2006), being converted into glutamate by OAT (Ventura *et al.* 2009) and supplying the substrate for GS-mediated ammonia detoxification. Cationic amino acid transporters are expressed at both, the luminal and the abluminal sides of the BBB (O'Kane *et al.* 2006), allowing ornithine to flow from the blood and to the BBB. In addition, ornithine transport from the blood and into the brain increases in rats with liver disease accompanied by hyperammonemia (Albrecht *et al.* 1994), providing evidence that ornithine supplementation would cross the BBB to act as a substrate for GS-mediated ammonia detoxification. Increasing GS activity during hyperammonemia with ornithine would help to prevent spillover of ammonia into the brain.

The reaction catalyzed by OAT favors glutamate production (Ventura *et al.* 2009), while GDH reaction normally favors α-ketoglutarate formation, due to the enzyme's affinity for ammonia (Ginguay *et al.* 2017; Plaitakis *et al.* 2017). However, 1 mM of ammonia has

been shown to stimulate glutamate synthesis by GDH in brains from mice (Voss *et al.* 2021). Even so, although endothelial cells consumed α-ketoglutarate over time (Supp. Data), α-ketoglutarate was not directed to glutamine production. It is possible that the ammonia levels were not enough to induce glutamate formation from α-ketoglutarate in an *in vitro* setting. In fact, far higher concentrations of ammonia can be used in astrocytes (Bartolić *et al.* 2016; Jayakumar *et al.* 2008). However, endothelial cells exposed to ammonia concentrations higher than 1 mM have reduced cell viability (data not shown). Without forming glutamate, alpha-ketoglutarate might be used for other cellular processes such as to provide energy by fueling the TCA cycle instead of producing glutamate to fuel GS. It is known that exposure to ammonia causes cataplerosis of the TCA cycle and impaired energetic metabolism (Davuluri *et al.* 2016). In agreement, treatment with 1 mM of ammonia reduced labeled glutamine formation from alpha-ketoglutarate vs. control (data not shown), supporting the role of alpha-ketoglutarate in energy maintenance.

Most differences in glutamine production between ornithine, glutamate and alpha-ketoglutarate were found in total or extracellular glutamine levels. Glutamine efflux from the endothelial cells is supported by the expression of glutamine transporters (Lee *et al.* 1998). Likely, most of the labeled glutamine generated by GS exits the cells though those transporters, resulting in high extracellular labeled glutamine. In addition, natural isotopes in the cell's media contribute to the higher ^{13}C extracellular and total glutamine levels. *In vivo*, glutamine formed by GS would exit the endothelial cells reaching the blood (Lee *et al.* 1998).

Ammonia lowering strategies such as ornithine-phenylacetate (OP) and L-ornithine L-aspartate (LOLA) focus on ammonia clearance by muscle GS. Treatments such as LOLA and OP rely on ornithine as the best option to stimulating GS-mediated ammonia

detoxification, since it's believed that ornithine enters the muscle easily (Jover-Cobos *et al.* 2013; Rose *et al.* 1999). Moreover, ornithine is considered a safer approach compared to glutamate, which might cause excitotoxicity (Montana *et al.* 2014). Agreeing with that, our results point to ornithine being the best substrate for GS in endothelial cells of the BBB. Since LOLA upregulates GS activity in muscle of hyperammonemia rats with acute liver failure (Rose *et al.* 1999) and together with OP protecting against neurological complications in animal models and patients with hyperammonemia, it is tempting to speculate that BBB's GS might also play a protecting role by increasing glutamine production and subsequent ammonia detoxification.

In conclusion, GS is expressed in endothelial cells of the BBB possibly playing a role in attenuating or delaying ammonia entry into the brain since ammonia stimulates an increase in GS activity. Ornithine supplementation has the potential to increase GS activity by providing substrate for ammonia detoxification.

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Tables

Table 2. Assessment of GS's substrates efficiency

Total labeled glutamine

Time	1 mM of ammonium chloride			
	α-ketoglutarate	Glutamate	Ornithine	p value
1h	5290.7 ± 107.2	6177.2 ± 760.4	6017.2 ± 266.9	*#p<0.05
8h	2773.7 ± 377.5	6242.5 ± 461.1	5893.4 ± 403.2	*#p<0.001
16h	1662.2 ± 196.8	5468.6 ± 226.8	5117.1 ±49.2	*#p<0.001
24h	1447.4 ± 194.9	4942.7 ± 223.2	5912.3 ± 199.4	*#p<0.001
	0.5 mM of ammonium chloride			
	α-ketoglutarate	Glutamate	Ornithine	
1h	4985.6 ± 186.7	6196.5 ± 239.3	6134.5 ± 690.2	*p<0.05, #p<0.01
8h	2712.1 ± 315.1	6216.4 ± 115.4	6030.9 ± 1093.9	*#p<0.001
16h	1709.1 ± 188.9	5369.3 ± 255.1	5229.5 ± 250.3	*#p<0.001
24h	1422.1 ± 189.5	4445.0 ± 508.3	4913.0 ± 307.2	*#p<0.001
	Untreated			
	α-ketoglutarate	Glutamate	Ornithine	
1h	5109.6 ± 313.5	6275.3 ± 741.0	6056.4 ± 375.0	*p<0.05, #p<0.01
8h	2801.6 ± 441.9	6340.2 ± 216.0	5677.0 ± 419.6	*#p<0.001
16h	1645.4 ± 109.2	5661.0 ± 242.7	5259.7 ± 432.7	*#p<0.001
24h	1446.4 ± 217.8	5035.3 ± 307.5	5911.4 ± 302.2	*#p<0.001, $p<0.05

	Intracellular labeled glutamine			
	1 mM of ammonium chloride			
	α-ketoglutarate	Glutamate	Ornithine	
1h	98.6 ± 3.5	110.6 ± 32.9	151.7 ± 71.1	
8h	116.1 ±30.5	99.4 ± 19.3	125.1 ± 55.5	
16h	99.7 ± 9.3	99.7 mM ± 9.3	247.3 ± 78.7	$p<0.05, *p<0.01
24h	97.9 ± 12.5	97.9 mM ± 12.5	246.0 mM ± 100.3	*p<0.01
	0.5 mM of ammonium chloride			
	α-ketoglutarate	Glutamate	Ornithine	
1h	151.1 ± 15.5	149.3 ± 29.5	224.2 ± 112.1	
8h	152.0 ± 38.8	137.7 ± 12.5	183.5 ± 77.3	
16h	121.8 ± 16.7	165.3 ± 8.3	256.7 ± 82.9	*p<0.05
24h	123.0 ± 5.4	169.8 ± 11.2	266.0 ± 56.8	*p<0.01
	Untreated			
	α-ketoglutarate	Glutamate	Ornithine	
1h	189.3 ± 13.1	167.6 ± 13.1	221.6 ± 62.9	
8h	185.8 ± 32.1	172.8 ± 18.9	198.0 ± 34.4	
16h	137.1 ± 25.2	183.6 ± 11.7	243.9 ± 30.9	*p<0.01
24h	116.0 ± 100.1	168.7 ± 33.5	263.8 ± 100.1	*p<0.001, $p<0.05
	Extracellular labeled glutamine			
	1 mM of ammonium chloride			
	α-ketoglutarate	Glutamate	Ornithine	p value
1h	5192.1 ± 110.4	6066.5 ± 731.8	5865.5 ± 331.7	#p<0.05
8h	2657.6 ± 405.3	6143.1 ± 462.2	5768.3 ± 454.4	*#p<0.001
16h	1562.5 ± 202.8	5339.0 ± 211.8	4869.8 ± 36.9	*#p<0.001
24h	1349.5 ± 201.2	4781.4 ± 225.7	5666.3 ± 291.2	*#p<0.001, $p<0.05
	0.5 mM of ammonium chloride			
	α-ketoglutarate	Glutamate	Ornithine	
1h	4834.4 ± 201.2	6047.2 ± 212.0	5910.3 ± 798.4	*#p<0.05
8h	1560.1 ± 347.4	6078.7 ± 125.4	5847.4 ± 1170.3	*#p<0.001
16h	1587.3 ± 196.8	5203.9 ± 257.7	4972.8 ± 223.5	*#p<0.001
24h	1299.1 ± 189.3	4275.3 ± 515.5	4647.0 ± 360.0	*#p<0.001
	Untreated			
	α-ketoglutarate	Glutamate	Ornithine	
1h	4920.3 ± 303.0	6107.8 ± 735.5	5834.8 ± 413.7	*p<0.05, #p<0.01
8h	2615. 8 ± 473.2	6167.5 ± 201.0	5479.0 ± 433.8	*#p<0.001
16h	1508.3 ± 133.0	5477.4 ± 248.0	5015.8 ± 407.9	*#p<0.001
24h	1330.3 ± 211.6	4866.7 ± 287.2	5647.5 ± 385.7	*#p<0.001

* α-ketoglutarate vs. ornithine, # α-ketoglutarate vs. glutamate, $ glutamate vs. ornithine. N = 3 independent experiments. Two-way ANOVA, numbers expressed as means ± SD, significance reached when p<0.05.

Figures

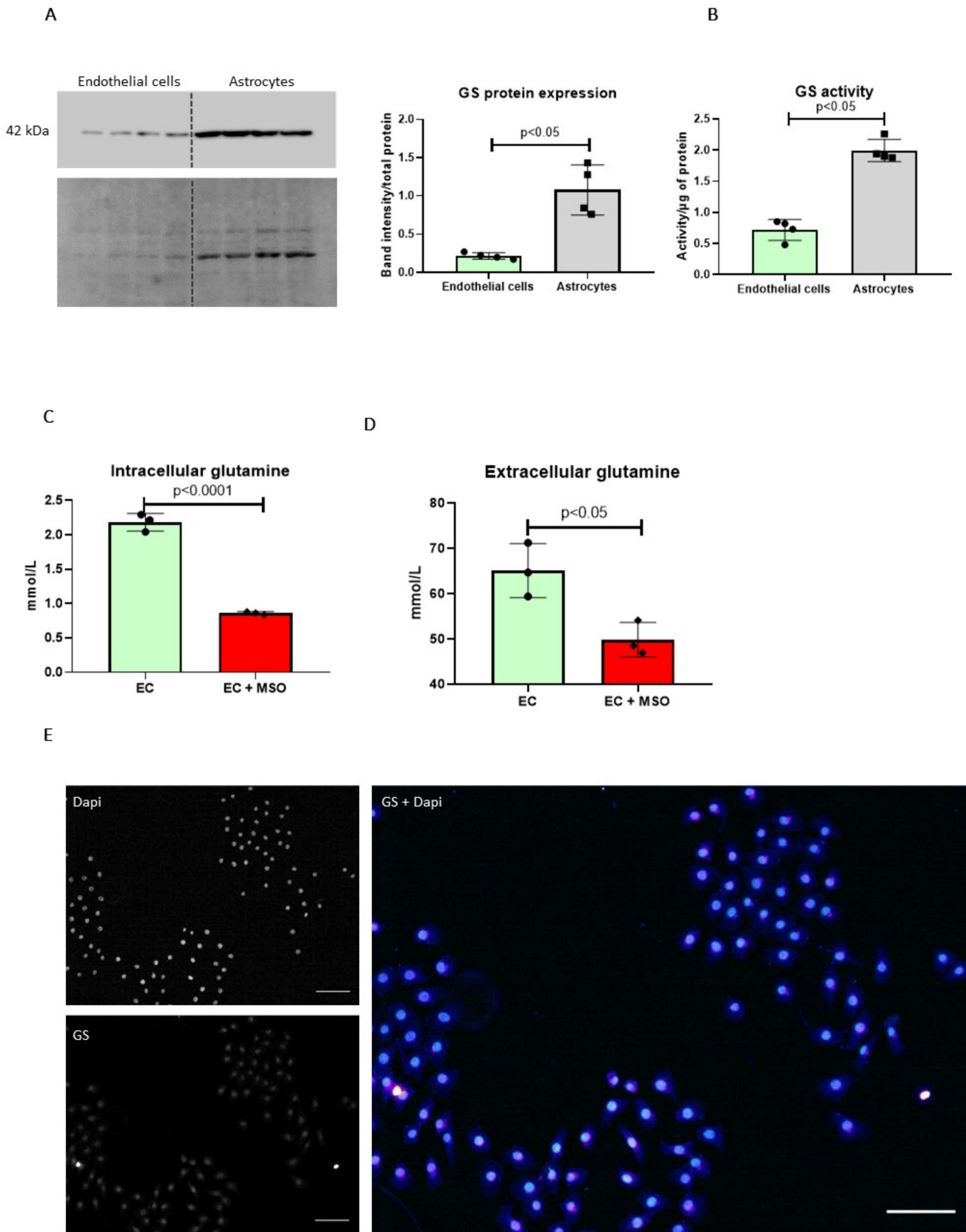


Fig. 8. GS expression on primary rat brain microvascular endothelial cells. Endothelial cells of the BBB had protein expression of GS (Fig. 8A) and GS activity (Fig.

8B), although both, protein levels and activity were lower compared to astrocytes. Intracellular (Fig. 8C) and extracellular (Fig. 8D) labeled glutamine was decreased in endothelial cells treated with MSO. GS expression was also shown by immunofluorescence (Fig. 8D) in cultured endothelial cells of the BBB. Scale 10 μM. N = 3-4 independent experiments per group. Student t-test, numbers expressed as means ± SD, significance reached when $p<0.05$.

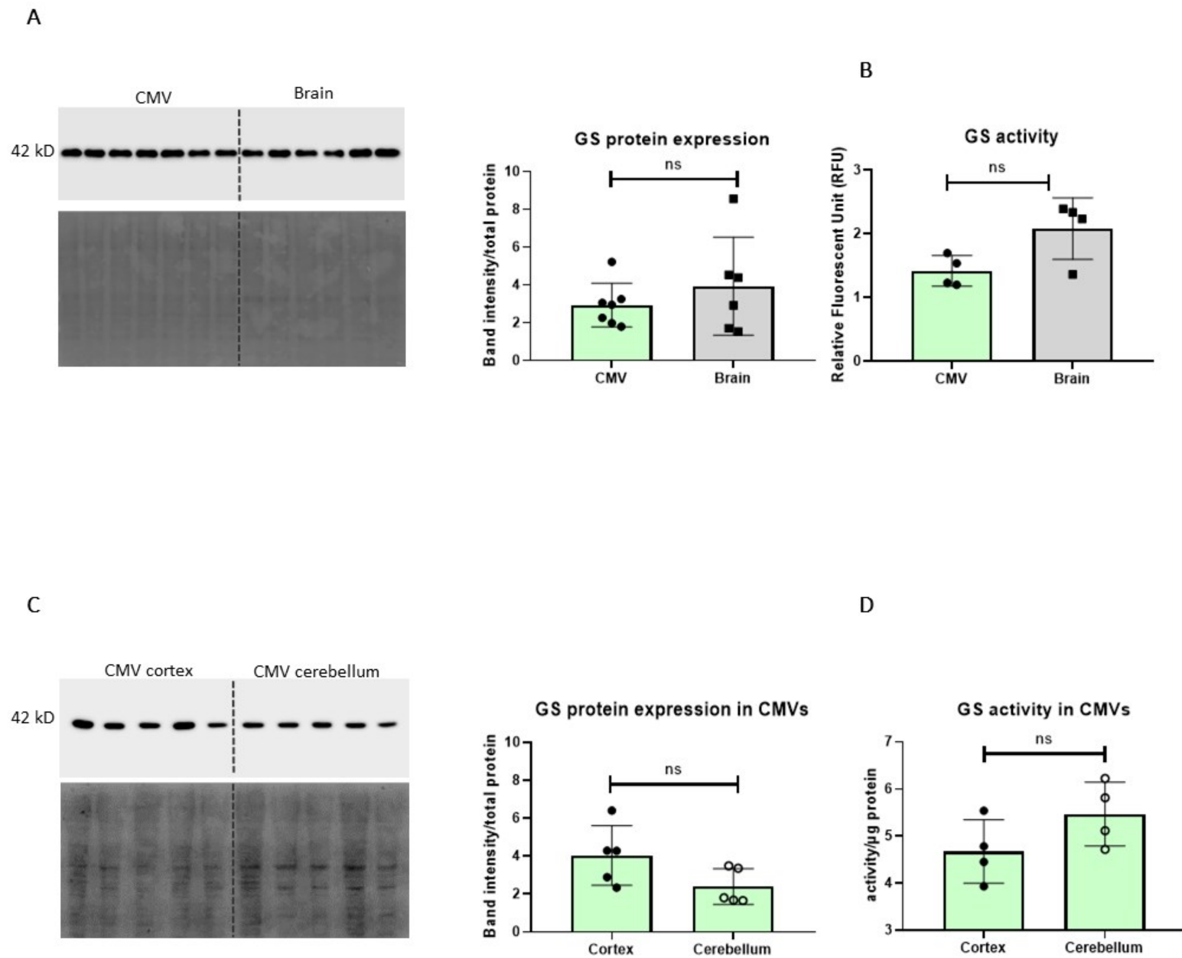
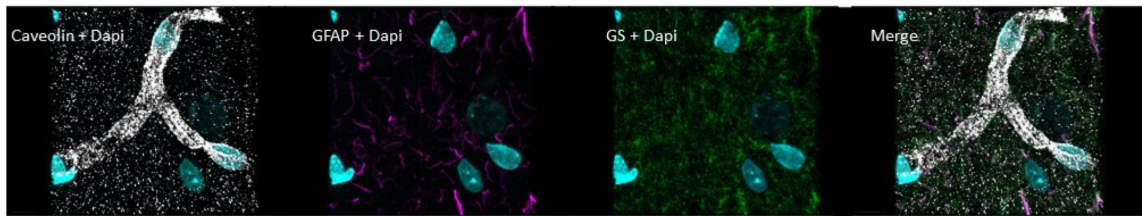


Fig. 9. GS expression in *ex vivo* endothelial cells of the BBB. GS protein expression (Fig. 9A) as well as GS activity (Fig. 9B) is present in isolated cerebral microvessels (CMV) from naïve rats in similar levels as brain tissue. There is no difference between GS expression (Fig 9C) as well as GS activity (Fig. 9D) in CMV of brain cortex vs. cerebellum. N= 5-7 rats per group. Student t-test, numbers expressed as means ± SD, significance reached when $p<0.05$.

A



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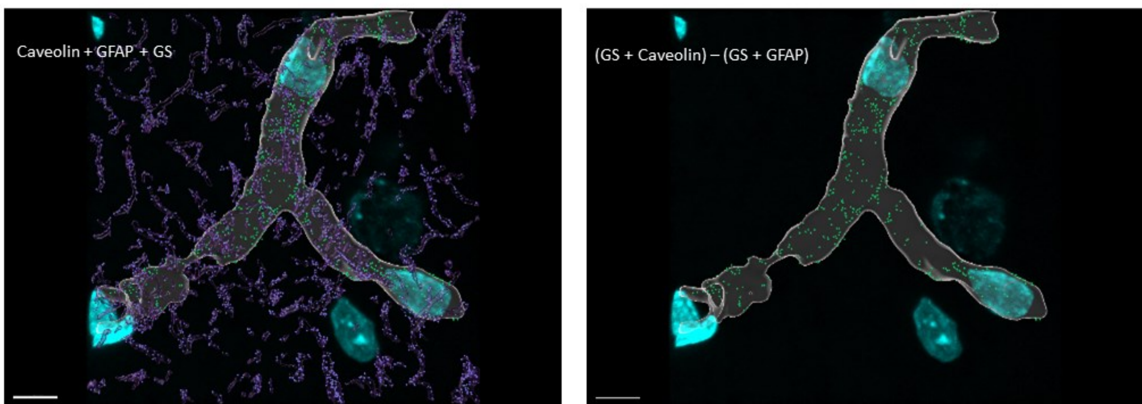

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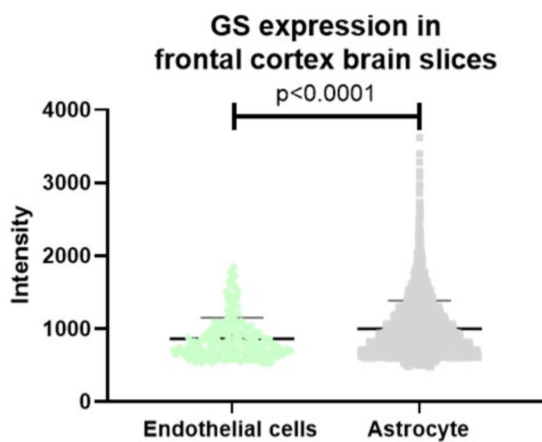

D

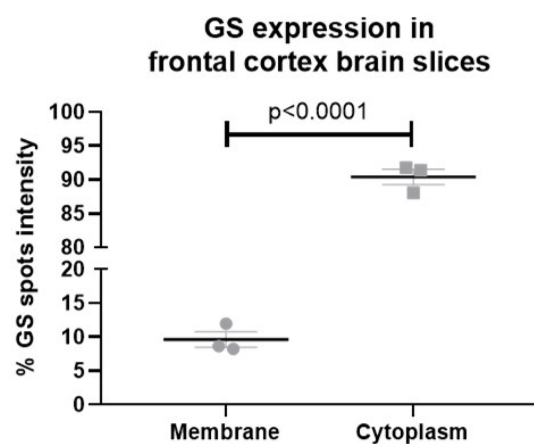

Fig. 10. *Ex vivo* localization of GS in the BBB. Specific localization of GS in endothelial cells of the BBB was demonstrated *ex vivo* in brain slices of naïve rats by confocal

microscopy (Fig 10A). After 3D reconstruction (Fig 10B) GS quantification show that endothelial cells of the BBB indicate lower GS intensity compared to astrocytes. GS in endothelial cells is localized mostly in the cytoplasm (90%) while only a small amount (10%) is membrane bound (Fig. 10C). Scale 7 μM. Student t-test, numbers expressed as percentages or means ± SD, significance reached when $p<0.05$.

Second article: Sex is associated with differences in oxidative stress and susceptibility to severe hepatic encephalopathy in bile-duct ligated rats

Sex is associated with differences in oxidative stress and susceptibility to severe hepatic encephalopathy in bile-duct ligated rats

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The authors have read the ARRIVE guidelines, and the manuscript was prepared and revised according to the ARRIVE guidelines.

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Abstract

Background: Hepatic encephalopathy (HE) is a debilitating neurological complication of chronic liver disease (CLD). Hyperammonemia plays an important role in the pathogenesis of HE but other pathogenic factors, such as systemic oxidative stress, can have synergistic effect with ammonia. During CLD, muscle plays a compensatory role in detoxifying ammonia and therefore loss of muscle mass leads to an increase in risk of developing HE. With the majority of animal studies involving males, the impact of sex on development of CLD and associated complications, such as HE and muscle mass loss, remains unknown.

Aims: Identify the impact of sex on CLD, HE and muscle mass loss in a rodent model of CLD.

Methods: The neurophenotype (anxiety, memory, motor-coordination and activity) of both male and female bile-duct ligated (BDL) rats (and respective SHAMs) were evaluated and ammonia-precipitated severe HE (lethargy/loss of righting reflex) was assessed. We also assessed liver injury markers, hyperammonemia, systemic oxidative stress and muscle mass and function including muscle ammonia clearance. Finally, to confirm the role of ROS on sex- based differences on HE, we treated male BDL rats with the antioxidant allopurinol (100mg/kg) and assessed susceptibility to ammonia-precipitated severe HE.

Results: Female and male BDL rats both developed CLD (aspartate transaminase; all $p<0.001$); alkaline phosphatase $p<0.001$; bilirubin $p<0.001$) and HE (impaired motor-coordination $p<0.05$; and night activity $p<0.05$) compared to respective SHAMs. Furthermore, degree of hyperammonemia (NS) as well as muscle ammonia clearance (NS) were also similar between sexes. Contrary to males, female rats did not develop

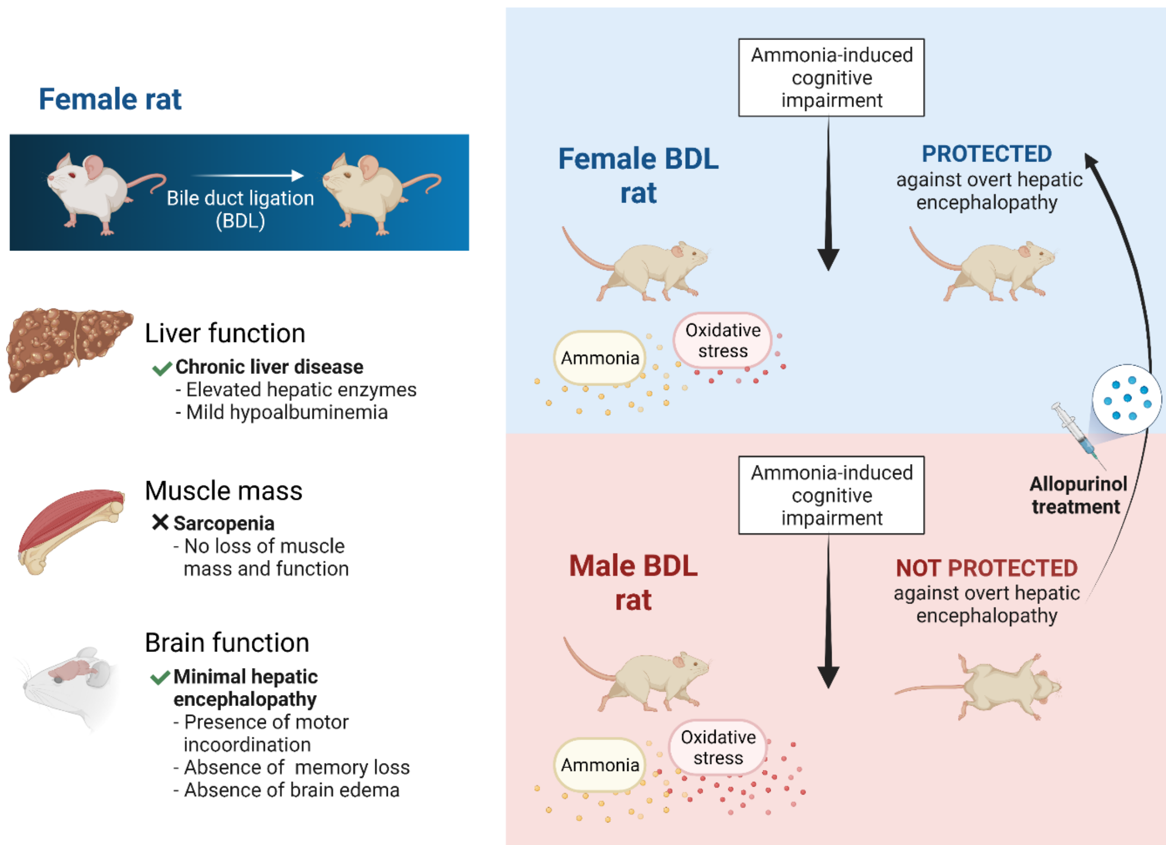
muscle mass loss (NS), brain edema (NS) and short-term memory loss (NS) vs. respective SHAMs. In addition, female BDLs had lower systemic oxidative stress ($p<0.05$), higher blood albumin ($p<0.01$) levels and were completely protected against ammonia-precipitated overt HE ($p<0.001$) compared to male BDLs. Finally, male BDL rats treated with the antioxidant allopurinol were protected against ammonia-precipitated overt HE ($p<0.05$) compared to untreated male BDL rats.

Conclusion: Female and male BDL rats develop distinct features of CLD and HE, with systemic oxidative stress playing a key role in the susceptibility to ammonia-induced overt HE.

Keywords: Ammonia; Glutamine synthetase; Sarcopenia; Sex; Brain edema

Core tip: The impact of liver disease and its complications such as cognitive problems (known as hepatic encephalopathy) is different between males and females. Although both males and females develop liver disease, only females can maintain lower oxidative stress. Because of that, females are protected against brain edema and severe cognitive problems, while males are at higher risk of developing hepatic encephalopathy. Lowering oxidative stress from males gave them similar protection and therefore it should be considered as a potential therapeutic target for the prevention of hepatic encephalopathy.

Graphical abstract



Introduction

Hepatic encephalopathy (HE) is a frequent neurological complication that develops during chronic liver disease (CLD). This neuropsychiatric syndrome manifests with a wide range of symptoms: from subclinical (covert HE (CHE)) such as impaired memory, decreased reaction time and motor incoordination to clinically detectable (overt HE (OHE)) such as lethargy, ataxia, gross disorientation and coma. As many as 80% of patients with CLD suffer from CHE, and more importantly, this underestimated phenomenon leads to a 4-fold increased risk of developing OHE with a 30% risk within the first year (Montgomery

and Bajaj 2011; Patidar et al. 2014). HE remains the primary cause of hospital readmissions, which in addition to accounting for a substantial amount of costs, is also associated with poorer prognosis and higher mortality compared to other complications of cirrhosis (Jepsen et al. 2010). The burden of HE is multidimensional imposing a significant economic charge to the patient, patients' caregivers, healthcare systems, and society.

The pathogenesis of HE is complex and multifactorial. Amongst the various pathogenic factors involved in the development of HE, ammonia toxicity is central. Since the ailing liver during liver disease has reduced capability of detoxifying ammonia via the urea cycle, hyperammonemia arises (Vierling et al. 2016). Ammonia freely crosses and diffuses through biological membranes and rapidly enters the brain causing deleterious effects. Elevated ammonia is shown to cause changes in pH, membrane potential and cell metabolism and is associated with the presence and the worsening of HE (Bosoi and Rose 2009; Vierling et al. 2016; Ong et al. 2003). Ammonia neurotoxicity has been demonstrated to be associated with an increase in brain water in patients with HE (Cudalbu and Taylor-Robinson 2019; Shah et al. 2008; Winterdahl et al. 2019). Similarly, in a rat model of CLD, our group has shown an increase in brain water in 6-week bile-duct ligated (BDL) male rats which is prevented following attenuation of elevated blood ammonia (Bosoi et al. 2011). Subsequently, ammonia lowering therapies are the mainstay strategy for the treatment of HE (C. F. Rose 2012).

In addition to ammonia, other factors such as oxidative stress (ROS) which arises from the imbalance of pro-oxidant and antioxidant capacity, play an essential role in the onset of HE (Giménez-Garzó et al. 2018; Görg et al. 2010). Systemic oxidative stress has been synergistically implicated with hyperammonemia in the presence of HE in

patients as well as the development of brain edema in male animal models of CLD and HE (Montoliu et al. 2011; Bosoi et al. 2012).

The muscle plays an important compensatory role for ammonia detoxification during CLD since it houses glutamine synthetase (GS), an ammonia removing enzyme which amidates glutamate to glutamine. However, sarcopenia, defined by loss of muscle mass and function, is another common complication occurring during CLD (Nardelli et al. 2019). Subsequently, sarcopenia further decreases the capacity to clear ammonia in CLD and is therefore associated with a higher risk of HE, a worse prognosis and higher mortality (Bhanji et al. 2018; Nardelli et al. 2019).

Although our understanding on the underlying pathophysiological mechanisms of HE has increased considerably over the last decades, the impact of sex on the natural course of CLD and HE remains undefined. This is partially due to the fact that the number of females inflicted with CLD (and HE) is lower compared to males. Therefore the impact of sex becomes statistically difficult to evaluate in clinical studies (Dickinson, Adelson, and Owen 2012), even though liver disease of etiologies such as primary biliary cholangitis is more prevalent in females (Smyk *et al.*, 2012). As a result, the majority of clinical studies investigating CLD and HE involve male patients (Xie et al. 2018; Ong et al. 2003; Poveda et al. 2010). Similarly, in pre-clinical studies in CLD and HE, most include male animals (Clément et al. 2021; Ochoa-Sanchez et al. 2021).

Therefore, we aimed to use the well characterized BDL rat (male) model of CLD and HE in female rats and to evaluate the impact of sex on the pathogenesis of CLD, HE, muscle mass loss as well as the susceptibility of HE.

Material and methods

The experimental design is summarized in Fig. 11.

Bile duct ligation (BDL)

Female and male Sprague-Dawley rats (200-225g) were purchased from Charles River and kept two per cage in a 12h light/dark cycle, with free access to water and rodent chow (TD.2819, 18% protein – Envigo, USA). BDL or SHAM surgery was performed as described by Bosoi et. al. (Bosoi et al. 2011). After 48 hours of acclimation, rats were anesthetized with isoflurane (4% induction and 2.5% maintenance, oxygen at 0.9L/min). The rats were shaved, the incision site was sterilized and infiltrated with the local anesthetic bupivacaine 2mg/kg. Then, a midline incision was done to open the skin and muscle, and the common bile duct was identified, isolated, and a ligature was placed distally. After the first ligature, 50µl of formalin was injected inside the bile duct to prevent the duct inflation, and a proximal ligature was rapidly done. A resection of the bile duct was made between the ligatures, and the rat abdominal wall was closed in layers with 4.0 (muscle) and 6.0 silk (skin). SHAM rats underwent the same procedure, except for the formalin injection, placement of ligatures, and resection of the bile duct. After surgery, rats were allowed to recover in an incubator at 27 °C for at least 1 hour. Rats received analgesic treatment before and up to 72 hours after the surgery with buprenorphine (sc. 0.05 mg/kg, every 12 hours), carprofen (sc. 5mg/kg, every 24 hours), and gabapentin (orally, 30mg/kg every 12 hours). All studies were approved by the Institutional Animal Care and Use Committee at the CRCHUM

Chronic liver disease assessment

Plasmatic liver markers

During euthanasia at week 6 after surgery, blood from the left heart ventricle from male and female BDL and SHAM rats anesthetized with isoflurane (4% induction and 3% maintenance, oxygen at 0.9L/min) was collected with a heparinized syringe. Blood samples were centrifuged at 4800 rpm for 5 minutes, and plasma was snap-frozen and kept at -80°C until analysis. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALKP), bilirubin, and albumin were measured using the COBAS system (c111, Roche, USA). Plasma ammonia was measured with a kit (Randox Laboratories, USA).

Liver histology

At euthanasia, the liver was dissected and weighed. The right lobe was dissected and a small piece was immersed in 10% formalin for 24h. Fixed liver tissue was blocked in paraffin, sliced (4μM) and stained with hematoxylin-eosin (H&E) and evaluated for bile duct proliferation, hepatocytes coverage, and activated Kupffer cells.

Covert HE assessment

Behavioral tests

All behavioral tests were conducted during the light phase (with exception of the activity test), at 5 weeks following either BDL or SHAM surgery in female rats.

Anxiety

The anxiety tests are based on the rat's expected conflict between exploring a new environment and fear of an unprotected open space. Thus, an anxious animal will avoid the open areas and stay close to the walls or in hidden areas. The Elevated plus maze (EPM) and the open-field (OF) test were used to assess anxiety. Both anxiety tests were

conducted with female BDL and SHAM rats, with dim light conditions, and the rats' cages were placed in the room to acclimate for 1 hour before the tests.

The elevated plus maze (EPM) test

The elevated plus maze arena consists of a cross-shaped maze with four 45cm^2 arms, two open and two closed. The maze was divided into three areas: open arms, closed arms, and center. For the test, rats were placed in the center, and their ambulatory movements were recorded for 5 minutes and analyzed with the SMART video tracking system (Panlab). Total time (seconds) and %distance in the open arms were used to assess anxiety.

The open-field (OF) test

One hour after the EPM test, rats were placed in the OF arena, consisting of a black square box (90 cm^2). The box was divided into three areas: walls, a center, and an intermediate area. For the test, the rats were placed in the corner, facing the wall, and their ambulatory movements were recorded for 5 minutes and analyzed with the SMART video tracking system (Panlab). Total time (seconds) and %distance in the center of the OF were used to assess anxiety.

Novel object recognition (NOR) test

The novel object recognition test was used to evaluate short term memory (STM). After 1 hour of acclimation in a dim-lit room, rats were placed in an empty open field arena (60x45x33cm) for 5 minutes for habituation. One hour later rats were placed in the same arena containing two identical objects (A + A) for 5 minutes for familiarization. One hour later, one of the objects was changed (A + B) and the rats were returned to the arena for STM assessment. The STM assessment was recorded by a video camera and is

expressed as % time exploring (sniffing, touching) the novel object (B) divided by the total exploration time (A + B).

Rota-rod test

Motor coordination was measured by the rota-rod test. The rota-rod device consists of a cylinder (7 cm of diameter) that turns with increasing speed and has a sensor that records rats' falls (ROTOR-ROD^{TM} System, San Diego Instruments). On the first day of rota-rod, rats were first habituated and trained to the equipment. Rats were placed on the cylinder without rotation for 5 minutes, and then the rota-rod was started, with a linear increase of speed reaching 40 rpm over 5 minutes. The trial was finished when the rat fell, and each rat was allowed four trials with 3 minutes of rest between trials. On the following day (test day), the rats were submitted to the same protocol, and the latency to fall (the highest from the four trials) was recorded.

Activity test

Rats were placed in plexiglass boxes (42x42x22 cm) surrounded by an infrared bean system to record ambulatory activity (Omnitech-electronic Inc). Rats were provided with a thin layer of bedding material and free access to food and water and were allowed to acclimate for 4 hours before the start of the measurements. The activity was recorded for 12 hours (from 18h30 to 6h30) during the dark (active) phase, and total activity was measured as total distance (centimeters) over 12 hours.

Brain edema

Brain water content from the frontal cortex of female BDL and SHAM rats was measured 6 weeks after surgery by densitometry as described by Marmarou et al. (Marmarou et al. 1978). Briefly, kerosene and bromobenzene gradient density columns were prepared and calibrated with different concentrations of potassium sulfate (known

densities). The frontal cortex from fresh brains was dissected on ice, and $2mm^3$ pieces (4 pieces for each rat) were placed and allowed to stabilize for 1 minute in the columns. Water content was measured as the tissue density, using the average of the four pieces.

<u>*Body parameters*</u>

Bodyweight and food intake

Bodyweight and food consumption of female BDL and SHAM rats were measured weekly until the 6th week after surgery. Since rats were housed two per cage, food intake was given as the mean of the cage per week for each rat.

Body composition

Fat and lean mass of 6 weeks-female BDL and SHAM rats were measured before euthanasia in non-sedated rats by EchoMRi 700® Body Composition Analyzer (R & D EchoMRi LLC).

Muscle strength

Muscle strength was measured using a digital force gauge (Chatillon® DFE-010; AMETEK TCI Division). Rats were supported at the thorax and the base of the tail, placed with the limbs gripping the mesh pull bars, and slowly pulled backward until releasing the bar. The mesh pull bars for forelimb measurements were placed horizontally, while for forelimb strength, the mesh pull bars were placed at a 45-degree angle. Muscle strength was recorded as the maximum strength after three trials.

Muscle circumference and weight

During euthanasia, the right gastrocnemius muscle from isoflurane anesthetized (4% induction and 5% maintenance, oxygen at 0.9L/min) male and female rats was localized the circumference was measured using a measuring tread. After dissecting the

gastrocnemius, wet weight was measured using a precision scale. Gastrocnemius muscle was then collected, snap-frozen and kept at -80°C.

GS activity

The glutamine synthetase (GS) activity assay is based on the detection of inorganic phosphate from adenosine 5-triphosphate (ATP) by GS according to Gawronski et. al. (Gawronski and Benson 2004). First, gastrocnemius muscle tissue was lysed with zirconia beads in RIPA buffer (trisaminomethane (Tris) 50mM, sodium chloride 150 mM, ethylenediaminetetraacetic acid (EDTA) 1 mM, sodium dodecyl sulfate (SDS) 0.1% and proteinase inhibitor cocktail 1/500), at 3000 RPM during 6 minutes at 4°C and protein concentration was determined using the Bio-Rad DC protein assay kit (Bio-Rad Laboratories). Following protein dosage, 40 µg of muscle lysate or potassium phosphate dibasic were incubated with Tris 10 mM –pH 7.5, magnesium chloride 5 mM, monosodium glutamate 25 mM and ammonium chloride 5 mM. The reaction was started by adding ATP 10 mM, and after 5 minutes, 12 % ascorbic acid and 2 % ammonium molybdate tetrahydrate were added. After 5 minutes, the reaction was stopped with 2 % sodium citrate tribasic dihydrate and 2% acetic acid, and absorbance was read at 655 nm on a microplate reader (Bio-Tek Synergy HT). Results are expressed as GS activity by nmol of phosphate.

Muscle ammonia clearance and glutamine production

Two days after recovery from the ammonia challenge, female and male BDL rats were anesthetized with isoflurane (4 % induction and 2.5 % maintenance) with an oxygen flow rate of 0.9L/min, and the left femoral vein and iliac artery were catheterized for blood

collection. Blood was taken in heparinized tubes, centrifuged at 4800 rpm for 5 minutes, and plasma was snap-frozen and kept at -80°C until analysis.

Plasma ammonia was measured by a kit (Randox Laboratories), and glutamine was measured by the EnzyChrom Glutamine Assay kit (BioAssay Systems).

Plasmatic oxidative stress

Reactive oxygen species in plasma were measured in female and male rats using the 2',7'-dichlorofluorescein diacetate (DCFDA) test (Sigma-Aldrich). Briefly, DCFDA (10 µM) was incubated with hydroxylamine (1 M, pH 8.5) for 30 minutes and then added to 10 µl of plasma in triplicates in a 96 well plate. Fluorescence (λ_{exc}485 ηm and λ_{emi} 520 ηm) was measured every 2 minutes, up to 10 minutes, and the slope of time/readings was calculated. Results are expressed as relative fluorescent units (RFU).

Brain oxidative stress – total antioxidant capacity

During euthanasia, brains were carefully dissected and kept at -80 °C until experiments. Total antioxidant capacity was measured by the total antioxidant capacity kit (Sigma-Aldrich) according to manufacture's instructions. Briefly, brains' cortex and hippocampus were lysed with assay buffer and, after protein dosage using the Bio-Rad DC protein assay kit (Bio-Rad Laboratories), 150 µg of protein was incubated with ABTS solution and hydrogen peroxide at the advised concentrations for 3 minutes. Stop solution was added and absorbance was read at 405 ηm. Results are given as total antioxidant capacity in mM.

Ammonia-induced overt HE

An ammonia challenge was performed to induce overt HE in female and male BDL rats as well as male BDL rats treated for 10 days (from day 25 to day 35 after BDL surgery) with either vehicle (saline) or allopurinol (100mg/kg intraperitoneally) (Bosoi et al. 2012). After baseline blood sampling, rats were injected with 6 mmoles/kg of ammonium acetate (subcutaneously). Mental status was evaluated every 5 minutes after the injection for mild lethargy (lack of spontaneous ambulatory movement, but capable of moving when manipulated), severe lethargy (inability to perform ambulatory movement even if manipulated, righting reflex delayed but present), and loss of righting reflex. Severe lethargy and loss of righting reflex were defined as an episode of overt HE. Blood was taken from the saphenous vein and tail vein in heparinized tubes at baseline and during episode or at the peak of mental status impairment for the rats that did not had an episode. All samples were collected up to 60 minutes after injection and average time of blood sampling was not different between the groups. Blood samples were centrifuged at 4800 rpm for 5 minutes, and plasma was snap-frozen and kept at -80°C until analysis.

Statistics

Data are expressed as mean ± standard deviation (SD) or percentages, and *p*-values < 0.05 were considered statistically significant. Normality was assessed graphically. Statistical significance was tested using parametric t-test for all variables except body weight, food intake and incidence of OHE episode. The chi-square test was used to compare the incidence of OHE episodes. Repeated measures two-way ANOVA was used to compare body weight and food intake. If the interaction between time and group was found significant, groups (BDL vs. SHAM) were compared at each time point

using Sidak's multiple comparisons test to control for multiplicity. NS= non significant. Statistical analysis was done using GraphPad Prism 8 (La Jolla, CA, USA).

Results

BDL-induced CLD

Six-weeks following surgery, female BDL rats developed elevated plasma AST ($p<0.001$), ALKP ($p<0.001$), bilirubin ($p<0.001$) and lower albumin ($p<0.001$) vs. female SHAM controls. ALT was also elevated but was not significant different (NS). Blood ammonia ($p<0.01$) and ROS ($p<0.05$) levels were also significantly higher in female BDLs vs SHAM controls (Table 3). Accordingly, liver histology revealed tissue disorganization, with reduced number of hepatocytes, proliferation of bile ducts and activated Kupfer cells in female BDLs (Fig. 12A) vs. female SHAMs (Fig. 12B).

Neurophenotype

Female BDL rats showed impaired motor coordination evaluated using the rota-rod test, with lower average latency to fall compared to female SHAM controls (135 ± 25 seconds vs 200 ± 68 seconds; $p<0.05$) (Fig. 13A). Night activity, total distance travelled, was also lower in female BDL rats compared to female SHAMs (17033 ± 6454 cm vs 31180 ± 8310 cm; $p<0.05$) (Fig. 13B). Level of anxiety as well as short-term memory were similar in female BDL rats vs female SHAMs (Fig. 13C-3E). Additionally, female BDL rats did not acquire brain edema with similar degrees of brain water found in both female BDL and SHAMs (77.93 ± 0.30 % and 77.95 ± 0.30 % respectively; NS) (Fig. 13F).

Food consumption and muscle mass

The results from body weight and food intake are reported in Table 4. There was a significant interaction between time and surgery (BDL and SHAM) for body weight

(p<0.001) and food intake (p<0.05), so we compared the groups at each time point. When we fixed the time, there was no difference observed between BDL and SHAM for either body weight or food intake (NS) (Fig. 14A). Female BDL rats had higher lean mass compared to SHAM (241.7 ± 16.2 g vs 221.7 ± 16.5 g respectively; p<0.05), while fat mass was lower in BDLs vs SHAMs (33.3 ± 7.4 g vs 56.4 ± 13.6 g respectively; p<0.001) (Fig. 14B). Female BDL and SHAM rats showed no difference in gastrocnemius muscle circumference (BDL 4.3 ± 0.2 cm vs. SHAM 4.7 ± 0.5 cm; NS) and weight (BDL 1.8 ± 0.2 g vs. SHAM 1.9 ± 0.2 g; NS) (Fig. 14C), as well as similar muscle strength in forelimbs (BDL 1680 ± 295 g vs. SHAM 1758 ± 167 g; NS) and hindlimbs (BDL 1289 ± 337 g vs. SHAM 1400 ± 214 g; NS) (Fig. 14D).

Liver, ammonia, ROS and muscle

Compared to female BDL rats, male BDL rats presented lower gastrocnemius muscle mass (92.7 ± 12.2 % vs 69.8 ± 10.7 % respectively; p<0.01) (Fig. 15A). Nevertheless, levels of hyperammonemia were similar between male and female BDLs (127.6 ± 81.7 µmol/L vs. 104.7 ± 37.9 µmol/L respectively, NS) (Fig. 15B). Interestingly, muscle GS activity was upregulated in male BDL rats vs. BDL females (6.62 ± 1.33 ηmol of phosphate vs 4.78 ± 0.79 ηmol of phosphate; p<0.01) (Fig. 15C). Ammonia clearance (male BDL; 21.40 ± 10.80 µmol/L vs. female BDL; 10.07 ± 17.18 µmol/L; NS) and glutamine production across the muscle (0.124 ± 0.306 mM for male BDLs vs. -0.118 ± 0.231 mM female BDLs; NS) (Fig. 15D) were not significantly different between male and female BDL rats. Lower levels of systemic ROS were found in female vs male BDLs (7.16 ± 2.67 RFU vs 26.43 ± 15.09 RFU respectively; p<0.05) (Fig. 15E) while brain ROS levels were not significantly different between female and male BDLs (pre-frontal cortex female

BDL; 0.21 ± 0.03 mM vs male BDL; 0.21 ± 0.07 mM; NS, and hippocampus female BDL; 0.20 ± 0.07 mM vs. male BDL; 0.21 ± 0.06 mM; NS) (Fig. 15F).

Ammonia-induced episode of OHE

Upon the acute injection of ammonia, 67% of the male BDL rats developed severe HE (OHE episode) while 0% of the female BDL rats incurred an episode ($p<0.001$) (Fig. 16A). During the episode, the rise in plasma ammonia were similar (non-significant) in male and female BDL rats (610.0 ± 200.2 μmol/L in females vs. male BDL; 918.6 ± 361.7 μmol/L; NS) (Fig 16B). Whereas, plasma ROS levels during the ammonia challenge were significantly lower in female BDL rats compared to male BDL rats (16.79 ± 9.13 RFU vs 98.53 ± 31.07 RFU respectively; $p<0.001$) (Fig. 16C). To further understand the role of ROS in the onset of ammonia-induced OHE, male BDL rats were treated with the antioxidant allopurinol, previously shown to be effective in reducing systemic ROS in BDL rats (Bosoi et al. 2012). Allopurinol treatment in male BDL rats lead to protection against ammonia-induced episodes compared to vehicle treated male BDL rats (25% developed episodes in allopurinol-treated vs. 67% in vehicle-treated male BDL rats; $p<0.05$) (Fig. 16D).

Discussion

Six weeks following BDL, female rats developed CLD, comparable to what has been previously reported in male BDL rats. Furthermore, female BDL rats developed similar parameters of neurological impairment as previously observed in males except for anxiety, loss of short-memory and brain edema for which females were safeguarded. Comparable levels of hyperammonemia were found in both female and male BDLs even though females did not endure a loss of muscle mass as observed in males. Female BDL

rats were protected against ammonia-induced episode of OHE, whereas male BDL rats were not. Systemic oxidative stress was found to play a deciphering role in protecting female BDL rats since systemic ROS were significantly higher in male BDL rats and when treated with allopurinol (antioxidant; xanthine oxidase inhibitor), male BDL rats did not develop severe neurological impairment following ammonia-challenge. Increased antioxidant capacity in female BDL rats, possibly due to higher levels of albumin vs. male BDL (data not shown), reduces the sensibility to ammonia-induced overt HE. This sex-specific increased antioxidant capacity found in females and resistance against HE suggests reducing oxidative stress with antioxidants could be an important treatment strategy for the prevention of HE in males, who are at higher risk.

The use of female animals in pre-clinical research is often underrepresented, resulting in significant lack of knowledge on the impact of sex in sickness and therefore creating a care gap. Historically, female animals were rarely chosen for studies since it was believed they would lead to increased variability compared to males. Subsequently, the lack of inclusion of female models in pre-clinical research became a growing concern (Beery and Zucker 2011), and it was later proven that the variability found in females is in fact not higher than in males (Becker, Prendergast, and Liang 2016). However, what became clear was that there are fundamental differences between males and females in response to disease and that addressing these differences would result in sex-specific management and therapeutic interventions. In liver disease, differences between male and females have been found in patients with CLD, including etiology of disease, mortality predictors, liver transplantation access, and gut microbiota (Saboo et al. 2020; Sarkar et al. 2015). However, the impact of sex-specific differences in the onset of CLD and HE remains largely unknown.

In this study, we described for the first time, a female rat model of CLD and HE. Our group has ample experience with this well characterized animal model of CLD and HE in male rats. Even though female BDL rats established CLD and HE after 6 weeks, they developed distinct features compared to male BDL rats. Since the liver is the main metabolic organ, liver disease negatively impacts body composition by changing energy homeostasis and inducing a hypermetabolic state. Consequently, sarcopenia develops which leads to increased mortality and higher susceptibility to episodes of OHE (Bhanji et al. 2018). While sarcopenia is a well-established condition in male patients with CLD and HE, there is evidence that female patients are less affected (Bhanji et al. 2018; Tandon et al. 2012). In pre-clinical research, our group already demonstrated male animal models of CLD and HE develop loss of muscle mass (Bosoi, Oliveira, Ochoa-Sanchez, et al. 2017). In the current study, supporting the lower incidence of sarcopenia in female patients, female BDL rats did not develop loss of muscle mass and function. It is known that muscle plays an important compensatory role in clearing ammonia during CLD and therefore, a reduction in muscle mass or function leads to a further decline in the capacity to clear ammonia. However, despite no loss of muscle mass in female BDLs, blood ammonia levels were comparable between female vs male BDLs. Furthermore, muscle ammonia clearance and subsequent glutamine production via GS was not higher in female BDL compared to males. However, male BDLs do develop a compensatory overexpression of GS in the muscle (M. Jover-Cobos et al. 2014). These sex-specific differences could explain the similar systemic ammonia levels between males and female BDLs. Diverse factors could explain the increased expression of GS in muscle of male BDLs. First, muscle mass loss in males could stimulate the upregulation of muscle GS in response to preventing ammonia levels from increasing (M. Jover-Cobos et al. 2014). Secondly, GS

expression could be regulated by androgenic hormones, such as testosterone. Indeed, in a study assessing sex-specific hepatic GS, its protein expression and activity were found to be higher in male vs. female rats (Sirma, Williams, and Gebhardt 1996). In agreement, removing male sex-hormones by gonadectomy in male rats decreased hepatic GS to levels similar to females (Sirma, Williams, and Gebhardt 1996).

CHE is defined by a broad spectrum of subtle neurological changes arising due to liver dysfunction encompassing impairments in memory, motor coordination, activity and mood. However, little is known about the impact of sex on CHE. Levels of anxiety were similar in female BDLs vs SHAMs, contrary to what has been demonstrated in male BDLs (Clément et al. 2021) whereas female BDLs had reduced overall activity, similar to what has been observed in male BDLs (Bosoi et al. 2011). There is evidence that female patients with cirrhosis and CHE have worse quality of life, poor sleep quality and increased anxiety compared to male patients (Popović et al. 2015; Christian Labenz et al. 2020; C. Labenz et al. 2018).. However, the methods for evaluating anxiety in rodents and patients are evidently different, which could also explain the discrepancy. Furthermore, anxiogenic behaviour in rats can be influenced by hormonal changes caused by the estrous cycle (ter Horst et al. 2012). However, when female BDL and SHAM rats were grouped by estrous cycle, anxiety levels did not differ between phases (Suppl. Fig. 1A-B). In addition, the breadth of CHE symptoms and behavioral variances can manifest differently in male vs. female BDL rats. Recognition memory evaluated by the NOR test encompasses the identification of a familiar stimulus, assessing episodic memory, thus the ability to recall specific episodes. Contrary to what has been previously demonstrated in males (Ochoa-Sanchez et al. 2021), female BDL rats did not develop impairment in short-term memory. It is plausible that the difference is due to the type of memory assessed, since females

have better performance on episodic memory, while males perform better on spatial memory tests (Loprinzi and Frith 2018). Since the neurophenotype of female and male BDL rats differs (females; lack of anxiety, brain edema and short-term memory loss), it would be interesting to evaluate the assessment of sex-specific cognitive tasks which could increase sensitivity and specificity to CHE testing.

Brain edema is considered to be implicated in the pathogenesis of HE. However, its precise role is unknown since the incidence of brain edema in HE patients is not uniform (Winterdahl et al. 2019; Joshi et al. 2014). Therefore, not all manifestations of CHE are related to brain edema. For example, not all animal models of HE develop brain edema. The portal-cava anastomosis (PCA) rat (type B model of HE) shows neurological impairments characteristic of HE without the presence of brain edema (Bémeur et al. 2016). In male BDL rats, brain edema is present (Bosoi et al. 2014), an entity which we did not observe in female BDL rats. Interestingly, in animal models of traumatic brain injury, females are partly protected due to their hormonal profile, developing delayed brain edema compared to males (O'Connor, Cernak, and Vink 2006). However, in our female BDL model, the estrous cycle, which is governed by the hormonal profile, is impaired (Suppl. Fig.1E). This suggests a hormonal imbalance, which is a known complication of CLD resulting in lower estrogen levels in females (Mahmoud 2018; Välimäki et al. 1984). The impact of hormonal dysregulation on brain physiology in cirrhosis remains to be thoroughly investigated. Our group has demonstrated that the onset of brain edema in male BDL rats is a result of the synergistic interaction between hyperammonemia and systemic oxidative stress (Bosoi et al. 2012; Bosoi, Tremblay, and Rose 2014; Bosoi and Rose 2013). We demonstrated that either reducing blood ammonia levels with an ammonia lowering agent or reducing ROS with an antioxidant, lead to a prevention of

brain edema in male BDL rats. Since the hyperammonemia levels were similar in male vs female BDL rats, our data suggest the lower systemic ROS levels in females is the plausible explanation for the protection against brain edema.

Many of the precipitating factors of OHE in clinic, such as constipation and gastrointestinal bleeding, lead to an increase in blood ammonia (Vilstrup et al. 2014, 2014). Therefore, we induced an episode of OHE with an acute injection (s.c.) of ammonium acetate. The majority of male BDL rats developed severe HE (severe lethargy and loss of righting reflex) following the ammonia challenge. Whereas none of the female BDL rats developed OHE. This protection was independent of ammonia levels (baseline; pre-injection and during episode) since in both cases, ammonia levels were similar in male and female BDLs. However, systemic ROS levels were significantly higher at baseline (pre-injection) and significantly higher during the hyperammonemia episodes in male BDL rats. To strengthen the causal role of ROS in precipitating OHE, we treated male BDL rats with allopurinol, an antioxidant acting as a xanthine oxidase inhibitor, which our group has previously shown to reduce systemic oxidative stress (Bosoi et al. 2012). Treatment of allopurinol to male BDL rats lead to a significant decrease in episodes of OHE compared to vehicle-treated male BDL rats. This strongly suggests that systemic oxidative stress plays an important role in the development of OHE.

The further induction of ROS following ammonia-injection in male BDL rats is evidently ammonia-dependent. Our group has demonstrated that the onset of CHE involves systemic ROS and hyperammonemia as independent factors (Bosoi and Rose 2013). However, it has been shown that higher levels of blood ammonia, as seen during acute liver failure (ALF) due to hepatic devascularization in rats or exposure to high concentrations of ammonia *in vitro* (higher than 500uM), can cause ROS levels to rise

(Bosoi and Rose 2013). Nevertheless, the ammonia-injection only lead to an increase in systemic ROS and the onset of OHE in male BDL rats. This suggests, together with lower systemic ROS levels at baseline (pre-ammonia injection), that females contain a higher antioxidant capacity in order to promptly neutralize the generated ROS induced from the acute injection of ammonia. Antioxidant capacity refers to the cumulative antioxidant action in a biological system and is impacted by the quantity of antioxidants produced and available in regards to generation of ROS in the system. The increased quantity of albumin observed in female BDL rats reflects a higher antioxidant capacity. In circulation, albumin is an effective antioxidant which has the capacity to control ROS in two distinct ways. First, albumin contains methionine and cysteine residues that actively bind to reactive oxygen and nitrogen species. The reduced cysteine groups in albumin are considered the largest pool of thiols in circulation, stressing albumin's importance as a potent antioxidant (Roche et al. 2008). Secondly, albumin binds to ligands such as iron and copper and prevent the formation of ROS (Roche et al. 2008). Increased albumin in female vs. male BDL rats is likely due to innate higher albumin in female rats, with higher albumin being found in female SHAMs compared to male SHAMs (data not shown). Other antioxidant enzymes, molecules and radicals as part of the antioxidant system might also play a role in the sex-specific protection against brain edema and OHE but this remains to be investigated.

In addition to attenuating a rise in ROS levels leading to protection against OHE episodes in female BDLs, the lack of brain edema must not be neglected as key factor involved in the reduced susceptibility to severe HE. Male BDL rats with brain edema (Bosoi et al. 2011) are susceptible to ammonia-toxicity as they developed OHE episodes. However, male BDL rats treated with allopurinol which leads to a decrease in brain water (Bosoi et al. 2012), were also protected against OHE episodes. Understanding the role of

brain edema in the susceptibility or risk of developing OHE remains to be thoroughly investigated.

Without a doubt, ammonia remains a key pathogenic factor in the pathogenesis of HE and therefore reducing ammonia remains a primary therapeutic strategy. However, we have demonstrated in our study that ROS can increase the sensitivity of ammonia toxicity and therefore reducing systemic oxidative stress should also be a treatment target. Interestingly, therapies such as vitamin C and zinc supplementation, considered to have antioxidant effects, have been shown to be superior compared to lactulose to treat HE in patients (Mousa et al. 2016). However, the effect of the treatments by sex was not evaluated.

In conclusion, the female rats following BDL develop similar hepatic damage/injury compared to the well-characterized male BDL rat. However, even though both female and male BDL rats develop HE, females have distinct features such as lack of anxiety and brain edema and intact short-term memory. In addition, sex-dependent differences were found at the muscle level since even though similar levels of hyperammonemia were found in female and male BDL rats, females were safeguarded against muscle mass loss while males developed a loss of muscle together with an upregulation in GS activity. A higher systemic antioxidant capacity found in females protected female BDL rats from enduring an ammonia-induced episode of OHE. Our results suggest that the occurrence and development of CLD-induced complications differs between male and females and that sex-specific management of patients merits further attention.

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Tables

	BDL	SHAM	P value
Liver weight (g)	29.1 ± 3.9	9.1 ± 0.8	***p<0.001
Blood markers of liver injury			
ALT (U/L)	40.9 ± 20.9	26.3 ± 8.5	NS
AST (U/L)	331.2 ± 140.9	68.7 ± 18.7	***p<0.001
ALKP (U/L)	361.3 ± 193.5	81.7 ± 30.87	**p<0.01
Blood markers of liver function			
Albumin (g/L)	29.9 ± 5.8	49.3 ± 8.4	***p<0.001
Bilirubin (µmol/L)	192.0 ± 30.4	1.1 ± 0.8	**p<0.001
Ammonia (µmol/L)	111.8 ± 51.8	50.7 ± 8.7	**p<0.01
ROS (fluorescence)	41.36 ± 20.9	5.24 ± 4.8	*p<0.05

Table 3: Markers of liver disease in female rats. Liver weight and liver enzymes at week 6 after BDL or SHAM surgery in female BDL rats. N=8 rats per group. Parametric t-test, numbers expressed as means ± SD, significance reached when p<0.05. NS = non significative. ALT, alanine aminotransferase; AST, aspartate transaminase; ALKP, alkaline phosphatase.

Body weight			
Week	**SHAM**	**BDL**	**p value**
0	221.9 ± 11.8	223.1 ± 12.7	NS

1	237.8 ± 10.9	229.5 ± 14.9	NS
2	257.8 ± 11.9	261.3 ± 15.5	NS
3	272.6 ± 15.2	283.8 ± 16.1	NS
4	286.3 ± 16.6	303.1 ± 20.7	NS
5	295.0 ± 22.5	312.9 ± 23.3	NS
6	292.1 ± 23.4	309.8 ± 23.6	NS
Food intake			
Week	**SHAM**	**BDL**	**p value**
1	20.26 ± 3.19	18.19 ± 3.69	NS
2	22.65 ± 3.94	21.43 ± 1.11	NS
3	23.01 ± 3.77	22.01 ± 1.27	NS
4	21.39 ± 1.09	22.50 ± 1.39	NS
5	21.11 ± 0.82	21.99 ± 1.92	NS
6	17.69 ± 1.31	19.93 ± 1.64	NS

Table 4: Results of the repeated measures two-way ANOVA for body weight and food intake in female SHAM vs. BDL rats. P-values come from the pairwise comparisons between groups at each time point after the interaction from the repeated measures ANOVA was significant (interaction $p < 0.001$ for body weight and $p<0.05$ for food intake). N=8 rats per group, numbers expressed as means ± SD, significance reached when $p<0.05$. NS = non significative.

Figures

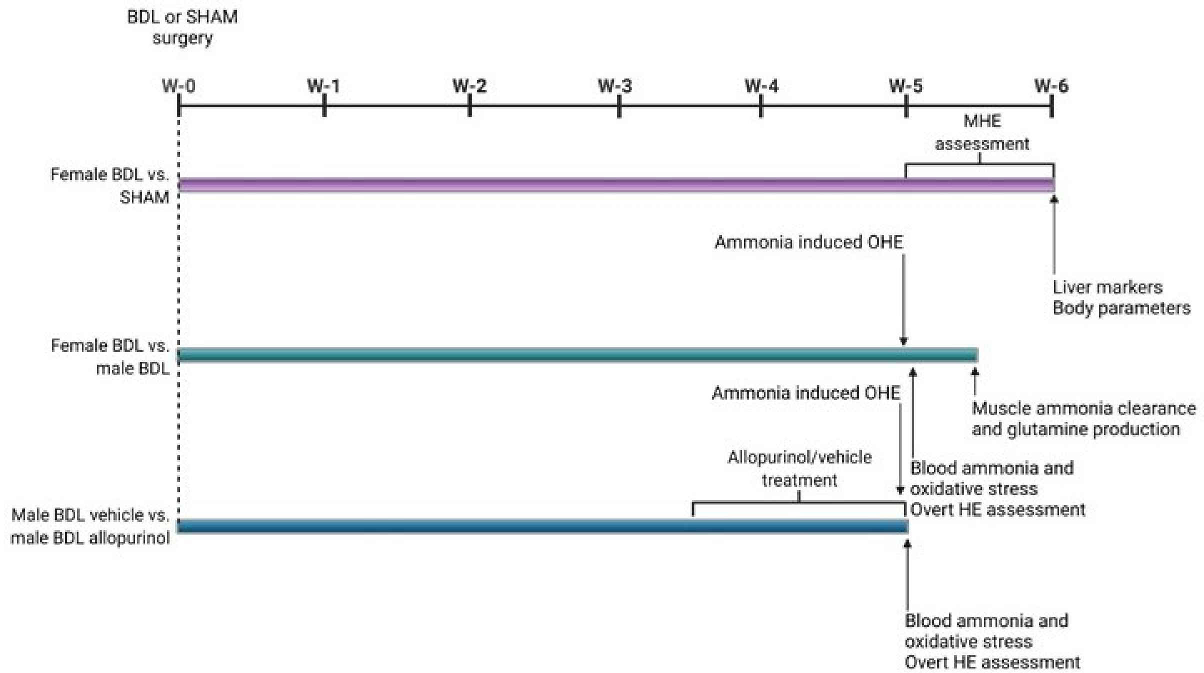

Fig. 11. Experimental design.

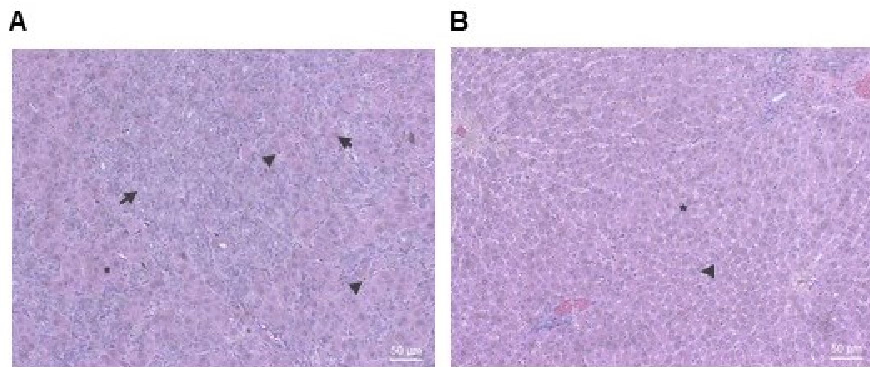

Fig. 12. Liver histology of female rats. Hematoxylin and eosin staining in livers of female rats after 6 weeks of bile duct ligation (A) or SHAM (B) surgery. = ★ hepatocytes, ➘ = bile ducts and ▼ = Kupfer cells.

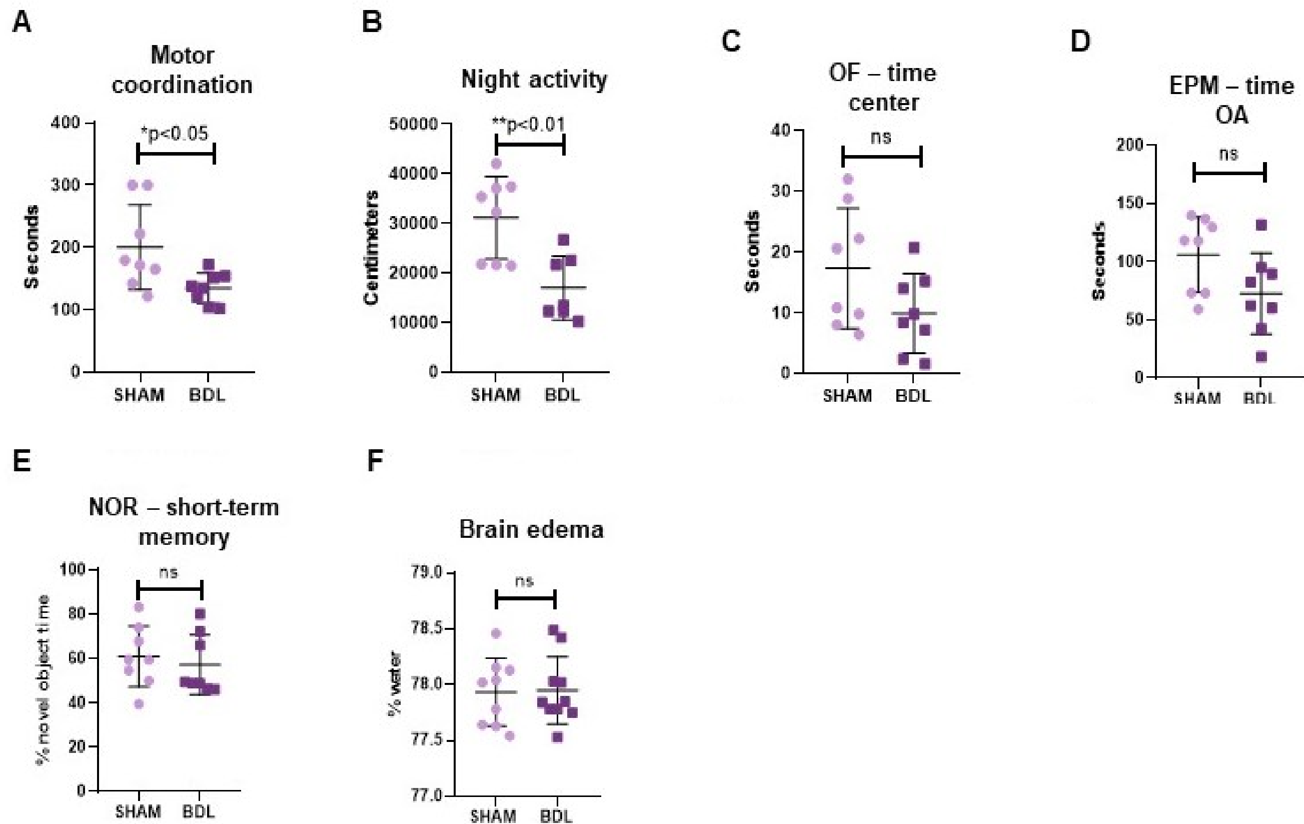

Fig. 13. Hepatic encephalopathy assessment after BDL or SHAM surgery in female rats.

In the rota-rod test, female BDL rats showed altered motor coordination (A) and impaired night activity (B), compared to female SHAM rats. Female BDL rats had no anxiety measured by the open filed (OF) and the elevated plus maze (EPM) tests vs. SHAM rats (C and D). Short term memory was not impaired in female BDL vs. SHAM, (E). Brain edema (F) was not present in female BDL rats compared to female SHAM rats, N=7-10 per group. Parametric t-test, numbers expressed as means ± SD, significance reached when p<0.05. NS = non significative.

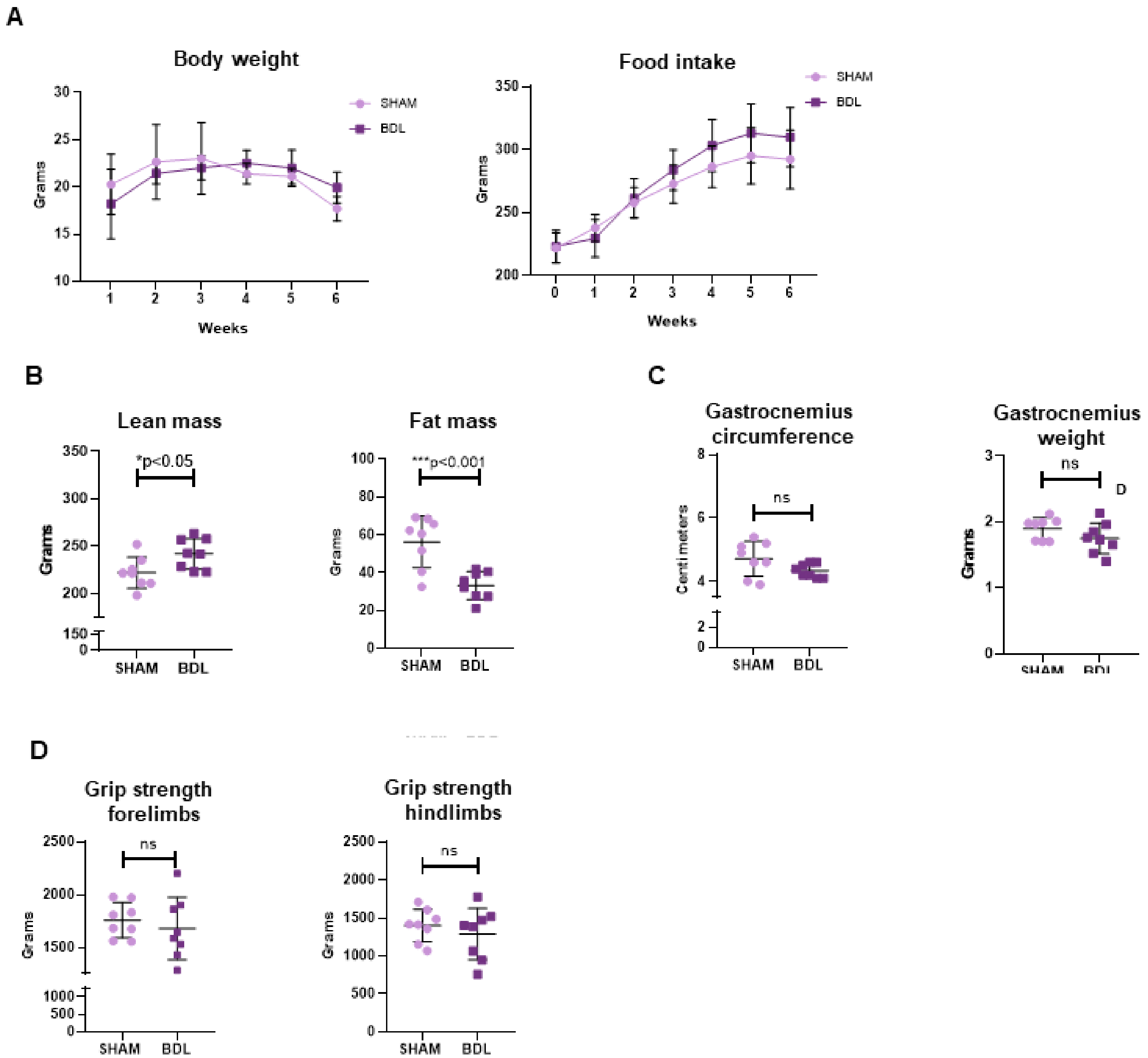

Fig. 14. Body parameters in female rats after BDL or SHAM surgery. Female BDL rats showed no changes of body weight and food intake (A) vs. female SHAM through the duration of the model. Body composition analysis (B) showed higher lean mass and lower fat mass in female BDL vs. SHAM. No muscle loss (C) assessed by weight or circumference was observed in female BDL rats vs SHAM controls as well as no loss of muscle strength (D) from either forelimbs or hindlimbs. N=8 rats per group. Two-Way

ANOVA and parametric t-test, numbers expressed as means ± SD, significance reached when p<0.05. NS = non significative.

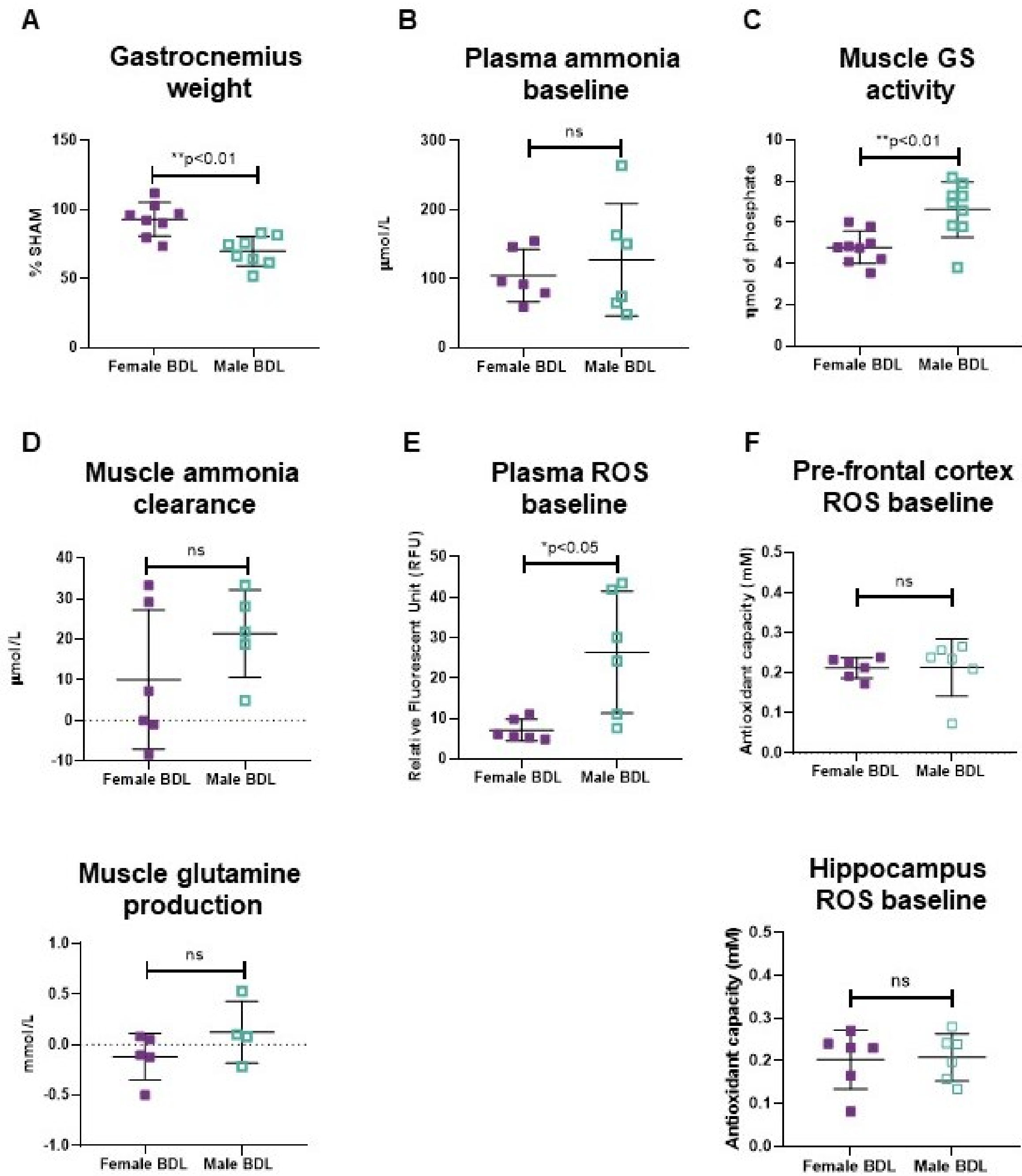

Fig. 15. Higher muscle mass does not result in lower ammonia in female BDL vs. male BDL rats. Male BDL rats had lower percent muscle mass vs. female BDL rats, normalized to respective SHAM groups (A) N=8, but no changes in baseline ammonia levels (B). Muscle GS activity (C) was higher in BDL male vs. female, and muscle ammonia clearance and glutamine production (D) were not different. Female BDL rats have lower baseline oxidative stress with lower baseline ROS) and higher baseline albumin levels (E) compared to male BDL rats. N=4-9 rats per group. Parametric t-test, numbers expressed as means ± SD, significance reached when $p<0.05$. NS = non significative.

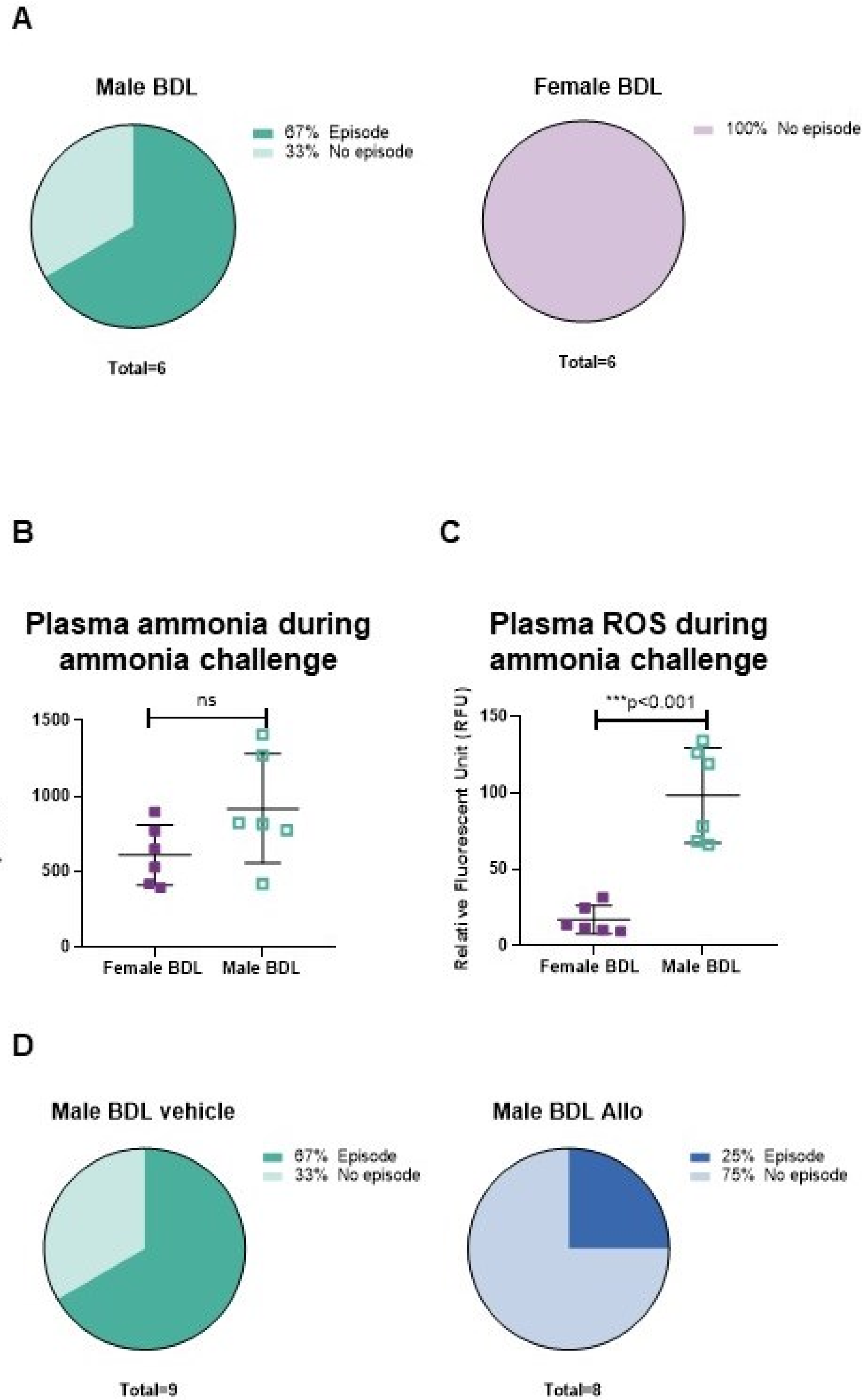

Fig. 16. Lower ROS protects against ammonia induced OHE. When challenged with ammonia, female BDL rats were protected against neurological impairment compared to

male BDL ($p<0.001$) (A). Female rats had similar plasma ammonia (B) and lower plasma ROS levels during the ammonia challenge (C) compared to male BDLs. In addition, male BDL rats treated with the antioxidant allopurinol were protected against ammonia induced neurological impairment compared to male BDL vehicle rats ($p<0.05$) (D). N=6-9 rats per group. Parametric t-test and Chi-square test. Numbers expressed as means ± SD or percentages, significance reached when $p<0.05$. NS = non significative.

Discussion

Highlights:

For the first time, we demonstrated the expression of GS in endothelial cells of the BBB, both *in vitro* and *ex vivo*. We also demonstrated that ammonia did not increase glutamine production, but supplementing GS with ornithine increases glutamine production and ammonia detoxification.

We also showed that although female BDL rats develop similar degrees of liver damage/impairment and CHE to male rats, females develop unique features of CLD such as lack of sarcopenia, normal brain water, and preserved short-term memory. We also found that female BDLs are protected against ammonia-precipitated OHE episodes, primarily due to lower systemic ROS compared to males. Overall, our results demonstrate that CLD-induced complications differ between males and females.

Glutamine synthetase and the BBB

Ammonia metabolism and the BBB

We elucidated that GS is expressed in endothelial cells of the BBB, confirming that ammonia is metabolized. It has been shown that GS in astrocytes can be regulated by ammonia or oxidative stress during CLD and HE (Görg *et al.*, 2007; Cudalbu *et al.*, 2012), and therefore further investigations into the regulation of GS in endothelial cells of the BBB are warranted.

How HE pathogenic factors cross the BBB

Systemic molecules can cross, bypass, or affect the BBB via different routes, influencing and impacting the brain. During disease, the overexpression of endothelial receptor-mediated transcytosis receptors or influx transporters or the downregulation of luminal efflux transporters increases the crossing of molecules (such as ions or amino acids) that normally cross the BBB. Increased expression of receptors and transporters leads to an increase in BBB permeability with an increase in the number but not the nature of the molecules that cross into the brain.

In contrast, the physical disruption of the BBB allows the entrance of molecules that would usually not cross into the brain. A physical breakdown of the BBB indicates a severe dysregulation of the tight junction, meaning the downregulation of protein expression or the disorganization of the tight junction proteins assembly. Consequently, this allows molecules that normally are restricted to paracellular transport to cross into the brain.

The breakdown of the BBB can occur by direct effects of harmful systemic molecules such as ROS and endothelial cell activation by inflammation. Activation of the endothelial cells occurs when proteins from the immune system (i.e., leukocytes) interact with the endothelial luminal membrane. In turn, this interaction will trigger intracellular signaling pathways, which will cause the opening (breakdown) of the BBB and facilitation of the leukocyte infiltration into the brain.

The interaction between the systemic circulation and the brain can also occur via areas that lack the BBB, like the circumventricular organs (CVO), or through direct interaction with the *vagus* nerve (Morita *et al.*, 2016). The *vagus* nerve originates at the brainstem, and it spreads widely, innervating most of the thoracic and abdominal cavity organs, from the esophagus to the colon. So, while the CVO provides systemic circulation access to specific brain areas, including the pineal gland, part of the pituitary, and the postrema area, the *vagus* nerve directly communicates to the brain from multiple organs. Molecules can reach the brain through the blood-CSF barrier, which is less restrictive than the BBB (even in health), allowing for more molecules to cross freely (reviewed by (Redzic, 2011)).

GS interacts with ammonia ions (NH_4^+). However, most NH_3 (gas) will be converted into NH_4^+ to establish equilibrium under physiological pH. Therefore, ammonia can reach GS in endothelial cells of the BBB by diffusion and transporters. Systemic ROS can activate endothelial cells and induce local oxidative stress (Widlansky and Gutterman, 2011), potentially reducing GS activity. Cytokines can cross the BBB via receptor-mediated transcytosis and affect the BBB via endothelial cell activation, leading to leukocyte migration. In both cases, cytosolic GS would not be in contact with either cytokines or leukocytes since the former would be wrapped around a membrane vesicle, and the latter would cross in between endothelial cells. However, our group, as well as others (Eelen *et al.*, 2018) demonstrated that GS is also membrane-bound, although at lower

levels than those found in the cytoplasm. Therefore, the interaction between GS and cytokines or leukocytes is possible, but this requires further investigation.

The BBB has polarity, with differential transporters expressed in its luminal (blood-facing) and adluminal (brain facing) sides. Generally, systemic molecules only have access to transporters in the luminal (blood-facing) side of the BBB unless they are allowed to cross the BBB. However, when the BBB is physically broken, the loss of the tight junction between the cells allows molecules to contact the adluminal side of the endothelial cells. Therefore, during the physical breakdown of the BBBs, factors can interact with GS by entering the endothelial cells via luminal or adluminal transporters. In addition, all systemic factors might interact with membrane-bound GS. Cytokines can interact with the vagus nerve, impacting the brain by causing inflammation among other effects (Houser and Tansey 2017). Also, all molecules can cross into the CVO, bypassing the BBB (and GS). However, by crossing via the CVO, molecules are restricted to the areas that surround the CVO, not strongly impacting other areas of the brain.

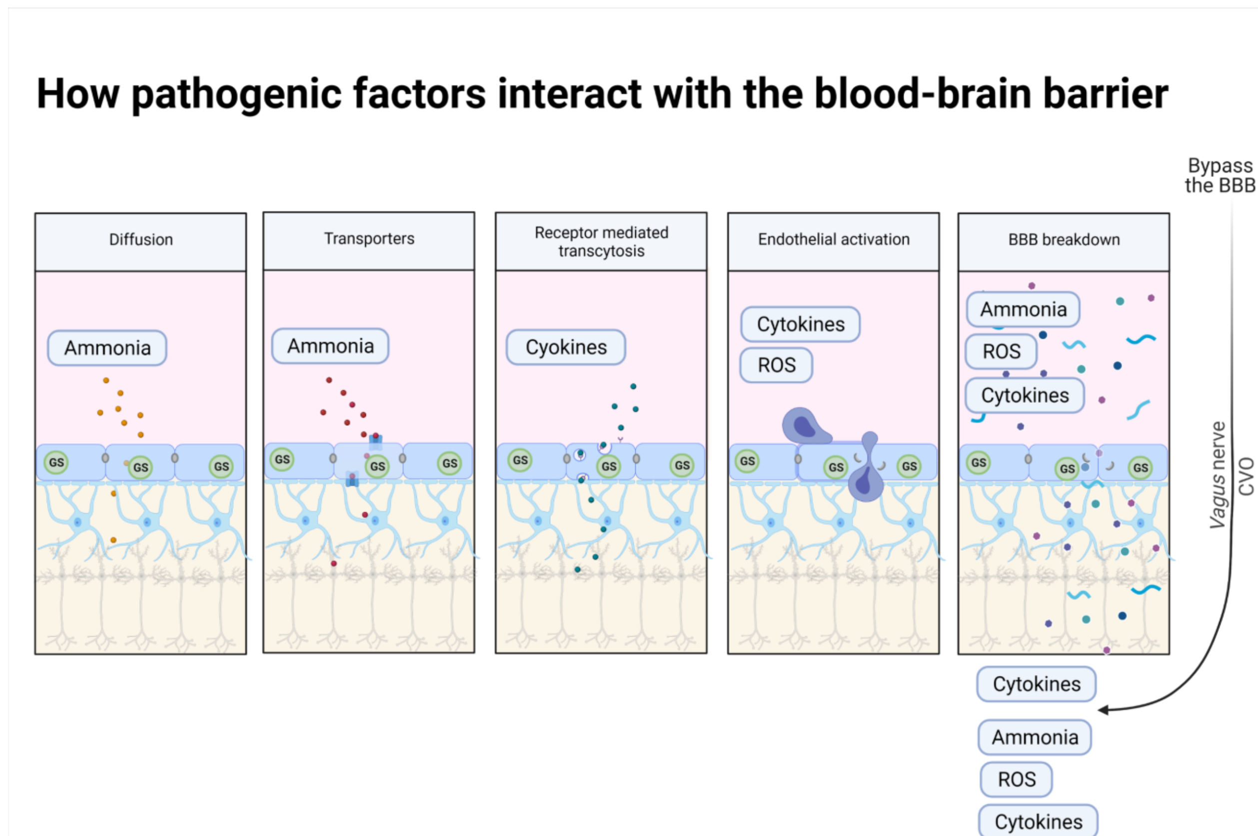

Figure 18. – How systemic pathogenic factors interact with the BBB

Ammonia crosses the BBB by simple diffusion as a gas and via transporters as an ion, and GS can detoxify ammonia. Inflammation mediators (cytokines) cross via receptor-mediated transcytosis, while inflammation and oxidative stress can induce endothelial activation and allow leukocyte migration. ROS from endothelial activation likely affects GS. However, interaction with inflammatory cytokines is unknown. All molecules or ions can easily cross a physically disrupted BBB, entering the endothelial cells by luminal and abluminal transporters and interacting with GS. Inflammation can reach the brain via signaling within the *vagus* nerve while all factors may reach the brain through circumvetricular organs (CVO), bypassing endothelial cells metabolism, including GS.

Hyperammonemia impacts the BBB

Breakdown of the BBB might allow ammonia to bypass the endothelial cells of the BBB, reaching directly to the brain and reducing ammonia's detoxification. However, although increased blood ammonia impacts the permeability of the BBB in type B and C HE models, it is likely without BBB breakdown. It has been shown that PCA rats have increased BBB permeability to the permeability marker horseradish peroxidase (Laursen and Westergaard, 1977). In addition, *in vitro* studies have shown a physical breakdown of the BBB. Exposure of a monolayer of cultured brain microvascular endothelial cells to ammonia increased the permeability to sodium fluorescein, which normally does not cross the BBB (Skowrońska *et al.*, 2012). However, far more compelling evidence exists that ammonia does not cause the BBB's breakdown. In rats, PCA surgery and infusion of ammonium acetate, which further elevated blood ammonia levels, increased the BBB's permeability to specific amino acids without physically affecting the BBB (Mans, Biebuyck and Hawkins, 1983). In addition, Bosoi et al. found no permeability to Evans blue and sodium fluorescein (molecules that do not cross the BBB) in PCA and BDL rats (Bosoi *et al.*, 2012). Even without the BBB's physical breakdown, the presence of ammonia in the brain is associated with neuropsychological impairments; therefore, preventing its entry into the brain is critical.

In conclusion, HE pathogenic factors can access the brain through several routes. Ammonia increases BBB's permeability, but it does not cause a physical breakdown, and ammonia can cross the intact BBB via diffusion or transporters in the endothelial cells, being detoxified by GS.

GS in disease

GS is essential for normal metabolism, including ammonia detoxification, neurotransmission, and energy metabolism. Therefore, its dysregulation is associated with disease states. It remains unclear whether or how GS is dysregulated in the BBB endothelial cells in HE and the potential consequences. However, the impairment of GS in the brain has been documented in several neurocognitive conditions, including HE.

Alzheimer's disease

Alzheimer`s disease is the most common type of dementia, with progressive memory loss and cognitive functions. Brains from patients are marked by the accumulation of β-amyloid plaques, tau proteins dysfunction, and impairment of glutamatergic pathways (Mattson *et al.*, 1992; Domingues *et al.*, 2007). Astrocytes are rich in GS and responsible for regulating glutamatergic neurotransmission (uptake and turnover) through the glutamate-glutamine shuttle.

Post-mortem brains of patients with Alzheimer's showed differential GS expression, with a shift in GS expression from astrocytes to pyramidal neurons, which generally do not express GS (Robinson, 2000). Those changes likely impact glutamate homeostasis in the CNS, but further research is required to understand the consequences fully.

In a transgenic animal model of Alzheimer`s, studies found reduced numbers of GS positive astrocytes compared to control mice (17% reduced at one month of age and 27% reduced at 6 and 9 months of age) medial PFC. The lower number of GS positive cells was accompanied by reduced GS protein starting at 6 months of life (Kulijewicz-Nawrot *et al.*, 2013). In agreement, another study using a transgenic mouse model found similar results (around 30% reduced number of GS positive astrocytes and 20% reduced GS protein expression) in two regions of the hippocampus (dentate gyrus and CA1) at 12 and 18 months of age. In addition, the number of GS-positive astrocytes were significantly reduced in areas with β-amyloid deposits (47% reduced) vs.

β-amyloid free areas (23% reduced) (Olabarria *et al.*, 2011). Furthermore, GS has been shown to increase in CSF and blood from patients with Alzheimer`s and in patients with other neurodegenerative conditions (to a lesser extent) (Tumani *et al.*, 1999).

These changes indicate that plaque deposition is linked with GS dysfunction. In addition, Alzheimer's brains are known to have higher inflammation and ROS, which also impacts GS function (Gella and Durany, 2009; Kinney *et al.*, 2018). Decreased GS expression might underlie the impairments on glutamate neurotransmission contributing to impaired glutamate homeostasis impacting mood, cognition, and memory via dysregulated synapsis (Kulijewicz-Nawrot *et al.*, 2013).

Epilepsy

The role of GS on the pathogenesis of epilepsy also involves glutamatergic neurotransmission, and GS is deficient in the hippocampus of patients with temporal lobe epilepsy. Consequently, this leads to higher glutamate levels in the hippocampus and might contribute to the initiation and propagation of seizures. Patients with temporal lobe epilepsy and hippocampal sclerosis (atrophy and neuronal loss in the hippocampus) have further astrocytic impairments with lower GS immunoreactivity and protein levels and higher gliosis associated with neuronal loss vs. patients with temporal lobe epilepsy without sclerosis (van der Hel *et al.*, 2005).

Mice with MSO treatment via microinfusion in the hippocampus showed that GS inhibition (82-97% vs. saline) resulted in recurrent seizures lasting for weeks. In addition, some of the mice showed signs of hippocampal sclerosis, similar to what is found in temporal lobe epilepsy. However, animals had GS dysfunction even in the absence of hippocampal sclerosis (Eid *et al.*, 2008). In addition, in a genetic model of epilepsy in rats, seizures arise at one month of age, accompanied by increased GFAP expression with reduced GS expression in the thalamus, while GDH was also increased. These modifications preceded the development of seizures, confirming that astrocytic dysfunction, including reduced GS, are involved in the pathways leading to epileptic seizures (Dutuit *et al.*, 2000).

GS inborne mutations

According to GS's essential role in neurotransmission, GS deficiency has severe effects on the CNS. Inborne GS deficiency is an ultrarare condition that results in severe epileptic encephalopathy followed by early death. Patients with GS deficiency have brain malformations, including brain atrophy and hypomyelination of the white matter. In addition, they present hyperammonemia with lower plasmatic levels of glutamine, although no consistent changes in plasma glutamate (Spodenkiewicz *et al.*, 2016). Therefore, the pathogenesis of this syndrome lies beyond glutamate excitotoxicity, namely glutamine's importance in energy metabolism. In agreement, supplementation with glutamine showed positive, although limited, results as a treatment option (Häberle *et al.*, 2012).

Similarly, inhibiting GS in mice pups during synaptogenesis in the hippocampus causes lower glutamatergic neurotransmission and spatial memory impairment during adulthood (Song *et al.*, 2019). In addition, selective GS deletion in the whole brain of mice results in a severe decrease in brain glutamine levels, mild hyperammonemia, and neonatal death (He, Theodorus B M Hakvoort, *et al.*, 2010). Deletion of GS restricted to the cerebral cortex results in a milder phenotype, with a 4-fold glutamine increase and no early death, although the animals suffer from decreased locomotion, neurodegeneration and seizures (Zhou *et al.*, 2019).

Other diseases

GS was also downregulated in the brain in other diseases such as traumatic brain injury and major depression disorder (Zhao *et al.*, 2003; Choudary *et al.*, 2005). However, although GS is downregulated in several neurological conditions, the opposite has been demonstrated. In the cerebellum of patients that died from acute hypoxic-ischemic episodes, assessment of brain GS showed upregulated GS activity vs. non-hypoxic neurological disease (Dao, Ahdab-Barmada and Schor, 1991). Likewise, rats submitted to 30 minutes ischemia and euthanized after 24 hours had higher GS measured by immunohistochemistry, with enlargement of GS positive cells and higher number of GS positive cells (Petito *et al.*, 1992).

Hepatic encephalopathy

In HE, brain GS plays a vital role in ammonia detoxification besides regulating neurotransmission. The brain is a sensitive organ and therefore susceptible to ammonia's toxicity. Therefore, the brain relies heavily on astrocytic GS to detoxify ammonia and prevent neurological dysfunction. However, the consequences of GS regulation in the brain during hyperammonemia and HE are unclear.

High brain glutamine levels indicate an increase in GS activity during hyperammonemia, especially in animal models of type A HE (Takahashi *et al.*, 1991; Cudalbu *et al.*, 2012). In addition, increased brain GS mRNA was found in a TAA-induced type A HE model (Thomas *et al.*, 1988). Increased GS activity and, consequently, glutamine levels are considered problematic. Since glutamine is a vital osmolyte, increased during brain edema in HE, studies evaluated the effect of GS inhibition on brain edema. Inhibition of GS with MSO protected mice against increased brain water and intracranial hypertension in acute hyperammonemia models (PCA + ammonium acetate or ammonium acetate only) (Blei *et al.*, 1994; Willard-Mack *et al.*, 1996; Tanigami *et al.*, 2005). However, treatment with MSO causes increased brain ammonia levels, leading to toxicity (Hawkins *et al.*, 1993). Furthermore, MSO is a non-specific drug and interacts with other substrates besides GS, such as glutamate-cysteine ligase and ornithine decarboxylase (Richman, Orlowski and Meister, 1973; Di Giacomo *et al.*, 1997), and therefore its effects cannot be directly associated with changes in GS.

Other studies related reduced GS levels during hyperammonemia in PCA in rats (Girard, Giguère and Butterworth, 1993; Desjardins *et al.*, 1999) or cultured astrocytes exposed to ammonia (Leite *et al.*, 2006). Differences in time and dose of the ammonia exposure (acute higher dose vs. chronic lower dose) possibly dictate the GS response and outcomes. In addition, other factors such as ROS in CLD and HE contributes to post-translational modifications in GS, such as tyrosine nitration, reducing its activity (Häussinger *et al.*, 2005; Görg *et al.*, 2007). In this setting, increasing GS levels would reduce ammonia levels, therefore protecting the brain.

In conclusion, GS in the brain is reduced in different neurocognitive disorders, contributing to dysregulation of glutamatergic synapses, glutamine energy metabolism, and ammonia

detoxification. In the BBB, reduced GS expression or activity might allow for a faster ammonia entrance into the brain.

GS treatments

Our study demonstrated that ornithine is an excellent target to enhance GS activity. Accordingly, ammonia scavenger therapies such as OP and LOLA have been shown to detoxify ammonia by GS specially in muscle. These treatments act by increasing GS activity by supplying glutamate for GS. However, the amount of GS in a cell might restrict its potential stimulation, and other strategies that increase GS expression can be used.

Gene therapy

Muscle targeted

Different methods for gene therapy were created to increase GS and protect against hyperammonemia. Baculovirus is a well-known vector used in vaccines for animals and humans (Fabre *et al.*, 2020). A study assessed the potential of cDNA from the GS gene delivery by a baculovirus. *In vitro*, epithelial cells, which do not express GS, when transfected with GS by this method showed higher ammonium chloride consumption than non-transfected cells. In addition, rats were injected with the GS cDNA intramuscularly and challenged with an ammonium acetate injection three days later. Rats injected with GS cDNA had two-times increased GS muscle expression as well as 66% higher ammonia detoxification compared to control rats (181.4 µmol/L for GS vs. 532.8 µmol/L for controls) (Torres-Vega *et al.*, 2015).

Using the BDL rat, another study showed the efficacy of the intramuscularly injected GS baculovirus during CLD. Three weeks after surgery, GS baculovirus (incubated three days before) caused decreased blood ammonia (128.3 µmol/L BDL-GS vs. 276.7 µmol/L BDL-control) (Espíritu-Ramírez *et al.*, 2018). Other studies showed that therapies involving similar delivery methods (baculovirus*) in vivo* last from 1 week to several months (Pieroni, Maione and La Monica, 2001; Luo *et al.*, 2013). However, in the BDL model, the ability to detoxify ammonia was lost at four weeks of BDL (10 days after the GS inoculation), showing that the GS vector in BDL rats does not last long (Espíritu-Ramírez *et al.*, 2018). Unfortunately, re-administration of baculovirus was

shown to cause innate and adaptative immune responses, and it is therefore not recommended (Luo *et al.*, 2013). Therefore, GS-baculovirus therapy does not seem the best strategy for CLD.

Another study used an electroporation device to deliver GS DNA plasmid to skeletal muscle by low-voltage electropermeabilization. Two days after GS inoculation in the muscle of mice, an ammonia challenge was performed. GS treatment caused a 30% decrease in ammonia levels compared to controls. In addition, two days after GS gene therapy, mice were submitted to ALF by TAA injection. GS treatment reduced ammonia levels (366.3 µmol/L for GS vs. 641.1 µmol/L for control) and reduced mortality by 50% on the fourth day after TAA injection (Khoshnejad *et al.*, 2020). Although the ammonia reduction after the challenge was less than GS treatment with baculovirus, GS DNA plasmid can be repeatedly delivered without immune response, allowing for multiple interventions (MacGregor *et al.*, 2000), constituting a better option for GS delivery.

Liver targeted

Intravenous injection of an adenoviral vector with GS expression under the control of a liver-specific expression cassette was done in mice to cause specific overexpression of hepatic GS. Upon ammonia challenge, four weeks after the vector inoculation, mice that received GS had a 39% reduction of ammonia levels and increased glutamine compared to controls. In addition, when inoculated in a mouse model of UCD (Cps-1 deficient), GS vector reduced ammonia after challenge and at baseline. The GS expression was specifically seen at the pericentral hepatocytes, confirming the target of the GS vector (Soria *et al.*, 2019). This treatment was not tested in models of CLD, and the loss of hepatocytes in a cirrhotic liver might impose a challenge for the enzyme's target, reducing its chance of success.

Enzyme therapy

In 3 week BDL rats supplemented with hyperammonemia inducing diet, treatments with a recombinant GS enzyme intraperitoneally resulted in lower ammonia levels than vehicle-treated rats (144 µmol/L for vehicle vs. 91 µmol/L for GS). In addition, GS treatment reduced HE-related brain impairments (brain edema) (Song *et al.*, 2019). Similarly, in a hyperammonemic mouse model of UCD, GS injection caused reduced plasma ammonia compared to vehicle-treated mice.

The authors showed that GS activity increases in plasma and the liver after injection, where GS can be found in high levels in the pericentral hepatocytes (Song *et al.*, 2019).

Encapsulated protein delivery

GS can be encapsulated in erythrocytes to protect it from early degradation and increase the duration of the ammonia lowering effect. After mice were injected with ammonium acetate intraperitoneally, the group that received erythrocytes loaded with GS showed around 50% lower ammonia levels than those with control erythrocytes (Kosenko *et al.*, 2008). However, since erythrocytes are not permeable to glutamate, ammonia lowering capacity would be limited. Fortunately, it is possible to add other enzymes of the ammonia metabolism to boost ammonia detoxification and allow for longer treatment times when encapsulating red blood cells (Protasov *et al.*, 2019). Based on our results, the addition of OAT to the erythrocytes, coupled with an ornithine treatment, would provide glutamate to stimulate GS in the erythrocytes, muscle, and endothelial cells of the BBB.

GS therapy for the BBB

The GS treatments discussed here aimed to stimulate ammonia detoxification by GS in muscle, liver, and plasma. In addition to these targets, it would also be possible to develop a system to stimulate GS in the endothelial cells of the BBB to protect the brain against ammonia's toxicity. Gene therapy using peptides that target low-density lipoprotein (LDL) receptors is being developed to manage the delivery of drugs through the BBB via receptor-mediated transcytosis (Molino *et al.*, 2017). This type of therapy is promising for successfully delivering genes *in vitro* and in animal models. However, different from therapies aimed at the CNS, the vector would have to cross the luminal endothelial barrier without crossing the abluminal barrier, preventing transport into the brain to increase GS in the BBB. Technologies increasing gene expression on the cells of the BBB are being developed, but the brain is still the primary target (Wu *et al.*, 2020). Therefore, more studies are needed to increase GS expression on the endothelial cells of the BBB.

However, increasing GS expression in the brain or BBB might be challenging since high GS can impact normal function. GS overexpression in the brain might dysregulate the glutamine-glutamate cycle, impairing neurotransmission. In addition, glutamine is a crucial osmolyte, and

increased glutamine formation by GS is associated with cytotoxic brain edema, with astrocyte swelling, in hyperammonemic models (Takahashi *et al.*, 1991; Jayakumar *et al.*, 2006). Swelling of endothelial cells of the BBB has been demonstrated before (Krueger *et al.*, 2019); therefore, GS overexpression could cause endothelial swelling.

In addition, since GS overexpression would be transitory, there is a risk of ammonia rebound in the brain or BBB treated with GS therapy. The enzyme glutaminase, found in neurons and possibly in endothelial cells of the BBB (Lee *et al.*, 1998), can re-convert the formed glutamine back into glutamate, releasing ammonia. Treatment with phenylacetate could prevent ammonia rebound by binding with glutamine and stopping reconversion by glutaminase. However, phenylacetate crosses into the brain only at small levels (Sandler *et al.*, 1982), and it is unknown if it enters or bypasses the endothelial cells of the BBB. In addition, the enzyme responsible for the conjugation of glutamine and phenylacetate into phenylacetylglutamine, phenylacetyltranferase, might not be present both in the brain and in the endothelial cells of the BBB. Finally, even if the enzyme exists, it is necessary to ensure the efflux of phenylacetylglutamine from the brain and into the blood, preventing accumulation.

In conclusion, GS therapies effectively lower ammonia in pre-clinical research, especially in the liver and muscle, but their use for brain and BBB's endothelial cells still need further studies.

Sex differences

We demonstrated that female BDL rats had similar liver injury than male BDL rats, even though they had lower plasma ALT. Although we have unveiled sex-based differences in CLD and HE, there are still several parameters that can be evaluated between males and females, both in health and disease.

Liver

The liver is considered a sex-dimorphic organ (Buzzetti *et al.*, 2017), and differences in hepatic morphology and metabolism in health might determine how male and female BDLs respond to liver disease, including the CLD-associated complications. In addition, although we only evaluated

sex differences in the BDL rat, a model of cholestasis, there is evidence that sex impacts other etiologies of liver disease.

Morphology

Hepatic morphology is also unique among males and females. Although studies in humans are lacking, studies in rodents showed that female rat livers have more Kupffer cells and hepatocytes per gram of tissue. That might be due to estrogen's role in cell proliferation and potentially impacts the liver detoxifying capacity (Atchley, Wei and Crenshaw, 2000; Marcos *et al.*, 2016). The localization of the Kupffer cells was also different. In female rats, fewer Kupffer cells were close to stellate cells (Marcos *et al.*, 2016). That is important since Kupffer cells can stimulate the activation of stellate cells and vice versa, affecting the progression of fibrosis during hepatic injury, potentially presenting a less inflammatory phenotype in females.

The liver is one of the few organs with polyploid cells. Around 30% of hepatocytes are polyploid in the human liver, and in rodents, it is close to 80%. The polyploidy of hepatocytes came from increased DNA per nucleus and an increased number of nuclei per cell (Donne *et al.*, 2020). The liver from females has fewer polyploid cells, which might be related to its regeneration capacity since diploid hepatocytes are known to divide quickly for regeneration (Gupta, 2000). In addition, polyploid cells are more susceptible to cancer (Donne *et al.*, 2020), which might partly explain the higher predisposition of males to hepatic cancer.

Unlike other organs, the liver is highly regenerative, and studies showed that the female rat liver has a higher capacity for regeneration (Tsukamoto and Kojo, 1990; Kitagawa *et al.*, 2009). In rodent models of ALF submitted to hepatectomy, a more significant portion of the female liver needs to be removed to cause the same phenotypic changes as males (Imamura *et al.*, 1999). However, little is known in humans, and more research is needed to unveil the potential differences in liver regeneration.

In the BDL rat, differences in liver morphology might change the liver disease progression. At 6 weeks post-BDL, we saw that female rats developed comparable degrees of liver damage, histologically assessed with hematoxylin-eosin. However, it is still unknown if the hepatic injury progresses at the same rate in males vs. female BDL. Comparing changes in markers of liver injury

and function and histology at early time points between male and female BDLs would answer this question. Three weeks would be a good time-point for histological analysis since male BDLs show severe signs of cirrhosis (Tarcin *et al.*, 2011), and female protection would be easier to identify. In addition, it is vital to analyze the markers as changes from same-sex SHAMs, so inherit differences will not be confounders.

<u>Drug metabolism</u>

In vitro hepatocytes from males and females exhibit different responses to drug toxicity. A study with human hepatocytes from female and male volunteers showed significant sex-based differences, with a drug-specific sensitivity to hepatotoxins. The calcium channel blocker verapamil caused higher toxicity to hepatocytes of women (pre and post-menopause). However, pre-menopausal women were protected against chlorpromazine and acetaminophen's toxicity compared to males and post-menopausal females (Mennecozzi *et al.*, 2015). The toxicity of these drugs seems to be linked to the antioxidant response to toxins, which is also sex dependant. While hepatocytes from females had a higher antioxidant capacity and lower ROS accumulation after acetaminophen treatment than men, the contrary was true for treatment with verapamil, which caused lower ROS in males (Mennecozzi *et al.*, 2015). The sex differences found in drug metabolism are mainly due to the differential expression of cytochrome P450 enzymes, which are differentially expressed between males and females (Yang *et al.*, 2012).

Although the BDL rat is not a drug-induced model, females had lower oxidative stress associated with higher albumin levels compared to males in our study. However, we did not measure hepatic oxidative stress, and we did not evaluate the differences in the hepatic antioxidant systems between male and female BDL rats.

Studies in rats confirmed sex-specific expression of cytochrome P450 enzymes, with differentiation between P450-male and P450-female (Kato and Kamataki, 1982; Kato and Yamazoe, 1992). In agreement, a study that compared CYP mRNA tissue distribution showed that females have 7% more CYPS in the liver (Renaud *et al.*, 2011). Also, the P450 complex is regulated by growth hormone (GH), which has a sexually dimorphic release from the pituitary. While males have a pulsatile pattern of GH release, females have a nearly continuous release (Waxman and

Holloway, 2009), resulting in specific gene expression and phenotype. Female rats are more susceptible to anesthetic drugs such as barbiturates, needing half of the dose for the same effects than males (Barron, 1933). However, although the metabolism of some drugs is slower in female rats vs. males, differently than humans, female rats are protected against several hepatotoxins (see later).

Etiology of liver disease

We elucidated sex-based differences only in the BDL rat, a model of cholestasis. However, other etiologies also impact males and females differently.

Acute liver failure

Generally, while men have a higher prevalence of CLD, females have a higher prevalence of acute drug-induced hepatitis, with females responsible for 74% of the ALF due to acetaminophen and 67% of the cases by other drugs (Lee *et al.*, 2008). In a study, almost all patients that developed fulminant hepatic failure due to drugs (89%) were women (Andrade *et al.*, 2005). Drug-induced ALF shows a substantial sex bias toward women in the US, with 73% of cases being females, with a mortality rate around 80% (Miller, 2001; Ostapowicz *et al.*, 2002).

Although differences between males and females are clear in animal models, results seem to disagree with what is found in humans. Female mice are protected against acetaminophen-induced liver failure, having less liver injury after administering similar doses than males (Du *et al.*, 2014; Mohar *et al.*, 2014). In the TAA-induced model of ALF, females also have a higher survival rate than males. It was also proven that the males' (and not the females') hormonal profile plays a vital role in the susceptibility to ALF by TAA administration. Gonadectomy in both males and females before TAA injection caused no changes in females, but protected males against ALF, showing that testosterone has a deleterious effect on ALF progression (Koblihová *et al.*, 2020).

Viral hepatitis

The incidence of viral hepatitis varies according to the region of incidence. For hepatitis C virus, men are more affected than women, with higher prevalence, morbidity, HCC, and mortality

(Buzzetti *et al.*, 2017). Females with hepatitis have symptomatic disease, with jaundice being a frequent symptom. Females also have higher spontaneous viral clearance than males (Bulteel *et al.*, 2016), which could be due to the differences in immune systems between males and females or estrogen receptor-mediated effects. Hepatitis B virus is sex-responsive. Androgen receptor activation increases mRNA production (Wang *et al.*, 2009), while estrogen receptor activation reduces mRNA levels (Wang, Chen and Yeh, 2015). The rate of fibrosis progression in patients with hepatitis is higher in men, independent of their alcohol intake (Poynard, Bedossa and Opolon, 1997), again showing that women are better protected against CLD from viral hepatitis. Lower fibrosis in women is likely due to estrogens and increased hepatic antioxidants that reduce stellate cell activation (Shimizu *et al.*, 2007). However, females have a higher incidence of adverse events such as anemia and more frequently need dose adjustments with treatments such as pegylated-interferon and ribavirin (Narciso-Schiavon *et al.*, 2010).

In rodent models of hepatitis B acute infection by injection of HBV genome, females showed lower HBV DNA and protein levels than males. That was paired with functional differences in T lymphocytes. The same differences were seen in other models of HBV (the woodchuck hepatitis virus transgenic and an HBV-tolerant immunocompetent mouse model), in which males had worse virus control than females (Yuan *et al.*, 2016; Kosinska *et al.*, 2017). The few studies that explore sex differences on the impact of hepatitis virus on liver injury show that in transgenic mice, the expression of HCV proteins induced liver cancer, which was lower or absent in females (Lerat *et al.*, 2002; Sekiguchi *et al.*, 2012).

NAFLD

It is not clear if the prevalence of NAFLD is higher in males or females. While female sex is still considered a risk factor for some authors (Neuschwander-Tetri and Caldwell, 2003), it appears that NAFLD prevalence is dependent on the hormonal cycle in females, with women being protected prior to menopause and being more at risk after menopause (Hashimoto and Tokushige, 2011), which might imply protection from estrogens in women. In agreement, a decrease in estrogen during menopause is accompanied by increased visceral adipose tissue, a risk factor associated with NAFLD (Völzke *et al.*, 2007). Knowing that estrogens results in the differences in body composition promoting fat storage in the gluteo-femoral area and not in

visceral tissue, the protection of pre-menopausal women might be due to protection against metabolic syndrome, which is connected to NAFLD.

Female mice are more susceptible to NAFLD. Female mice with NAFLD from fructose ingestion (16 weeks) have higher hepatic inflammation than male mice (Spruss *et al.*, 2012). In addition, 8 weeks with a choline-deficient high fat diet induces similar levels of liver injury (ALT, AST, ALP) in both sexes. Still, differently from males, female mice gain more bodyweight and have higher hepatic hydroxyproline than regular diet matched controls, suggesting a worse development of NAFLD in females (Heintz *et al.*, 2020). Although estrogen levels were not measured, the mice's age (10 weeks old) and the mild severity of the liver injury suggest the females were normally cycling and with normal estrogen levels. Therefore, it is likely that the mechanisms protecting pre-menopause women from NAFLD, such as fat distribution, do not hold the same weight in mice models of NAFLD.

Our lab characterized a model of NASH and cholestasis mixing the effects of BDL with a high-fat diet in male rats to produce a model of CLD and HE with fast progression and severe hepatic damage (compared to NASH alone). Obese male BDLs had faster HE progression, with motor coordination impaired at 3 weeks after surgery (vs. 5 weeks in lean BDL), with worse liver injury, but no muscle mass and function changes. Although we did not evaluate the effects of BDL + high-fat diet in females, we anticipate that liver disease might progress faster, and females might have a worse liver injury, which would cause worse HE, vs. lean female BDLs.

Alcohol-related liver disease

Women are more susceptible to alcohol-related liver disease than men. Even though men have higher alcohol intake, women have over twice as high a risk of developing alcohol-related cirrhosis (Guy and Peters, 2013). Controlling the progression of liver injury seems to be particularly challenging in women. Fibrosis progression is faster in women (Poynard *et al.*, 2003). In addition, a study showed that alcohol abstinence protects against progression to cirrhosis in men but not in women (Parés *et al.*, 1986).

As in humans, female rodents are more susceptible to liver injury due to alcohol than men. Female mice submitted to 3 months exposure to increasing concentrations of ethanol (up to 20%) in

drinking water had a discrete presence of liver steatosis (small droplets) and increased liver/body weight ratio, which was not found in male mice (Alharshawi *et al.*, 2021). In agreement, an intragastric alcohol challenge (6 g/kg bodyweight) caused 3x higher hepatic lipid levels in female mice than males, although both sexes showed increased plasma transaminases and acute hepatic steatosis (Wagnerberger *et al.*, 2013). Inflammation and plasma endotoxin upon challenge with lipopolysaccharides (LPS) are higher in females than males that received alcohol and contribute to hepatic damage, with estrogen playing an important role (Thurman, 1998). A sub-lethal dose of LPS (in control rats) caused death when administered in estrogen pre-treated rats. In addition, death was prevented by exposure to gadolinium chloride, a Kupffer cell toxicant. Levels of plasma TNF-α correlated with mortality, showing that exposure to estrogen treatment causes Kupffer cell sensitivity to endotoxin and contributes to a more pro-inflammatory profile (Ikejima *et al.*, 1998).

Since models of pure alcohol-induced liver disease are long and cause only mild liver injury, our lab is currently developing a HE model from mixed etiology, using the BDL rat coupled with ethanol exposure (intragastric) in males. We saw that adding ethanol caused further brain impairment in the male BDL (memory and motor coordination), without further liver injury compared to male BDL vehicle treated rats. However, we did not explore the effects of BDL+ethanol in female rats yet. Based on the literature, we would expect to see faster liver disease progression in female BDL+ethanol compared to male BDL+ ethanol.

Cholestasis

The two leading causes of cholestasis, PBC and PSC, have a sex-dependent prevalence. However, since both conditions are not common, little is known about the differences in disease pathogenesis by sex since it is difficult to obtain enough patients for sex comparisons. For biliary cirrhosis, males are less symptomatic than females. Pruritus abdominal pain/discomfort are more common than in females, while GI bleeding and jaundice are more common in males (Durazzo *et al.*, 2014).

PSC has a male predominance, with seven male patients for three females, with no known treatment besides liver transplant (Buzzetti *et al.*, 2017). Contrary to that, PBC affects women 10x more than men. In females, UDCA is the only treatment for PBC, with liver transplant being the

final option for advanced disease. Females also have pruritus as the first symptom of PBC (Smyk *et al.*, 2012), while men with PBC are older, have more severe disease, and do not respond to UDCA (Carbone *et al.*, 2013).

In rats, we showed that BDL causes comparable liver disease between males and females with impaired histology and markers of liver injury (AST and bilirubin) and impaired liver function (albumin and ammonia). However, some differences were found. BDL surgery increased ALT in males but not in female BDLs. Although we do not know why ALT is not increased in our 6 weeks model, another study showed that 2-week female BDLs had increased ALT levels (Chang *et al.*, 2013). Therefore, females might have higher ALT levels early in liver disease progression, and weekly blood measurements of liver injury markers would help answer that question. In addition, albumin levels were higher in male BDL than female BDL. However, this is primarily due to inborne differences in albumin since female SHAMs also had higher albumin than male SHAMs (data not shown).

In conclusion, the etiology of liver disease impacts the differences between males and females. In cholestasis, female BDL rats have similar liver injury compared to male BDLs after 6 weeks. However, the progression of the liver disease still needs to be evaluated.

Complications from liver disease

We evaluated the impact of sex on the incidence of complications of liver disease in the BDL rat for the first time. Our study found differences in the incidence and severity of GI bleeding and ascites in males vs. female BDLs (Annex 3). However, there is little data (pre-clinical or clinical) on the incidence and management of such complications between the sexes.

Portal hypertension

Portal hypertension is a driver of other complications from CLD, such as ascites and GI bleeding. There is no data on the prevalence of portal hypertension in males vs. females. However, a study showed that porto-pulmonary hypertension, a complication of portal hypertension, affects 6% of patients with advanced liver disease, with the majority being women (Kawut *et al.*, 2008). Other studies have shown that females are more susceptible to pulmonary hypertension (Lahm, 2021), indicating that portal hypertension is more severe in women. However, studies in different

populations (not restricted to CLD) showed that females with pulmonary hypertension have better hemodynamics than men (Ventetuolo *et al.*, 2014), meaning that females cope better with portal hypertension, developing fewer symptoms.

There is no data on the impact of sex on portal hypertension in pre-clinical studies. In our study, the absence or lower incidence of complications that arise from portal hypertension in female BDLs indicates two things. Either female rats are protected from the development of complications that arise from portal hypertension, or they are protected against the development of portal hypertension itself.

Ascites

Little is known on the prevalence of ascites in men and women since most studies do not separate the participants by sex. However, a study with 100 patients with alcohol-related liver disease evaluated the incidence of complications by sex. The study showed a higher incidence of ascites in women (30%) vs. men (12%). However, alcohol-related liver disease might be more severe in women. In addition, ascites in the study were presented together with jaundice and peripheral edema, characterizing a severe complication from liver disease (Morgan and Sherlock, 1977). Therefore, it is necessary to evaluate the impact of sex on ascites in cirrhosis due to other etiologies.

In our study, contrary to what is found in patients, females were protected against ascites. In male BDLs, 45% developed ascites, with an average of 5 ml of fluid in the abdominal cavity, while none of the females presented ascites (Annex 3).

GI bleeding

Compared to men, women with esophageal varices are protected against variceal bleeding (Haukeland *et al.*, 2020). A study with 100 patients with cirrhosis from alcohol abuse showed that men have a higher incidence of GI bleeding. While 9% of the men had GI bleeding, no females had it (Morgan and Sherlock, 1977). Women also have lower in-hospital mortality (Rubin, Sundaram, and Lai 2020). High alcohol consumption is also associated with an increased risk of death in patients with variceal bleeding (Haukeland *et al.*, 2020), and lower alcohol intake in women might be protective against death from variceal bleeding.

Portal hypertensive gastropathy (PGH) is a common complication of CLD, which causes GI bleeding. In a study with 110 patients, severe PHG (vs. mild PHG) was associated with the male sex. Moreover, there were more males in both mild and severe PHG. However, the numbers in this study may reflect the higher prevalence of male patients with CLD (Simbrunner *et al.*, 2020).

Similar to what is found in patients, we saw that male BDL rats developed a higher incidence of GI bleeding than females (Annex 3). In male BDLs, 73% had moderate to severe GI bleeding (measured as blood or reddish-brown food mass inside the stomach or intestines), while only 11% of the females had the same degree of bleeding.

In conclusion, the impact of sex on complications of CLD still needs to be further evaluated. In BDL rats, females had a lower incidence of all complications at 6 weeks after surgery. These results suggest that a sex-based approach to CLD might be beneficial.

Muscle

In BDLs, females are protected against loss of muscle mass and function, although we do not know if those differences are associated with innate muscle characteristics being different between males and females. Muscle is influenced by hormones and has a sex-dependent gene expression, with over 3000 genes being expressed differentially (Haizlip, Harrison and Leinwand, 2015a). Therefore, differences in muscle morphology and metabolism might explain the lack of sarcopenia in female BDL rats.

Morphology

Satellite cells are proliferative muscle cells, and therefore, the source of new myofibers. There are sex-based differences in satellite cells activation and proliferation. Male mice have a higher number and proliferation of satellite cells coupled with more mRNA related to differentiation and hypertrophy, such as myogenin and MyoD (Neal, Boldrin and Morgan, 2012). This effect is in part linked to hormonal regulation since the importance of testosterone on satellite cell proliferation was shown by several studies.

Reduced testosterone decreases satellite cell number and reduces muscle size, and supplementing testosterone in female rats and other animal models increases satellite cell

numbers (Mulvaney, Marple and Merkel, 1988; Joubert and Tobin, 1989). Moreover, treatment of satellite cells with serum from control males caused higher proliferation and differentiation than cells treated with serum from females or castrated males (Lee *et al.*, 2011), confirming the importance of testosterone for satellite cell proliferation and muscle mass. In addition, treatment with testosterone also increases muscle mass and the number of myonuclei in females (Gutmann, Hanzlíková and Lojda, 1970; Egner *et al.*, 2013).

In animal models, estrogen hormones are essential for muscle maintenance, and KO of estrogen receptors impairs muscle regeneration after injury in mice (LaBarge *et al.*, 2014). However, a decrease in estrogen is also paired with increased muscle mass. Female mice with gonadectomy have increased body weight and muscle mass, although a loss of muscle strength is paradoxically present (Moran, Warren and Lowe, 2006).

In women, hormonal changes from menopause are associated with muscle loss. Like animal models, supplementation with testosterone increases protein synthesis (by 50%) in postmenopausal women (Smith *et al.*, 2014). However, contrary to what is found in animal models, low estrogen due to menopause in women causes a decline in lean mass (Bea *et al.*, 2011). In agreement, hormonal replacement therapy with estrogens in women improves mobility, muscle power, and muscle mass (SIPILÄ *et al.*, 2001; Ronkainen *et al.*, 2009). That suggests that the effect of estrogens in muscle might be different in humans compared to animal models.

Unfortunately, we did not evaluate sex differences in muscle morphology between male and female rats. However, we would expect female SHAMs to have a lower presence of satellite cells, as it happens in mice.

In conclusion, female SHAM rats might have a lower amount of satellite cells in the muscle, associated with lower testosterone levels in females. However, that does not impact muscle mass and function in female BDL rats.

Function

Males have a higher percent muscle mass and strength than women, while women have higher resistance to muscle fatigue. Higher muscle strength in men is associated with overall larger

muscle fibers (Miller *et al.*, 1993). In women, higher muscle resistance allows them to keep muscle activity for longer while having continuous or intermittent low to moderate muscle contractions (Wüst *et al.*, 2008). A study showed that females' elbow flexor and knee extensor muscles are more resistant to a fatigue protocol than males (Albert *et al.*, 2006). Moreover, the lower endurance in men is not due to higher exerted force, since when both men and women have similarly exerted force, women still show higher endurance (11 min vs. 18 min) (Hunter, Critchlow and Enoka, 2004).

Because males have greater muscle size, blood flow restriction by the contraction of larger muscles in men could reduce blood flow and result in loss of energy supply, decreasing resistance compared to women. However, females maintain higher muscle endurance than males even when blood flow is similarly restricted (Wüst *et al.*, 2008).

Muscle fiber composition shows differences between sexes. Muscle fiber can be divided broadly into type I fibers (slow-twitch, oxidative), responsible for exercise endurance, and type II fibers (rapid-twitch, glycolytic), responsible for power and speed. While women's muscles have more type I fibers, men's have a predominance of type II fibers (Haizlip, Harrison and Leinwand, 2015b). Estrogens likely play a role in fatigue resistance since greater fatigue resistance was also reported in post-menopausal women with lower estrogen levels (Hicks and McCartney, 1996).

Similar results are found in muscle fibers differences in mice. Female mice have the higher maximal running capacity (measured as distance) and greater (20%) work to exhaustion than age-matched males. That is accompanied by increased type I and decreased type II fibers in females vs. males (Oydanich *et al.*, 2019). Sex hormones contribute to both differential function and fiber composition. Higher exercise endurance in mice is associated with estrogen hormones since gonadectomy in females resulted in the loss of the enhanced exercise capacity, and supplementing males with estrogens increased their endurance to similar levels compared to intact females (Oydanich *et al.*, 2019). Supplying estrogen to female rats with gonadectomy results in higher exercise endurance on a treadmill than untreated females with gonadectomy (Kendrick *et al.*, 1987).

We did not evaluate muscle endurance in our model. However, agreeing with the literature, we found lower muscle strength measured in the forelimbs (but not hindlimbs) by the grip strength test (data not shown) in female SHAMs than male SHAMs.

In conclusion, female SHAMs have lower muscle strength than males, and female SHAMs likely have higher endurance than males, associated with differences in muscle fiber composition.

Metabolism

Muscle metabolism is sex-dependent. While males have higher glycolytic capacity and dependency on glycolytic pathways, females have greater fat oxidation in muscle (Hicks, Kent-Braun and Ditor, 2001). Males and females have overall the same protein synthesis with mammalian target of rapamycin (mTOR) activation from stimuli such as eating a standard meal (15% as protein, 55% as carbohydrates, 30% as fat)(Smith *et al.*, 2009) or during high-intensity leg resistance exercise while fasted (Dreyer *et al.*, 2010). However, other studies found higher protein synthesis in females after protein supplementation (whey protein) (Horstman *et al.*, 2019) or increased in males after sprint interval training (Scalzo *et al.*, 2014). The different stimuli and techniques for protein synthesis measurement might explain why different results were found.

Although no clear conclusions can be drawn for protein synthesis, the pathways for protein degradation (ubiquitin-proteasome and autophagy pathways) are impacted differently between sexes. Women have lower mRNA related to ubiquitin-proteasome pathways and higher related to autophagy pathways for protein degradation than males (Rosa-Caldwell and Greene, 2019).

Female rats show higher total gastrocnemius muscle protein content (Colom *et al.*, 2007). However, whether that is due to higher protein synthesis, or lower protein degradation is not known. Sex hormones have an essential role in muscle energy metabolism. Males have higher ATP content and activity of creatine phosphokinase and myokinase enzymes than females, and gonadectomy decreases both ATP and enzymes in both sexes. Supplementation with testosterone reverses the effect in males, while in females, it enhances ATP content and enzymes activity to the same levels as males. Finally, estrogen supplementation reverses the gonadectomy effects in females but does not affect males (Ramamani, Aruldhas and Govindarajulu, 1999). In addition, females have more efficient mitochondrial metabolism, with higher mitochondrial

protein and markers of mitochondrial function (oxidative and phosphorylative machinery and activities) (Colom *et al.*, 2007).

We did not evaluate protein metabolism in male vs. female rats. However, higher muscle protein content and higher fat oxidation (vs. glycolysis) in the muscle of female rats could potentially protect against muscle loss in females.

In conclusion, females depend more on muscle fat oxidation, have a more efficient energy metabolism, and have higher protein content in muscle, which might protect them against muscle loss.

Sarcopenia

Differently from male BDLs, females are protected against sarcopenia due to CLD, and the sex differences in hormones, nutrition, energy metabolism, and response to certain disease stimuli might explain why.

Sarcopenia is a common complication of many diseases, and it seems to have distinct features depending on the disease driver (Wang and Pessin, 2013). Conditions such as cancer primarily affect glycolytic fibers (predominant in males), while muscle impairments from disuse atrophy impact oxidative fibers (predominant in females) (Talbot and Maves, 2016). In agreement, females are at higher risk of intensive care unit associated muscle weakness (De Jonghe *et al.*, 2002; Yang *et al.*, 2018) and are more likely to have associated complications, including death (Rosa-Caldwell and Greene, 2019).

In rats, little is known on the impact of sex on the different drivers of sarcopenia. Female rats have a more significant shift to smaller muscle fibers than males due to disuse atrophy (Callahan *et al.*, 2014). That is translated into higher muscle loss (soleus muscle) in females via protein ubiquitination (Yoshihara *et al.*, 2019).

Sarcopenia in cirrhosis

Patients with cirrhosis are often malnourished due to nausea, early satiety with ascites, delayed gastric emptying, impaired gut motility, and poor eating habits, especially in patients who consume alcohol, primarily males (Quigley, 1996; Bergheim *et al.*, 2003). Among cirrhotic

patients, the prevalence of malnutrition is lower in females (29%) compared to males (50%) (Riggio *et al.*, 2003). Moreover, while male patients with cirrhosis have reduced fat and fat-free mass, females have reduced fat mass only, even though both have similar energy expenditure (Alberino *et al.*, 2001). Females naturally have more fat to help them cope with the disease (Riggio *et al.*, 2003). Women with cirrhosis conserve their protein stores. A study found that while only 28% of the women with cirrhosis had significant protein depletion, more than 60% of the male patients had the same condition. In addition, the protein depletion was worse in men. Women lost 11% of their body protein stores, while men lost 20% of theirs, regardless of disease severity and etiology (Peng *et al.*, 2007).

Unsurprisingly, there is a higher prevalence of sarcopenia in men vs. women with cirrhosis (around 50% vs. 18% respectively) (Montano-Loza *et al.*, 2012; Tandon *et al.*, 2012; Hanai *et al.*, 2015; Kim and Jang, 2015). Nonetheless, the prevalence of sarcopenia is also dictated by other factors such as body weight. While in underweight patients with cirrhosis, the prevalence of sarcopenia is similar between males and females, in normal-weight and overweight patients, females have less sarcopenia than males (Hanai *et al.*, 2015).

In agreement with clinical data, our lab has shown that male BDL rats have profound changes in muscle proteins (Annex 1). Six weeks after bile-duct ligation, male rats have reduced body weight with lower lean and fat mass, lower gastrocnemius weight and circumference, and skeletal muscle morphological changes compared to SHAM. Male BDL rats also have impaired protein synthesis, specifically in skeletal muscle, and lower muscle strength vs. male SHAM rats (Annex 1). Female BDL rats, on the other hand, manage to maintain protein stores. After 6 weeks of bile-duct ligation, female rats have reduced only fat mass and not lean mass, with no changes in muscle weight, circumference, and strength, similar to women with CLD. Nutrition might have a small role in the muscle loss in the BDL model. While males generally have lower protein intake in the last week of the model (compared to SHAMs), female BDL rats maintain regular food intake throughout the 6 weeks of the model. However, fat and muscle loss start to decrease at three weeks after BDL surgery in males (data not shown), and therefore, other factors play a role.

Sarcopenia and ammonia metabolism

In liver disease, ammonia is primarily detoxified by GS in muscle. Therefore, lower muscle mass due to sarcopenia in males would reduce the potential for ammonia detoxification. In CLD, patients with sarcopenia have higher HE prevalence and higher systemic ammonia levels (Bhanji *et al.*, 2018; Gioia *et al.*, 2019). However, it was unclear if this effect was more substantial in females or males. Our BDL model showed that although males (and not females) have muscle loss due to CLD, they still have similar muscle ammonia detoxification vs. females, calculated as ammonia from the femoral artery vs. ammonia from the femoral vein. Upregulation of GS activity in males vs. female BDL contributes to similar muscle ammonia detoxification and muscle glutamine production, resulting in similar systemic ammonia levels.

Sex hormones impact muscle during disease

In patients, CLD frequently causes hypogonadism in males and females (Green, 1977; Cundy *et al.*, 1991). A decrease in testosterone in men is associated with decreased muscle mass, health problems, and early death (Khaw *et al.*, 2007). Furthermore, testosterone supplementation increases muscle mass in men with hypogonadism (Brodsky, Balagopal and Nair, 1996). Although impaired muscle mass and function are not always found in females compared to males, hormone replacement therapy after menopause improves lean mass and decreases the risk of injuries in women after menopause (Bea *et al.*, 2011).

In PCA rats, delayed growth and malnutrition from liver disfunction are apparent only in male rats. PCA rats have increased estradiol in both males and females, decreased testosterone in males, and increased testosterone in females (Smanik *et al.*, 1991). In females, estrogens might protect muscle by acting as an anti-inflammatory (Tiidus, 2003).

Effect of sex on sarcopenia in alcohol-related liver disease

Alcohol-associated liver disease is the etiology with the highest incidence of sarcopenia (80%) compared to other etiologies (30% to 60%) (DiMartini *et al.*, 2013). Undoubtedly, the higher prevalence of males in alcohol-related cirrhosis contributes to the higher overall incidence of sarcopenia amongst males. However, it is unknown if sex impacts the development of sarcopenia in patients with alcohol-associated liver disease.

In mice, acute alcohol intake decreases muscle function (contractile force) in both sexes, recovered upon 24-hour alcohol clearance in females but not in males (Laudato *et al.*, 2021). In addition, alcohol effects on protein synthesis are slightly different in males vs. females for chronic (but not acute) alcohol exposure. Rats submitted to alcohol by gavage (up to 75 mmol/kg body weight), and after 6 weeks, male rats had lower muscle protein synthesis than females. However, at 14 weeks, muscle protein synthesis was similar between sexes (Lang, 2018). Unfortunately, we did not evaluate muscle mass and function in our male BDL + ethanol model. However, we found that although the liver function is not worse, blood ammonia has a trend to be higher in male BDL + alcohol vs. BDL alone. Therefore, alcohol may accelerate sarcopenia progression in males, reducing ammonia clearance. In a model using female BDL + ethanol, even though female BDL are protected against sarcopenia and alcohol has a higher deleterious effect in males, the added injury might result in lower muscle mass in females compared to BDL without ethanol. However, we expect that sarcopenia would still be greater in males. Interestingly, the gut microbiota can also be affected by alcohol, increasing the number of urease bacteria and therefore, ammonia levels (Zuo et al. 2017).

In conclusion, female BDLs protection against sarcopenia is linked with higher body fat and the milder consequences from hormonal changes during CLD than males. However, protection against sarcopenia does not cause higher muscle ammonia detoxification.

Interventions to prevent muscle mass loss in CLD

Nutrition

Malnutrition is an important driver of sarcopenia due to cirrhosis, and as such, tailored nutritional interventions exist for patients. The most important recommendation to prevent sarcopenia due to cirrhosis is a high protein diet (1.2-1.5 g/kg/day) (Amodio *et al.*, 2013). There is no information on whether high protein intake is beneficial for both males and females during liver disease since no studies have compared the effects of high protein by sex.

Although females BDLs had no sarcopenia, their g of food intake (and protein intake) per kg of body weight was higher than male BDLs, even though females lost fat mass. Therefore, the

energetic needs of female BDLs likely increase at the end of the model, justifying the recommendation of a high-energy diet, but not necessarily a high-protein diet for females.

Patients should eat small, frequent meals and a high-protein late-night snack to avoid muscle wasting due to gluconeogenesis (Tsien, McCullough and Dasarathy, 2012).

Since females depend more on fat energetic metabolism while males depend on amino acids, the time between meals might impact females differently (loss of fat mass primarily) than males (loss of muscle mass primarily).

In addition, supplementation with branched-chain amino acids (BCAA) might be superior to restoring muscle mass compared to protein intake only. BCAA (valine, leucine, isoleucine) are essential amino acids metabolized by the skeletal muscle. Found in lower levels in patients with cirrhosis, BCAAs are often used as a nutritional supplement (Amodio *et al.*, 2013). In a study, patients with CLD in a high-protein, high-fiber diet (1.2 g/kg of protein and 30g of fiber) supplemented with BCAA (110g per day) for 6 months had increased muscle mass compared to their baseline while patients on high-protein, high-fiber diet alone did not (Ruiz-Margáin *et al.*, 2018). There was no evidence that the patients in this study were sarcopenic and, although the authors did not compare sexes, 83% of the patients were females, suggesting that BCAA therapy increases muscle mass in females.

In another study, with 74 % males, patients with cirrhosis received a standard diet (35 kcal/kg/day and 0.7 g of proteins/kg/day), supplemented with 30 g of BCAA for 56 weeks and showed increased mid-arm circumference compared to control patients (standard diet and 30 g of maltodextrin) (Les *et al.*, 2011). This result indicates that BCAA also has beneficial effects in males.

Therefore, therapy with BCAA might be beneficial for muscle mass in male and female BDLs, even though females have no sarcopenia. However, the importance of higher muscle mass in females during CLD and HE still needs to be established.

Ammonia lowering strategies

In liver disease, muscle ammonia detoxification by GS shifts ammonia detoxification towards a muscle-based process, consuming muscle glutamate. This might reduce the muscles' amino-acid

pool and impair energetic metabolism by diverting α-ketoglutarate from the TCA cycle to glutamate formation by GDH.

Because of that, ammonia lowering therapies that stimulate the muscle GS, such as LOLA and OP, improve ammonia detoxification while preventing muscle wasting. Males depend more on muscle for energy, and as a result, a study showed that in male PCA rats, treatment with LOLA prevented gastrocnemius muscle loss after 4 weeks of the model (Kumar *et al.*, 2017). Although no studies were done in females, protein exhaustion in females is not as crucial as in males since they depend on fat metabolism and have no sarcopenia.

In addition, ammonia was also shown to reduce muscle satellite cells in male PCA rats (Dasarathy *et al.*, 2011). Therefore, lowering ammonia would recover normal satellite cell numbers. Since female rats have fewer satellite cells than males, ammonia might be deleterious for females' muscle health. Even if female BDLs do not have sarcopenia, further impairment of satellite cells could result in reduced injury repair. In this setting, ammonia lowering therapies such as LOLA and OP would also benefit female BDL rats.

Exercise

Besides proper nutrition, exercise is one of the recommendations to maintain muscle mass in patients with CLD. So far, no studies have explored the benefits of exercise in male and female patients with cirrhosis separately. However, in healthy people, several studies showed differences in the impact of exercise.

A study with young active and sedentary adults and active older people showed that age and physical activity impacts muscle mass and function (measured by tomography, speed test, chair test, and handgrip) in males, but not in females (Rivera *et al.*, 2016). In males, muscle function correlated with age, activity, and muscle volume. In males, sedentarism determined loss of muscle mass starting at an early age, showing that physical activity throughout life could have a protective effect on late age sarcopenia (Rivera *et al.*, 2016). Muscle mass was correlated with activity and age in women, while muscle strength was correlated with age only. That shows that muscle mass is not directly related to function in females and that although age has an essential effect on muscle strength, exercise seems to have a minor role in young females (Rivera *et al.*,

2016). Other studies confirmed the lack of correlation between exercise and sarcopenia in younger adults. Another study showed that as the ratio and physical activity increased, sarcopenia decreased, with additive effects in the young male, but not young females (Cho *et al.*, 2020). However, the impact of exercise vs. no exercise in older females seems to be more complex. Different studies have shown that in older females (older than 50 years old or from 65 – 84 years old), mild or moderate exercise correlates with a lower risk of sarcopenia (Park *et al.*, 2010; Cho *et al.*, 2020).

Our laboratory has shown that progressive resistance training protects male BDL rats against sarcopenia (Annex 2). We submitted male BDL rats to a ladder-climbing apparatus five days per week for four weeks for this study. Male BDLs submitted to the exercise protocol had higher gastrocnemius muscle weight and circumference and higher grip strength than non-exercisers. As female BDL rats have no loss of muscle mass or function, it is unclear what benefits they would acquire with exercise. In healthy female rats and mice, exercise increased muscle mass (White *et al.*, 2016) and improved genetic markers of muscle atrophy and apoptosis-related to older age (GAO *et al.*, 2021). Like in humans, the effects of exercise in female rodents might appear only at an older age. Therefore, it would be interesting to 1) evaluate the presence of sarcopenia in older BDL rats and 2) implement an exercise protocol to counteract the sarcopenia if present in female BDLs.

In addition, females with cirrhosis might have other benefits from exercise that go beyond muscle health. Exercise reduces total colon transit time in women (but not in men), which naturally have longer transit time. In patients with cirrhosis, that might mean preventing constipation and related complications such as HE by decreasing potential ammonia absorption by the colon (Bong Kil Song and Kim, 2012).

Although the maintenance of muscle mass by exercise could contribute to long-term management of ammonia levels in patients with CLD due to muscle GS action, the muscle can also produce ammonia during exercise in a sex-dependent manner. Males have higher muscle ammonia concentration following exercise than females with the same exercise effort (Derave, Bouckaert and Pannier, 1997). In addition, plasma and muscle ammonia can increase significantly

from the onset and throughout the exercise (MacLean *et al.*, 1991). Blood ammonia during exercise increases due to the breakdown of adenosine nucleotides to inosine monophosphate (Lowenstein, 1990; MacLean *et al.*, 1991) during brief intensive exercise and from branched-chain amino acids metabolism during long term mild-moderate exercise (MacLean *et al.*, 1991).

In compensated patients with cirrhosis, muscle ammonia levels get higher than in healthy subjects during exercise (124 vs. 74 µM respectively), even though basal ammonia was similar between both groups (Dietrich, Bachmann and Lauterburg, 1990). There was no mention of HE development during the study, indicating that the transitory ammonia change was not enough to have deleterious effects. Therefore, it is likely that the benefits of exercise for compensated patients outweigh the potential complications, and exercise is considered protective for patients with CLD.

Unfortunately, we did not measure plasma ammonia levels in male BDL rats during exercise. Nevertheless, like in humans, we did not observe any overt behavioral changes that would indicate the presence of OHE due to ammonia increase.

In conclusion, nutrition, ammonia lowering strategies, and exercise in male BDL rats protect them against sarcopenia. In female BDLs, although sarcopenia is not present, the same interventions might help muscle health, although their impact in CLD and HE are less clear.

Pathogenesis of HE

In our female BDL study, we explored different aspects of HE between males and females, but the information is still missing on the impact of sex on the different factors on the pathogenesis of HE.

Ammonia

Female BDL rats have similar systemic ammonia levels compared to male BDL rats and similar muscle ammonia detoxification. However, other aspects of ammonia metabolism might differ between males and females.

Absorption

Ammonia metabolism is complex and involves the diet (amount and type of ingested proteins), GI function (digestion and absorption of ammonia and amino acids), portal vein transport, and hepatic and extra-hepatic ammonia clearance. However, data on sex-based differences for those processes are rare.

A cross-sectional population-based study in the UK showed that although there were no significant differences in protein intake between men and women, men are more likely to ingest less than the recommended protein intake (Bennett, Peters and Woodward, 2018). Also, women have slower post lag gastric emptying and colonic transit (measured by scintigraphy) (Degen and Phillips, 1996), which might aid protein digestion. In addition, total colon transit time is longer in healthy women (25.8 hours) compared to healthy men (7.4 hours) (Bong Kil Song and Kim, 2012). That means that females have extended absorption time in the colon than males, allowing more time for ammonia absorption. However, men have a higher portal vein diameter than females (Singh *et al.*, 2017), which means more blood from the intestines is transported to the liver. Therefore, more ammonia could be carried over time, and the ammonia levels that reach the liver might be similar between males and females.

In liver disease, GI transit times are slower than during health, especially in the small intestine. Lower intestinal motility in patients has been associated with the severity of liver disease, intestinal bacterial overgrowth, and HE (Fukui and Wiest, 2016). However, sex differences in GI dysmotility in liver disease are still unknown.

In rodents, female mice have a slower GI transit time (138.5 in females vs. 107.5 minutes in males) (Gallego *et al.*, 2020), although that is a smaller difference compared to humans. In female rats, gastric emptying and GI transit are also slower than males and are affected by sex hormones. Gonadectomized female rats that received estrogens showed slower transit time than males and gonadectomized vehicle females (Bond, Heitkemper and Perigo, 1996).

Even though transit time is different, factors such as diet, digestion, availability of amino acids, and the levels of intestinal glutaminase might impact ammonia absorption in males and females. In our BDL models, females ate around 70g of food /kg body weight while males ate around 55g

of food /kg body weight at the end of the model, resulting in higher protein intake in females, which could lead to higher ammonia absorption. However, more experiments are needed. Calculations of portal vein ammonia during a protein load in male and female BDL and SHAM rats would clarify if there are differences in ammonia absorption between sexes. Also, quantification of intestinal glutaminase levels would help to explain the differences (if present). In addition, quantifying hepatic vein ammonia levels would tell us if hepatic ammonia detoxification is different between sexes since systemic ammonia and muscle ammonia clearance are similar between males and females.

Gut microbiota

Recently, the importance of the gut microbiota in susceptibility to illness was brought to light. In CLD, gut microbiota might affect gut permeability and ammonia production by urease bacteria, both factors that impact the disease. Although the advancement of technology allowed us to assess gut microbiota extensively, there are still many confounding factors such as diet, drug intake, and sex to consider. There are differences in microbiota composition between males and females in healthy individuals. Studies showed sex-based differences in gut microbiota composition (Kim *et al.*, 2020), with women having a higher abundance of bacteria in the gut, with bacteria per body cells being 1.3:1 in males and 2.2:1 in females (Sender, Fuchs and Milo, 2016). In addition, differences in the microbiota contribute to regulating sex hormones (Markle *et al.*, 2013), influencing the immune system (Fransen *et al.*, 2017).

In liver disease, the impact of liver failure on microbiota dysregulation is well established. However, the impact of sex on microbiota dysregulation is often overlooked. Saboo and collaborators found that in patients with CLD and HE, microbiota composition is different between men and women, with differences in the abundance of several bacterial families (Saboo *et al.*, 2020).

Differences in gut microbiota are well explored in mice but vary across studies. A study using two different mouse strains (BALBc and B6) showed higher bacterial richness and diversity in females than males (Elderman *et al.*, 2018). In other studies, specific phyla such as Actinobacteria and Tenericutes were more abundant in male mice, while the Lachnospiraceae family was more

abundant in females (Org *et al.*, 2016). However, the consequences of these differences are not yet understood.

Our laboratory is currently exploring the role of microbiota in male BDL rats. We treated male BDL rats with either vehicle or fecal matter transplant from male SHAM rats, improving HE signs (motor coordination, memory), although not lowering systemic ammonia. We have no indication that these results would differ in female BDLs transplanted with female SHAMs fecal matter. However, since female intestinal time is longer and systemic ammonia is similar to males, females may have fewer urease bacteria, and therefore less ammonia production in the gut. Therefore, it would be interesting to transplant microbiota from female SHAMs into male BDLs (and vice versa) to explore the possibility that female's microbiota paired with male's shorter intestinal transit time are protective against ammonia production and absorption in the gut.

In conclusion, female's GI transit time and protein intake could increase ammonia absorption while the liver or the microbiota likely compensates for ammonia detoxification since female and male BDLs have similar systemic ammonia and muscle ammonia clearance.

Oxidative stress

Female BDL rats have lower systemic oxidative stress compared to males, which might be due to potential differences in antioxidant capacity between males and females.

The relationship between sex and oxidative stress is not always clear, but it points to a better antioxidant capacity in females. While a study with 195 healthy individuals showed that women had higher reactive oxygen metabolites, with no difference in antioxidant capacity, others found decreased oxidative stress in women (Brunelli *et al.*, 2014). Young men have higher oxidative stress measured by plasma thiobarbituric acid-reactive substances (TBARS), which measures lipid peroxidation products, compared to pre-menopausal women. However, there was no difference in plasma superoxide dismutase (SOD), catalase, and vitamin E (Ide *et al.*, 2002). Besides antioxidant enzymes, females might have antioxidant properties from other sources such as estrogen (Campos *et al.*, 2014). Total hysterectomy and oophorectomy (and subsequent estrogens decrease) caused a reduction in SOD and glutathione peroxidase mRNA in women, which was recovered with hormone replacement therapy (Bellanti *et al.*, 2013).

In animal models, the brains of female mice have higher glutathione peroxidase and SOD than males (Chen *et al.*, 2011). Other studies showed lower oxidative stress in females, mainly related to protection against cardiovascular diseases (Matarrese *et al.*, 2011; Bhatia *et al.*, 2012). In our BDL model of liver disease, systemic oxidative stress was lower in female rats than males between both SHAMs and BDLs. However, we found no differences in brain oxidative stress between males and females. In addition, our data support that lower systemic oxidative stress in females protected them against ammonia-induced OHE since the treatment of male BDL rats with allopurinol protected them from OHE.

Among the factors contributing to higher antioxidant capacity in females was higher albumin. The most abundant plasmatic protein, albumin, is an essential antioxidant due to its reduced cysteine groups and capacity to bind to ligands such as iron and copper and prevent ROS formation (Roche *et al.*, 2008). More studies are needed to evaluate other factors involved with the higher antioxidant capacity, such as estrogens and differential expression of antioxidant enzymes in females with CLD.

In conclusion, female BDLs have higher antioxidant capacity associated with higher albumin and possibly the antioxidant activity of estrogens, protecting them against ammonia-induced OHE episodes. The regulation of oxidative-stress enzymes in female vs. male BDLs remains to be seen.

Inflammation

Response to diseases (infectious or not) varies by sex, with males being more susceptible to infection. Accordingly, female BDLs have lower systemic and central inflammation than male BDLs (Annex 4). Those differences can be due to several factors, including genetic (X-linked gene expression), biological (hormonal regulation), and behavioral (risky behavior, exposure to pathogens), which will contribute to a sex-differential immune response.

Sex impacts the number and activity of immune cells. Males have higher natural killer (NK) cell numbers (Abdullah *et al.*, 2012), while females have neutrophils and macrophages with higher phagocytic activity (Spitzer, 1999). Females also have higher antibodies responses, immunoglobulin levels, and B cells abundancy than males regardless of age (Teixeira *et al.*, 2011; Abdullah *et al.*, 2012; Furman *et al.*, 2014).

In males, testosterone might act supressing the early immune response and activating late immune response, as it happens in sepsis (García-Gómez, González-Pedrajo and Camacho-Arroyo, 2013; Trigunaite, Dimo and Jørgensen, 2015). On the other hand, estradiol acts as an activator as progesterone acts as immunosuppressive in certain conditions such as pregnancy (Kalkhoven *et al.*, 1996; Hirano, Furutama and Hanafusa, 2007). As a result, in response to infections, females produce high levels of interleukins such as IL-4, IL-5, and IL-10 by T-helper 2 (Th2) cell responses while males produce high levels of TNF-a, IL-1β, IL-2, IL-6, and IL-8 by T-helper 1 (Th1) cells (Ackerman, 2006; Aulock *et al.*, 2006; McClelland and Smith, 2011). That response in males is often associated with negative outcomes such as sepsis and bacteremia (Blackwell and Christman, 1996). In agreement, female patients have a lower incidence of sepsis (62.2% men vs. 37.8% in women) and lower sepsis-associated mortality (Kondo *et al.*, 2021).

In addition, women during menopause have the immune response impaired. Post-menopause or oophorectomized females have lower levels of B lymphocytes and anti-inflammatory cytokines such as IFN-γ, and higher levels of pro-inflammatory cytokines production such as TNF-α, IL-1β, IL2, and IL-6 by NK cells (Kumru, Godekmerdan and Yilmaz, 2004; Marriott and Huet-Hudson, 2006; Yasui *et al.*, 2007) and the effects are improved, but not completely reversed with hormone replacement therapy (Giglio *et al.*, 1994; Giefing-Kröll *et al.*, 2015).

Similar to women, female mice have a privileged immune system. Antigen-presenting cells (APCs) from females are more efficient at presenting peptides than male cells (Weinstein, Ran and Segal, 1984). In response to LPS, males present an exacerbated response, leading to sepsis or septic shock (Li *et al.*, 2018). Males have a higher expression of TLR4 on the surface of their macrophages (Marriott and Huet-Hudson, 2006), which can trigger an uncontrolled inflammatory response. Removing androgens by gonadectomy in mice reduces the expression of TLR4 in macrophages, and testosterone replacement rescues this effect. In addition, macrophages cultured in the absence of testosterone have reduced expression and activity of TLR4, which results in lower TNF-a levels (Rettew, Huet-Hudson and Marriott, 2008).

In contrast, TLR7 is more expressed in women and is related to a higher response to viral infections and increased autoimmunity in women (Spiering and de Vries, 2021). The TLR7 gene is

encoded by the X chromosome, which might scape X-chromosome inactivation in around 20% of the cells and, therefore, is more expressed in women (Tukiainen *et al.*, 2017; Souyris *et al.*, 2018). Moreover, activation by TLR7 agonist caused peripheral blood cells from females to release more IFN-a than men (Berghöfer *et al.*, 2006).

Our BDL model showed that females have lower systemic and brain PFC levels of inflammation measured as TNF-α (Annex 4). BDL surgery disrupts the estrous cycle and affects estrogen response in females (Mahmoud, 2018). It is possible that the hormonal dysregulation caused by BDL in females is not enough to impair immunity or that the protection against inflammation might be due to reasons other than hormones. In addition, if female BDL rats have differences in immune cells as seen in healthy females (higher macrophage numbers and phagocytic capacity (Spitzer 1999)), the response to a potential infection could be more efficient in females, resulting in lower cytokine levels. The more efficient response to infection in females could also protect them against an OHE episode, since infection is one of the precipitating factors of HE.

In conclusion, female BDLs have lower inflammation than male BDLs, likely due to the more anti-inflammatory profile in females, including a more efficient immune response to infection than males.

Brain

Although female BDLs have CHE (impaired motor coordination and activity), they are protected against short-term memory loss, anxiety, and brain edema. However, the reasons for those differences are not entirely understood. Sex differences exist in brain morphology and function in health and other disease models, and uncovering those differences might help shed light on the results found in female BDLs.

Morphology and function

The volume differences between the brains of males and females (with males having greater brain volume) is a confounding factor when comparing structures from male and female brains since most structures from the male brain will have a greater volume than the female brain. However, there are still differences between males and females.

A metanalysis with different imaging techniques like functional magnetic resonance imaging (fMRI), positron emission tomography (PET), single-photon emission computed tomography (SPECT), and structural magnetic resonance imaging (MRI) in mentally healthy individuals confirmed that brain volume is greater in men than women. However, when controlling for total volume, women have more gray matter while men have more white matter (Cosgrove, Mazure and Staley, 2007). Furthermore, gray matter in different brain areas correlates with the intelligence quotient (IQ) in men and women. In men, IQ correlates with gray matter volume in the frontal and parietal lobes, whereas in women, IQ correlates with gray matter volume in the frontal lobe and Broca's area (Cosgrove, Mazure and Staley, 2007). That suggests that even though both sexes might achieve a similar score in a group of cognitive tasks, there is a dimorphic component of intelligence based on the unique brain structures in males and females. In agreement, another study showed that females had better episodic memory for more verbal tasks such as words, sentences, prose, nameable images, and locations, while men had better spatial episodic memory for abstract images and routes (Asperholm *et al.*, 2019).

Differences in gray matter were also found in specific brain regions. Men have higher gray matter in subcortical temporal structures, such as the amygdala, hippocampus, temporal pole, fusiform gyrus, primary visual cortex, and motor areas such as the premotor cortex, putamen, anterior cerebellum. Meanwhile, women have more gray matter in medial and lateral prefrontal areas, the superior temporal sulcus, the posterior insula, and the orbitofrontal cortex (Ruigrok *et al.*, 2014; Lotze *et al.*, 2019), and those differences are independent of brain volume. In addition, sex differences in gray matter are ruled at least in part by the expression of sex-chromosome genes.

A study coupled higher expression of sex-chromosome genes with the areas of differential gray matter between men and women (Liu *et al.*, 2020), confirming similar results from mice (Qiu *et al.*, 2018) and elucidating some of the mechanisms for brain dimorphism.

Sex differences in behavioral tests are strain-specific, and a study showed that sex differences between adolescent C57BL/6N, DBA/2, and FVB/N mice were also task-specific. Male FVB/N mice were more active than females and had higher climbing counts while female FVB/N mice showed less anxiety in the elevated plus-maze. C57BL/6N female mice were more active and had lower

anxiety than males in the open field test. In addition, no differences were found between sexes for any strain in other anxiety tests like the light/dark compartment test (Eltokhi, Kurpiers and Pitzer, 2020). In spatial and cued navigation tasks in the Morris water maze, although both male and female mice (C57BL/6) performed well in the spatial task, males performed better in the cued task than females (Berger-Sweeney *et al.*, 1995). That shows that differences on the same parameter (anxiety or memory) differ depending on the tests performed.

Memory-related long-term potentiation (LTP) in the CA1 region of the hippocampus depends on estrogen and activation of estrogen receptor-alpha in females but not males. In addition, signaling pathways for LTP are also different between males and females, with females having activation of two LTP-related kinases ((Src, ERK1/2) and of postsynaptic TrkB) (Wang *et al.*, 2018).

In the BDL model, both sexes had impaired motor coordination and activity, while males only had anxiety and short-term memory impairments. In addition, females were protected against brain edema and ammonia-induced OHE episode. Although the protection conferred to females came partly from lower oxidative stress, as shown in male rats treated with allopurinol, other aspects might play a role. Differences in brain morphology and processes might protect female BDLs against memory loss and anxiety, while it is also possible that these impairments exist and other behavioral tests are needed to perceive them in female BDLs.

In conclusion, differences in brain function and morphology likely protect female BDLs against memory loss and anxiety. However, other behavioral tests in females might unveil these impairments.

Neurotransmission

Neurotransmission is an essential part of brain functioning, which differs between males and females. Females have higher GABA levels in the brain cortex, which fluctuate with the menstrual cycle (Sanacora *et al.*, 1999; Epperson *et al.*, 2002), and higher brain glutamate levels in the striatum, cerebellum, cortex, and hippocampus (Grachev and Apkarian, 2000; Hädel *et al.*, 2013; Zahr *et al.*, 2013), even though men have higher blood glutamate (Zlotnik *et al.*, 2011).

In rats, a study assessed glutamate-mediated dopamine release by microdialysis, therefore bypassing systemic metabolism, showed that antagonism of AMPA receptors decreases dopamine in PFC in both sexes while NMDA antagonism increases dopamine in males but decreases in females. In addition, when both NMDA and GABA-B were simultaneously submitted to antagonism, males responded to GABA-B alone while females responded only to NMDA, stressing different mechanisms for neurotransmission interaction (Locklear *et al.*, 2016). Female rats are more responsive to the NMDA receptor antagonist ketamine (McDougall *et al.*, 2017) and are more sensitive to excitotoxic damage following an NMDA receptor antagonist (Wozniak *et al.*, 1998). This increase in NMDA sensitivity may result from increased receptor expression since female rats have higher NR1 and NR2B NMDA subunits (Wang *et al.*, 2015). Moreover, females also have higher basal levels of the metabotropic glutamate receptor system, with higher mGluR2/3 and mGluR5 within the hippocampus and increased mGluR5 in the PFC (Wang *et al.*, 2015).

There is a paucity of data on the impact of sex on neurotransmission on HE. However, in other neurocognitive disorders such as Alzheimer's, spatial memory impairments and inhibitory avoidance tasks appear earlier in females than in male mice (Clinton *et al.*, 2007). In Alzheimer's, excess glutamate is caused by blockage of glutamate uptake by β-amyloid plaques and can cause excitotoxicity and neurodegeneration (Mattson *et al.*, 1992; Domingues *et al.*, 2007). Female transgenic mouse models of Alzheimer's have even lower expression of GluA2-containing AMPR receptor, which might impact glutamate's toxicity. As a result, males and females present impaired working memory, short-term memory, and increased anxiety by 12 months old, while only females have impaired reference memory (Blázquez *et al.*, 2014).

Accordingly, the NMDA receptor antagonist memantine shows positive results in treating moderate to severe Alzheimer's in patients (Winblad *et al.*, 2007), although no differences between sexes were observed.

Interestingly, memantine was proven to improve cognitive function in acute animal models of HE (Vogels *et al.*, 1997; Cauli *et al.*, 2008), and it might be an essential tool to unveil potential sex differences in glutamatergic dysfunction during HE. Our experiments found that females

presented CHE with impaired motor coordination and activity similarly to male BDLs, but without memory impairments and anxiety present in males. We also found that brain glutamate (PFC and hippocampus) is not different between male and female BDLs (data not shown). However, we do not know if glutamate toxicity plays a more significant role in HE in females compared to male BDLs. Therefore, treating both with memantine and evaluating the protection against HE might help uncover sex differences in neurotransmission.

In conclusion, male and female BDLs have similar levels of glutamate, and the role of glutamate toxicity in males vs. females in the pathogenesis of HE remains to be seen.

Blood flow

Several studies showed that women have higher cerebral perfusion at rest and during cognitive activity (Gur *et al.*, 1982; Devous *et al.*, 1986; Cosgrove, Mazure and Staley, 2007), associated with a higher cerebral rate of glucose use (Baxter *et al.*, 1987). However, a higher glucose rate could be due to smaller brain sizes in females since individuals with smaller brains have higher glucose use rates (Yoshii *et al.*, 1988). Higher blood flow might impact the delivery and distribution of brain-directed drugs and result in higher central susceptibility to systemic factors in females. Higher blood flow might be due to the action of sex hormones since hormone replacement therapy with estrogens in menopausal women increased regional blood flow, especially in the hippocampus, parahippocampal gyrus, and temporal lobe (Maki and Resnick, 2000).

In HE, there is evidence that cerebral blood flow might be dysregulated, and patients might suffer from impaired cerebral blood flow autoregulation (O'Carroll *et al.*, 1991). Females would be protected against such complications by having a more efficient blood supply to provide energy for the brain. On the other hand, a higher perfused brain is more susceptible to systemic pathophysiological factors of HE. A study in patients with cirrhosis correlated cerebral blood flow values with ammonia brain perfusion. The authors showed that brain areas with higher perfusion, such as the thalamus, the lenticular nucleus, and the cerebellum, also had higher ammonia extraction into the brain (Ahl *et al.*, 2004).

Moreover, hyperammonemia in the cerebellum reduces GluA2 and increases GluA1 membrane expression (Cabrera-Pastor *et al.*, 2018). Higher GluA2 increases the risk for glutamate-mediated

excitotoxicity (Counts *et al.*, 2011), further impacted by an ammonia-mediated increase in extracellular glutamate (Rose, Kresse and Kettenmann, 2005). In females, that would mean that more ammonia, among other factors, would enter the female brain and have worse deleterious effects, leaving females more susceptible to HE. However, further studies are needed to determine how glutamate receptors respond to ammonia in females.

The regulation of cerebral blood flow is essential for delivering nutrients (glucose, amino acids) to the brain, and it is tightly regulated. No studies have explored the differences in brain perfusion between males and females. Our lab showed an impairment of cerebral blood flow autoregulation in the male BDL. The presence of CHE in BDLs and the induced hypotension (mimicking the blood loss from a transplant) caused impaired cerebral flow in male BDLs but not in SHAM rats, accompanied by neuronal degeneration (Clément *et al.*, 2021). In this setting, higher cerebral blood flow in female BDL could help them maintain normal cerebral blood flow autoregulation during hypotension, preventing neuronal impairment.

On the other hand, higher blood flow could mean that the brain is more susceptible to systemic ammonia in females. However, female BDLs had no OHE episode after the ammonia challenge, while male BDLs did. Still, measuring CSF ammonia levels during the ammonia challenge would help to elucidate if female brains are more exposed to ammonia due to higher blood perfusion. In addition, potential differences in brain ammonia detoxification could also play a role and should be considered.

In conclusion, higher cerebral blood flow in female BDLs might be either protective due to more efficient autoregulation or detrimental due to higher ammonia susceptibility.

Conclusions

The results present in this thesis demonstrate for the first time that:

- GS is present in endothelial cells of the BBB
- GS in endothelial cells of the BBB is expressed in lower levels compared to astrocytes
- Ornithine is a good target to enhance GS activity in endothelial cells of the BBB
- Female BDL rats develop liver injury similar to males
- Female BDL rats have fewer complications of liver disease than males, including sarcopenia
- Muscle ammonia detoxification and systemic ammonia levels are similar in males and females
- Female BDL rats develop CHE, with impaired activity and motor coordination, similar to males
- Female BDL do not develop anxiety and memory loss unlike from males
- Female BDL rats have lower systemic (but not central) oxidative stress than males
- Female BDL rats are protected against ammonia-precipitated OHE due to lower oxidative stress
- Antioxidant therapy in males protect against ammonia-precipitated OHE

We showed for the first time that the BBB is equipped to protect the brain against ammonia toxicity via the expression of GS in endothelial cells of the BBB. We also showed that ornithine is the most efficient substrate for GS activity, and therefore treatments with ammonia scavengers such as OP and LOLA potentially protect against HE ammonia through ammonia reduction by GS, not only in muscle but also in endothelial cells of the BBB.

Moreover, we demonstrated the impact of sex as a biological variable on the pathogenesis of CLD and HE. We showed for the first time that the female BDL rats have a similar hepatic injury to male BDL. However, complications of CLD are different between males and females. Female BDL had CHE with impaired activity and motor coordination, similar to male BDLs. However, female

BDL rats did not have short-term memory loss or anxiety. Unlike male BDLs, female BDLs had no loss of muscle mass and function, which did not translate into higher muscle ammonia detoxification. Also, female BDLs had lower oxidative stress, which protected them against ammonia-induced overt HE.

Our results elucidated two new findings that impact the understanding of CLD and HE. HE is a complex and heterogeneous syndrome; therefore, better understanding the pathogenesis of this disease will lead to better care. The role of the BBB in protecting the brain from ammonia needs to be further explored. Likewise, the sex-based unique response of CLD and HE merits to be considered when designing experiments in pre-clinical and clinical research.

Future directions

The role of GS in endothelial cells of the BBB

We showed that GS is expressed in the endothelial cells of the BBB and that ornithine could be used to increase its expression. However, the role of GS on the BBB is unknown.

In vitro

To ensure that ornithine can increase GS activity and ammonia detoxification, differently from our results from this thesis, we would treat brain endothelial cells with ornithine and ammonia and measure ammonia detoxification vs. ammonia only cells.

The role of GS in barrier tightness and protection against ammonia can be assessed *in vitro* by culturing endothelial cells of the BBB with astrocytes (to induce BBB's properties) and by silencing GS with siRNA. Because membrane-bound GS has a role on the endothelial organization (Eelen *et al.* 2018), in a transwell setting, endothelial barrier's permeability to small molecules (dextran) and expression of tight junction proteins by western blot can be evaluated.

In vivo

An inducible KO mouse model (specific GS-endothelial cells of the BBB-KO) can be used to uncover BBB's GS function *in vivo*. After GS deletion, BBB function (tightness with permeability to small molecules such as dextran, expression of tight junction proteins) would be evaluated. In addition, we would evaluate susceptibility to cognitive impairment upon ammonia challenge to assess BBB's GS role in protecting against ammonia. Finally, we could perform BDL surgery in the KO mice to assess the importance of BBB's GS in disease.

GS in the endothelial cells of the BBB - Sex differences

This thesis showed the importance of sex differences in response to disease. Therefore, GS's presence and role need to be explored in females (*in vivo* and *in vitro*). For that, experiments so far should be replicated using females, and future experiments should include both females and males.

The mechanisms for OHE in females

This thesis showed that female BDL rats are protected against an OHE episode while male BDLs are not. Although we showed that oxidative stress protects against OHE, more research is needed to uncover other aspects of sex and OHE.

Susceptibility to OHE

Ammonia is a critical factor involved in the pathogenesis of HE. However, females did not develop OHE from an ammonia challenge. To better understand that, we could measure CSF ammonia during the ammonia challenge in female vs. male BDL rats to determine whether females are protected due to lower brain ammonia or similar brain ammonia.

To uncover if other factors are more effective in precipitating OHE in females, we could stimulate a ROS or inflammation precipitated episode with diethyl maleate or LPS, respectively, in female and male BDL rats.

Role of hormones

To understand the role of hormones in CLD and HE, we could assess liver disease progression and complications (including sarcopenia and HE) in gonadectomized male and female rats. In addition, we could perform the ammonia challenge to assess the role of hormones on the protection of females and the susceptibility of males to the ammonia-precipitated OHE.

Other animal models

Finally, we could explore further the differences between males and females by characterizing other female animal models of liver disease and HE (type A and B).

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Annexes

Annex 1: The bile duct ligated rat: A relevant model to study muscle mass

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ORIGINAL ARTICLE

The bile duct ligated rat: A relevant model to study muscle mass loss in cirrhosis

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Abstract Muscle mass loss and hepatic encephalopathy (complex neuropsychiatric disorder) are serious complications of chronic liver disease (cirrhosis) which impact negatively on clinical outcome and quality of life and increase mortality. Liver disease leads to hyperammonemia and ammonia toxicity is believed to play a major role in the pathogenesis of hepatic encephalopathy. However, the effects of ammonia are not brain-specific and therefore may also affect other organs and tissues including muscle. The precise pathophysiological mechanisms underlying muscle wasting in chronic liver disease remains to be elucidated. In the present study, we characterized body composition as well as muscle protein synthesis in cirrhotic rats with hepatic encephalopathy using the 6-week bile duct ligation (BDL) model which recapitulates the main features of cirrhosis. Compared to sham-operated control animals, BDL rats display significant decreased gain in body weight, altered body composition, decreased gastrocnemius muscle mass and circumference as well as altered muscle morphology. Muscle protein synthesis was also significantly reduced in BDL rats compared to control animals. These findings demonstrate that the 6-week BDL experimental rat is a relevant model to study liver disease-induced muscle mass loss.

Keywords Experimental cirrhosis · Muscle mass loss · Protein synthesis · Ammonia · Hepatic encephalopathy

Introduction

Loss of muscle mass is the most common and clinically significant complication of chronic liver disease (cirrhosis). It is a major contributor to adverse clinical outcomes (both pre and post liver transplantation), including morbidity and mortality (Merli et al. 2002). In addition, muscle wasting leads to poor quality of life and increased susceptibility to infection (Metter et al. 2002; Pichard et al. 2004; Cosquéric et al. 2006; Millwala et al. 2007; O'Brien and Williams 2008; Montano-Loza et al. 2012; Tandon et al. 2012). It has been suggested that liver disease-induced muscle mass loss results from varying contributions including reduced protein synthesis, increased protein catabolism, and an impaired proliferation and differentiation of skeletal muscle progenitor satellite cells (Dasarathy et al. 2002). However, the precise pathophysiological mechanisms underlying the loss of muscle mass in cirrhosis remains to be elucidated.

Hepatic encephalopathy, another complication of liver disease which greatly impacts on patients' quality of life, is characterized by a constellation of symptoms, including cognitive, psychiatric and motor disturbances (Cash et al. 2010). Although the pathogenesis of hepatic encephalopathy is multifactorial including oxidative stress (Görg et al. 2010; Bosoi et al. 2012), inflammation (Shawcross and Jalan 2005; Shawcross et al. 2011; Montoliu et al. 2009), lactate (Bosoi et al. 2014) and altered gut microbiota (Bajaj et al. 2008), ammonia is a key player as blood-derived ammonia rises to toxic levels in the brain (Butterworth 2002; Bosoi and Rose 2009). The toxicity of ammonia is a result of its direct effect on pH, membrane potential and metabolism which independently or collectively cause cell

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dysfunction (Lai and Cooper 1991; Bosoi and Rose 2009). As the effects of ammonia are not brain-specific (Lai and Cooper 1991; Norenberg 2003), it has been shown that elevated concentrations of ammonia can also affect other organs and tissues (Kubota et al. 2004; Jia et al. 2014; Rose 2014).

The 6-week bile duct ligation (BDL) rat, a surgical model involving obstruction of the common bile duct, is a well-established experimental model which recapitulates the main features of cirrhosis including, liver failure, hyperammonemia, secondary biliary cirrhosis, ascites, jaundice, brain edema and hepatic encephalopathy (Butterworth et al. 2009; Bosoi et al. 2011; Bosoi et al. 2012). It has been previously suggested that impaired skeletal muscle protein synthesis is the primary reason for loss of muscle mass in rats with portacaval-systemic shunting (Dasarathy et al. 2011). However, muscle mass in the BDL rat has not been extensively evaluated. The present study aims to characterize body composition as well as muscle protein synthesis in the BDL rats with hepatic encephalopathy.

Material and methods

Animal model

Cirrhosis was induced in male Sprague-Dawley rats (200–225 g) (Charles River, St-Constant, QC) by BDL. The latter is created by obstruction of the common bile duct which reproduces the main features of human cirrhosis. Briefly and as previously described, rats were anaesthetized with isoflurane, and the common bile duct ligated and resected under a dissecting microscope. Sham-operated control rats, matched for weight, were similarly anaesthetized; a laparotomy was performed and the bile duct was isolated (Rose et al. 1999; Bosoi et al. 2011; Bosoi et al. 2012). Rats were maintained under controlled conditions (22°C, 12 h:12 h dark-light cycle) with free access to their food and water. Two experimental groups were tested; 1) BDL ($n = 5$) and 2) Sham-operated control rats (SHAM) ($n = 6$). Experiments were conducted following the guidelines of the Canadian Council on Animal Care and were approved by the Animal Protection Committee of the Centre de recherche du Centre hospitalier de l'Université de Montréal (CRCHUM).

Body weight and food intake

Body weight was measured every day of the 6 week experimental protocol using an electronic scale. Food consumption was also monitored every day by the weight of the food.

Body mass composition

Body composition in terms of lean and fat mass was assessed in conscious rats (full body) by in vivo scanning and magnetic resonance imaging (EchoMRI 100® Body Composition Analyzer) 6 weeks after the surgeries, according to the manufacturer's protocol. The instrument for composition analysis creates contrast between soft tissues by taking advantage of the differences in relaxation times of the hydrogen proton spins in different environments. Radio pulses cause protons to spin and emit radio signals which are then received and analysed. The amplitude, duration, and spatial distribution of these signals are related to properties of the material scanned. The high contrast between fat, muscle tissue, and free water is further enhanced by application of define composed radio pulses sequences (Nixon et al. 2010).

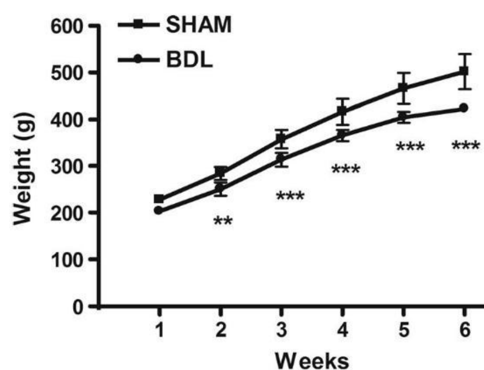

Fig. 1 Growth curve of rats with bile-duct ligation (BDL) compared to sham-operated controls. Mean of daily weights were averaged and expressed as weekly weight. The two -way ANOVA indicated an effect of time/week ($F_{5,\ 54} = 231.94$, $p < 0.001$), an effect of surgery ($F_{1,\ 54} = 85.93$, $p < 0.001$) and interaction ($F_{1,\ 54} = 2.47$, $p = 0.04$). $^{**}p < 0.01$, $^{***}p < 0.001$, significantly different from SHAM

Gastrocnemius muscle mass, circumference and morphology

At the end of the 6 weeks experimental protocol, rats were sacrificed and the gastrocnemius muscle was dissected and

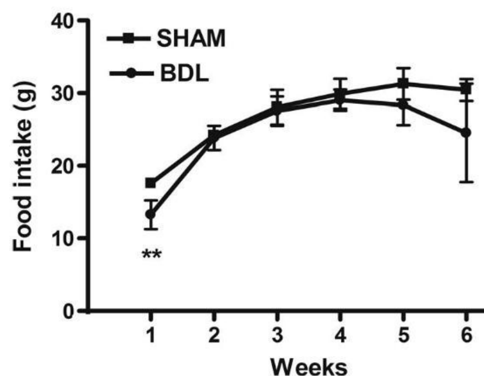

Fig. 2 Food intake of rats with bile-duct ligation (BDL) compared to sham-operated controls. Mean of daily food intake were averaged and expressed as weekly intake. The two -way ANOVA indicated an effect of time/week ($F_{5,\ 54} = 98.21$, $p < 0.001$) and an effect of surgery ($F_{1,\ 54} = 11.22$, $p < 0.001$), with no interaction ($F_{1,\ 54} = 1.47$, $p = 0.21$). $^{**}\ p < 0.01$, significantly different from SHAM

weighed. Muscle (gastrocnemius) mass and circumference were measured using an electronic scale and a scaled thread, respectively. Muscle samples were then fixed in 4% paraformaldehyde buffered with phosphate-buffered saline, decalcified with 10% formic acid, and embedded in paraffin. Longitudinal histology sections were cut with a microtome, and stained with hematoxylin-eosin. Tissue sections were then visualized using microscope.

Protein synthesis

Protein synthesis was quantified as the fractional and absolute protein synthesis rates in the dissected and homogenized muscle and other organs including the brain (frontal cortex), heart, intestine, kidney and liver, using the modified phenylalanine tracer pulse method (Zhang et al. 2002; Dasarathy et al. 2011). In brief, rats were given a small dose (0.5 mg/100 g body weight) of L-[ring-$^2H_5$]phenylalanine ip at t = 0 min, L-[1-^{13}C]Phenylalanine ip at t = 30 min and L-[^{15}N]Phenylalanine ip at t = 60 min. At t = 65 min, the rats were killed and blood and tissue collected. The calculation of the fractional protein synthesis was done by using the enrichment in tissue protein samples of L-[ring-$^2H_5$]phenylalanine, divided by the average enrichment in plasma (from area under the curve calculation of the curve, constructed from the three different phenylalanine isotopes). The enrichment of phenylalanine in plasma and tissue hydrolysates was measured by LC-MS/MS (Engelen et al. 2013; Luiking et al. 2015).

Ammonia

Ammonia levels were measured in arterial plasma using a commercial kit (Sigma, MO, USA). Ammonia levels were assessed based on the reaction with α-ketoglutarate and reduced nicotinamide adenine dinucleotide phosphate in the presence of L-glutamate dehydrogenase. Oxidation rate of reduced nicotinamide adenine dinucleotide phosphate was recorded by the absorbance decrease at 340 nm. Ammonia concentration was calculated according to manufacturer's protocol.

Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). Significance of difference was tested with unpaired t test or ANOVA followed by Bonferroni post-test using GraphPad Prism4 (La Jolla, CA, USA). Probability values of $p < 0.05$ were considered statistically significant.

Results

Bile duct ligation-induced cirrhosis leads to decreased gain in body weight

Body weight was significantly lower in BDL animals compared to SHAM from week 2 to the end of the experimental protocol (week 6) (Fig. 1). At 6 weeks, BDL rats weighed 422.4 ± 6.2 g compared to 509.7 ± 15.6 g for sham-operated animals ($p < 0.001$). Over the 6 weeks, daily food intake was similar (non-significant) between the two groups (area under the curve: 1018 g ± 34 g and 1078 ± 21 g in BDL and SHAM animals, respectively). However, there was a significant difference at week 1 between the two groups (13.4 ± 2.3 g vs 17.6 ± 0.5 g in BDL and SHAM animals, respectively; $p < 0.01$) (Fig. 2)

Bile duct ligation-induced cirrhosis provokes altered body composition

Six weeks after surgery, BDL rats displayed a significant decrease in lean (311.2 ± 12.1 g vs 376.5 ± 11.9 g; t(9) = 3.82, $p < 0.001$) and fat (33.9 ± 8.1 g vs 89.3 ± 9.7 g; t(9) = 10.15, $p < 0.001$) mass compared with SHAM animals (Fig. 3a, b), as measured by magnetic resonance.

Bile duct ligation-induced cirrhosis leads to decreased gastrocnemius muscle mass and circumference as well as altered muscle morphology

Compared with control rats, BDL animals had decreased gastrocnemius mass (1.92 ± 0.05 g vs 2.85 ± 0.10 g; t(9) = 7.60, $p < 0.001$) and smaller gastrocnemius circumference

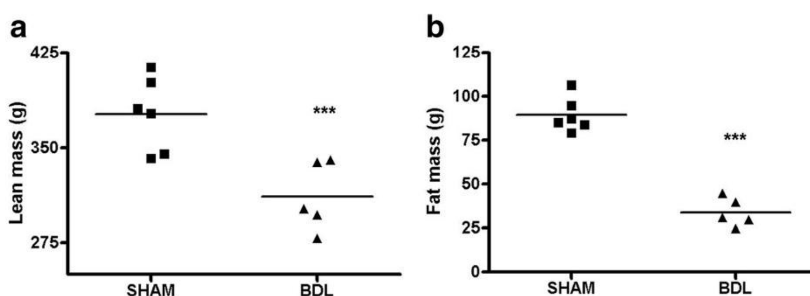

Fig. 3 **a** Lean mass and **b** fat mass in rats with bile-duct ligation (BDL) compared to sham-operated controls. *** $p < 0.001$, significantly different from SHAM

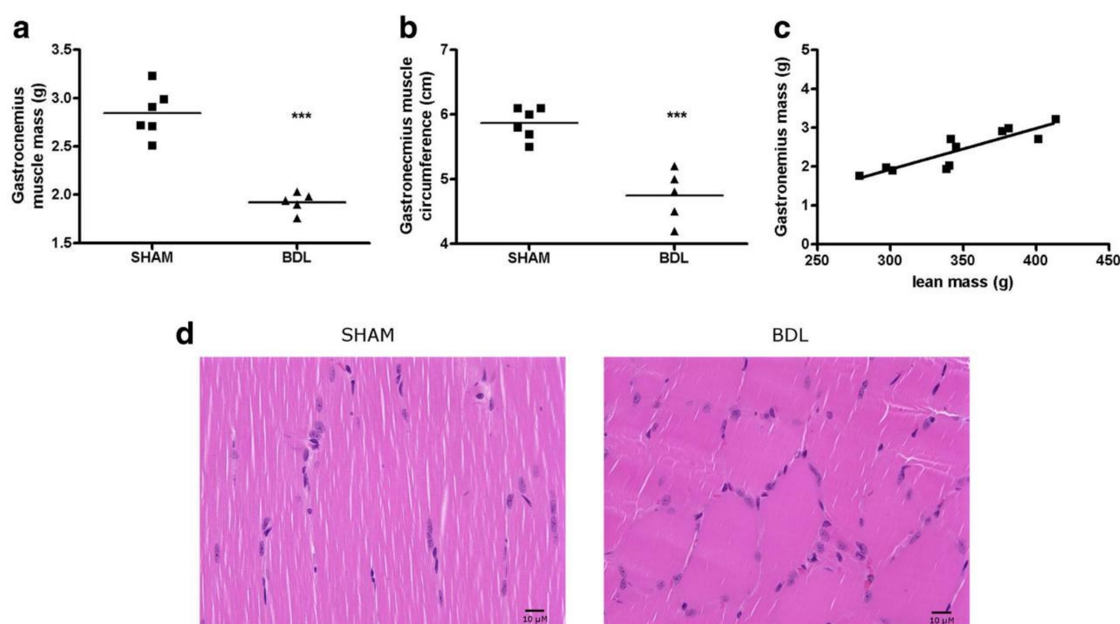

Fig. 4 **a** Gastrocnemius muscle mass **b**, muscle circumference **c**, correlation between lean mass and gastrocnemius mass and **d** muscle histology of rats with bile-duct ligation (BDL) compared to sham-operated controls. *** $p < 0.001$, significantly different from SHAM. Bar = 10 μm

(4.7 ± 0.2 cm vs 5.9 ± 0.1 cm; t(9) = 5.80, $p < 0.001$) (Fig. 4a, b). A strong correlation was observed between lean and muscle mass in both BDL and SHAM rats ($r = 0.8889$; $p = 0.0003$) (Fig. 4c). Gastrocnemius muscle morphology was analyzed by histological analysis of muscular tissues in SHAM and BDL animals at the end of the 6 week experimental protocol. Hematoxylin-eosin staining revealed disorganized fibres in BDL muscles (Fig. 4d).

Bile duct ligation-induced cirrhosis reduces muscle protein synthesis

Muscle protein synthesis, 6 weeks after surgery, was significantly reduced in BDL rats compared to SHAM animals (0.32 ± 0.02%/h and 0.17 ± 0.07%/h, respectively; t(10) = 1.81, $p < 0.05$), as evidenced by decreased fractional synthesis rate, whereas protein synthesis in other organs including the brain, heart, intestine, kidney, liver and lung was unaltered (Table 1).

Table 1 Protein synthesis of organs in rats with bile-duct ligation (BDL) compared to sham-operated controls. *$p < 0.05$, significantly different from SHAM

	SHAM	BDL
Frontal Cortex (%/hour)	0,63 ± 0,25	0,56 ± 0,07
Heart (%/hour)	0,68 ± 0,31	0,64 ± 0,14
Intestine (%/hour)	4,46 ± 1,21	3,42 ± 1,34
Kidney (%/hour)	2,34 ± 0,92	1,73 ± 0,29
Liver (%/hour)	3,45 ± 1,29	2,95 ± 0,67
Lung (%/hour)	1,08 ± 0,39	1,25 ± 0,31
Muscle (%/hour)	0,32 ± 0,19	0,17 ± 0,07*

*$p < 0.05$, significantly different from SHAM

Bile duct ligation-induced cirrhosis increases ammonia levels

Arterial ammonia significantly increased in BDL rats (129.0 ± 14.8 μM vs 42.1 ± 8.2 μM respectively; t(9) = 5.38, $p < 0.001$) (Fig. 5).

Discussion

Results of the present study demonstrate that BDL (6 week model) is associated with significant alteration in body composition as evidenced by decreased lean and fat mass, reduced gastrocnemius muscle mass and circumference as well as altered muscle morphology. The strong correlation observed

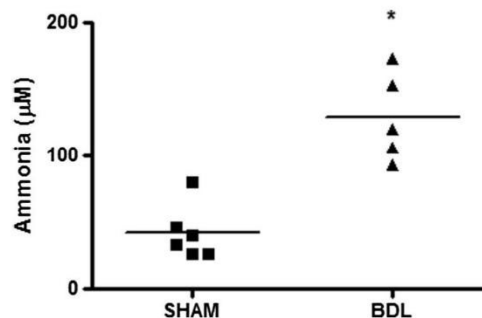

Fig. 5 Serum ammonia in rats with bile-duct ligation (BDL) compared to sham-operated controls. * $p < 0.05$, significantly different from SHAM

between gastrocnemius mass (weight) and overall lean mass (assessed by MRI) indicates that gastrocnemius is representative of muscle body composition. Interestingly, our results also indicate a significant decrease in fat mass in BDL compared to SHAM rats. This reduction could be a result of fat mass been used as an energy source in order to maintain muscle mass during liver disease. The mechanisms responsible for fat mass decrease in experimental cirrhosis remain to be elucidated.

We found altered muscle morphology in BDL compared to SHAM rats which is most likely due to collapse of a smaller muscle. The mechanisms responsible for this alteration remain elusive. In addition to significant changes in muscle morphology, our results also revealed muscle protein synthesis is significantly decreased in BDL animals compared to SHAM. Impaired muscle protein synthesis may represent a major cause for muscle mass loss in experimental cirrhosis. Interestingly, alteration in protein synthesis was specific to the muscle as the protein synthesis rate was unchanged in the brain, heart, intestine, kidney, liver and lung between the two experimental groups. We speculate that ammonia may exert a deleterious effect on the muscle and contribute to its dysfunction by altering protein synthesis. This is supported with studies demonstrating hyperammonemia induced by portacaval-systemic shunting is associated with reduced muscle mass synthesis (Dasarathy et al. 2011; Davuluri et al. 2016).

Our study also indicate that BDL-induced cirrhosis leads to decreased gain in body weight compared to sham-operated animals, whereas food intake remains similar from week 2 to week 6. The statistical difference in food intake observed at week 1 (a consistent observation found with our extensive experience with this model) (Rose et al. 1999; Bosoi et al. 2011; Bosoi et al. 2012) may be explained by the invasive nature of the BDL surgery along with the recovery phase required for such a surgical procedure. This suggests that nutritional problems occur during the setting of cirrhosis which may include, among other mechanisms, metabolic alterations, malabsorption of nutrients, increased intestinal protein losses, disturbance of substrate utilization and increased energy expenditure (Bémeur et al. 2010). Specifically, hypermetabolic state and increased energy-protein expenditure and requirements may occur in chronic liver disease. Indeed, the hyperdynamic circulation in cirrhosis leads to systemic vasodilation and an expanded intravascular blood volume which consequently leads to a greater use of macro- and micronutrients; hence causing a high energy expenditure and demand. Also, the inflammatory state associated with the inability of the liver to adequately clear activated proinflammatory mediators may result in hypermetabolism (Tilg et al. 1992; von Baehr et al. 2000). Regarding malabsorption, the cirrhotic liver may inadequately synthesize proteins and has diminished storage capacity and an impaired enterohepatic cycle. In addition, portal hypertensive enteropathy may lead to impaired absorption of essential nutrients. Moreover, pancreatic insufficiency, cholestasis, and drug-related diarrhea may all contribute to malabsorption in liver disease. Loss of proteins may result from complications of cirrhosis or from iatrogenic interventions including the use of diuretics for the treatment of ascites and fluid retention as well as the use of lactulose for the treatment of hepatic encephalopathy. Blood loss from oesophageal and gastric varices and from the intestinal lumen due to ulcers or portal enteropathy may also lead to increased protein loss in cirrhosis.

Several factors have been implicated in the pathogenesis of hepatic encephalopathy, including ammonia (Cooper and Plum 1987; Felipo and Butterworth 2002), oxidative stress (Görg et al. 2010; Bosoi et al. 2012), inflammation (Shawcross and Jalan 2005; Montoliu et al. 2009), lactate (Bosoi et al. 2014) and gut microbiota (Bajaj et al. 2008). Ammonia is considered the major factor in the pathogenesis of hepatic encephalopathy as hyperammonemia leads to toxic levels of ammonia in the brain (Cooper and Plum 1987; Felipo and Butterworth 2002). During liver disease, extra-hepatic ammonia metabolism is altered and, with muscle expressing an ammonia-lowering enzyme, glutamine synthetase, muscle plays a critical compensatory role in attenuating hyperammonemia. However, muscle wasting can exasperate the degree of hyperammonemia and may play a pivotal role in the risk of developing hepatic encephalopathy (Qiu et al. 2012; Montano-Loza et al. 2015; Rombouts et al. 2016). The underlying causes of muscle wasting in liver disease remain undetermined. However, paradoxically, it has been demonstrated that high ammonia concentrations leads to muscle dysfunction and reduction in protein synthesis (Qiu et al. 2012; Qiu et al. 2013). An understanding of ammonia and its removing vs pathophysiological pathways is totally unclear and remains to be thoroughly investigated.

We conclude that the 6-week BDL experimental model is an excellent model to study liver disease-induced muscle mass loss. Demonstration of similar alterations in human cirrhosis will potentially enhance our understanding of the mechanisms of muscle mass loss in liver disease. Importantly, understanding these mechanisms will allow the identification of therapeutic targets to prevent muscle mass loss and ameliorate the quality of life and the prognostic of patient suffering from liver disease.

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Annex 2: Progressive resistance training prevents loss of muscle mass and strength in bile duct-ligated rats

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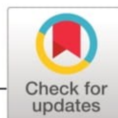

CIRRHOSIS AND LIVER FAILURE

WILEY Liver INTERNATIONAL

Progressive resistance training prevents loss of muscle mass and strength in bile duct-ligated rats

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Abstract

Background: Loss of muscle mass and strength is common in cirrhosis and increases the risk of hyperammonaemia and hepatic encephalopathy. Resistance training optimizes muscle mass and strength in several chronic diseases. However, the beneficial effects of resistance training in cirrhosis remain to be investigated. Bile duct-ligated (BDL) rats develop chronic liver disease, hyperammonaemia, reduced muscle mass and strength. Our aim was to test the effects of resistance training on muscle mass, function and ammonia metabolism in BDL-rats.

Methods: A group of BDL-rats underwent a progressive resistance training programme and a group of non-exercise BDL-rats served as controls. Resistance training comprised of ladder climbing with a progressive increase in carrying weights attached to the tail. Training was performed 5 days a week during 4 weeks. Muscle strength and body composition were assessed using grip strength and EchoMRI. Weight and circumference of the gastrocnemius muscle (normalized to bodyweight), plasma ammonia and glutamine synthetase protein expression and activity were assessed.

Results: BDL + exercise rats had significantly larger gastrocnemius circumference compared to non-exercise BDL-rats: ratio 0.082 vs 0.075 ($P < 0.05$). Gastrocnemius muscle weight was higher in exercisers than controls: 0.006 vs 0.005 ($P < 0.05$). A tendency towards a lower plasma ammonia in the exercise group compared to controls was observed ($P = 0.10$). There were no differences in lean body mass, GS protein expression and activity between the groups.

Conclusion: Resistance training in rats with chronic liver disease beneficially effects muscle mass and strength. The effects were followed by non-significant reduction in blood ammonia; however, a tendency was observed.

KEYWORDS
cirrhosis, exercise, hepatic encephalopathy, hyperammonaemia, muscle strength

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Abbreviations: BDL, bile duct ligation; BDL-rat, bile duct-ligated rat; GS, glutamine synthetase; HE, hepatic encephalopathy; TCA, tricarboxylic acid.

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1 | INTRODUCTION

Diminished skeletal muscle mass and strength are frequent complications in cirrhosis, and adversely influence clinical outcomes. Loss of muscle mass is an independent risk factor for complications and mortality in chronic liver disease.[1,2]

Glutamine synthetase (GS) is an ammonia-utilizing enzyme that catalyzes the synthesis of glutamine from glutamate. The activity of GS is low in muscle tissue but being the largest organ in the body, it plays a substantial compensatory role when ammonia detoxification in the liver is significantly reduced.[3] Hence, in relation to complications and especially the treatment of hepatic encephalopathy (HE), optimizing skeletal muscle mass may be an important target.[4]

Physical activity, especially resistance training, can be beneficial in optimizing skeletal muscle mass. To date, only few studies have investigated the beneficial effects of exercise on skeletal muscle in cirrhotic patients.[5] In addition, these studies focused solely on aerobic exercise. It was observed that aerobic exercise had a positive effect on endurance and on muscle mass.[6-8]

Resistance training is superior for muscle-building and -strengthening when compared to other types of exercise.[9] Resistance training accelerates protein synthesis, induces muscle hypertrophy and increases strength in healthy individuals.[10] Currently, no studies have investigated the effects of resistance training on muscle mass or strength in cirrhosis (both animal models and patients). Since the loss of muscle mass remains a highly prevalent complication in cirrhosis, intervention strategies including resistance exercise programmes to help optimize skeletal muscle during chronic liver disease are highly warranted.

Chronic liver disease following bile duct ligation (BDL) in the rat is a well-characterized model which leads to complications such as jaundice, ascites, portal hypertension, hyperammonaemia and HE. Additionally, BDL-rats develop muscle abnormalities with loss of muscle mass and reduction in force.[11,12] Previous animal studies suggest that hyperammonaemia may play an important part in the pathogenesis that lies behind the development of muscle abnormalities.[11-14]

The aim of this study was to evaluate the effect of 4 weeks of progressive resistance training on optimizing skeletal muscle mass and strength in BDL-rats. In addition, we tested the effects of resistance exercise on ammonia metabolism.

Key points

- The BDL-rat develops chronic liver disease including complications such as loss of muscle mass, muscle strength and high ammonia blood levels. BDL-rats tolerated progressive resistance training and as a result muscle strength and calf size were greater in BDL-exercise rats compared to BDL-controls (not exercising) at the end of the study.

2 | METHODS

2.1 | Animal model/Bile duct ligation

Bile duct ligation (BDL) was performed to induce chronic liver disease in male Sprague-Dawley rats (175-200 g; Charles River, QC, Canada). The rats were anesthetized by isoflurane. During laparotomy, the common bile duct was located, ligated and resected under a dissecting microscope.[11] All rats resided in controlled conditions (22°C, 12 hours:12 hours dark-light cycle) with free access to food and water.

Twenty-six rats were bile duct-ligated. One BDL-rat died during the week of convalescence. The remaining BDL-rats were randomly split into two groups before the habituation period and referred to as; BDL-exercisers (n = 13) and BDL-controls (n = 12). BDL-exercisers performed resistance training for 4 weeks while BDL-controls did not undergo resistance training (non-exercisers). All rats were sacrificed 5 weeks post-surgery.

All experiments were conducted following the guidelines of the Canadian Council on Animal Care and were approved by the Animal Protection Committee of the Centre de Recherche du Centre Hospitalier de l'Université de Montréal (CRCHUM).

2.2 | Exercise protocol

The resistance training equipment (a ladder) for rodents was previously described by Hornberger et al. The ladder measured 110 cm in height with 80-85° of inclination and had a housing chamber placed on the top.[15,16] Instead of steps, our ladder consisted of a wire mesh.

One week after surgery, a 2-day habituation period with equipment familiarization was introduced to BDL-exercisers. Day one of habituation; for the first climb, the rats were placed on the ladder 10 cm from the top (housing chamber), for the 2nd climb in the middle of the ladder and at the bottom of the ladder for 3rd-8th climb. During the 6th-8th climb, an empty plastic tube was attached to the tail with medical tape. Between each climb, rats rested for 2 minutes in the housing chamber. Day two of habituation was a repeat of day one except weights were inserted into the plastic tubes bound to the tail for the 3rd-8th climb. The maximal carrying load was determined by adding 30 g to each subsequent climb following the established baseline (50% of bodyweight) until exhaustion (when the rats could not complete the climb).

After the 2-day habituation period, 18 exercise sessions were performed 5 days per week until sacrifice. All rats were well qualified to perform resistance training after a 2-day habituation period. Each session consisted of climbing the ladder with a carrying a load and that every second climb increased progressively from 50%, 75%, 90% to 100% of the maximal load. If all eight climbs were

kerosene and bromobenzene mixtures and precalibrated with K_2SO_4 solutions of known densities. Eight sample measurements were averaged in each rat. Water content was calculated based on tissue density.[23]

2.11 | The open field test

The open field test is an indicator of exploratory behaviour, activity and anxiety. The open field test (software SMART® version 2.5, Panlab, Spain) consisted of an open black box (90 × 90 × 40 cm) placed in a quiet, dimly lit room and with a video camera equipped with an automatic movement tracking system connected to a computer and placed above the box. The open field area was divided in a central zone (45 × 45 cm) and a peripheral zone. The rat was placed in the room 30 minutes prior to testing, and at the start of the test placed in the periphery of the open field for 1 minute. Behaviour was recorded for the subsequent 5 minutes.

The total length of each tracked trail was measured. Time spent in central zone as well as the number of entries into the central zone were indicators of natural exploratory behaviour and anxiety.[24]

2.12 | Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM) or median with interquartile ranges (IQR). Significance of difference between the BDL-exercise and BDL-control groups was tested with unpaired *t* test or Wilcoxon Mann-Whitney test when appropriate. Correlation was assessed using Pearson's correlation. All tests were run using GraphPad Prism 6 (La Jolla, CA, USA). *P*-values < 0.05 were considered statistically significant.

3 | RESULTS

3.1 | Exercise

All BDL-exercisers completed the protocol. The average maximal carrying load progressed throughout 18 sessions without any decrease the last week from session 1:280 ± 6 g to session 18:760 ± 9 g.

3.2 | Body weight and food consumption

Both at baseline and at week one (start of resistance training), body weight was similar between groups. Both groups consumed an equivalent amount of food (isocaloric and isonitrogenous diet) every week (see Table 1 for details). The BDL-exercisers had a significant lower body weight compared to the BDL-controls during the last 2 weeks. Week 4:352 ± 8 g vs 381 ± 11 g (*P* = 0.05) and week 5:364 ± 10 g vs 402 ± 11 g (*P* = 0.02).

3.3 | Muscle size and grip strength

The BDL-exercisers had a significantly larger gastrocnemius circumference (mm) normalized to bodyweight (g) compared to controls: ratio 0.082 ± 0.001 vs 0.075 ± 0.002 (*P* = 0.01; Figure 1A). The weight of the gastrocnemius normalized to bodyweight (g) was also significantly higher in exercisers compared to controls: 0.0060 ± 0.0003 vs 0.0053 ± 0.0001 (*P* = 0.04; Figure 1B). A correlation was found between the gastrocnemius muscle weight and circumference; *r* = 0.61 (*P* = 0.001).

Grip strength was significantly stronger in BDL-exercisers vs BDL-controls in both forelimbs: 1310 ± 40 g vs 1064 ± 38 g (*P* < 0.01) and

TABLE 1 Results

	BDL- exercise	BDL- control	*P*-value
Food intake per week			
Week 1 (g)	43 ± 1	42 ± 1	0.49
Week 2 (g)	171 ± 5	177 ± 1	0.23
Week 3 (g)	149 ± 2	157 ± 5	0.18
Week 4 (g)	187 ± 4	196 ± 4	0.14
Week 5 (g)	160 ± 8	180 ± 10	0.13
EchoMRi			
Lean mass-to-bw ratio	0.77 ± 0.01	0.78 ± 0.01	0.82
Fat mass-to-bw ratio	0.10 ± 0.01	0.11 ± 0.004	0.36
Total water-to-bw ratio	0.66 ± 0.01	0.64 ± 0.004	0.15
Free water-to-bw ratio	0.05 ± 0.01	0.03 ± 0.01	0.38
Blood samples			
Ammonia, μmol/L	106 ± 16	142 ± 13	0.10
ALT, U/L	68 ± 7	56 ± 4	0.16
AST, U/L	297 ± 32	256 ± 17	0.27
ALP, U/L	455 ± 48	548 ± 79	0.32
GGT, U/L	23 ± 3	21 ± 1	0.53
Bilirubin, μmol/L	161 ± 2	175 ± 8	0.46
Albumin, g/L	20 ± 1	21 ± 1	0.50
GS			
Activity, unit/μg protein	0.18 ± 0.004	0.18 ± 0.004	0.49
Relative protein expression, GS band intensity/total band intensity	2.20 ± 0.18	1.98 ± 0.21	0.37
Brain water %	80.2 ± 0.5	79.7 ± 0.6	0.55

Values are given as mean ± SEM.
ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate transami-nase; BW, bodyweight; GGT, gamma-glutamyl transferase; GS, glutamine synthetase.

successfully completed, an extra 40 g were added to the 9th climb -setting a new maximal load. Each animal performed 8-12 dynamic movements/repetitions per climb.[17]

All BDL-exercisers successfully completed the resistance training programme without injuries or death. There was a whole day's rest between last exercise session and sacrifice.

2.3 | Body weight and food consumption

Body weight and food intake were measured weekly. Standard rodent chow (TD.2819, 18% protein, Envigo, Madison, WI, USA) and water were offered ad libitum. Rats were housed two rats per cage, and food consumption was measured as a mean per week for each rat.

2.4 | Muscle strength

Muscle strength was measured by a grip strength metre using a digital force gauge (Chatillon® DFE-010; AMETEK TCI Division, Largo, FL, USA) with mesh pull bars. The peak strength applied to the gauge was recorded in series of four and the mean value was calculated. The hindlimb strength was measured placing the mesh pull bar of the gauge in a 45-degree angle and facing the rat away from the gauge. While gently supported at the thorax and base of the tail, the rat was lowered to the gauge, grapping the bar firmly with both hind paws and then slowly pulled backwards until releasing the bar. The forelimb strength was measured positioning the gauge horizontally, both front paws were placed on the pull bar and the rat was gently pulled in a straight direction away from the bar until releasing it.[18]

2.5 | Gastrocnemius muscle weight and circumference

After sacrifice the right-sided gastrocnemius muscle was carefully exposed. Circumference of the muscle was measured by a scaled thread, while the muscle was still attached to the leg and after dissection the wet weight was measured using an electronic scale.

2.6 | Body composition

Whole body composition was estimated before sacrifice by EchoMRi 700® Body Composition Analyzer (R & D EchoMRi LLC, Houston, TX, USA) -a nuclear magnetic resonance (NMR) system. The non-sedated rats were placed in a plastic tube for 3-4 minutes. Whole body mass measures of fat, lean, total water and free water were measured.[19]

2.7 | Blood samples

At sacrifice, arterial plasma drawn from the heart was snap frozen and stored at −80°C. Blood samples were measured by the biochemistry department at CRCHUM, Montréal, Canada. Plasma ammonia μmol/L was measured using enzyme glutamate dehydrogenase (Randox Laboratories Ltd, Crumlin, UK) as a mean of three measurements.[20]

2.8 | Glutamine synthetase protein expression

Western blot assays were performed to assess GS protein levels. Briefly, after protein quantification using Bio-Rad DC protein assay kit (Bio-Rad Laboratories, Irvine, CA, USA), 5 μg of muscle lysates were mixed in Laemmli buffer, heated for 5 minutes at 100°C and loaded into 9% sodium dodecyl-sulphate polyacrylamide gels (SDS-PAGE resolving gel: 0.375 mol/L Tris pH 8.8; 0.1% sodium dodecyl sulphate; 9% acrylamide). After electrophoresis, proteins were transferred to a nitrocellulose membrane (Bio-Rad Laboratories) and blocked for 1 hour in a solution of Tris-buffered saline + Tween 20 (TBST)-Milk (1 mmol/L Tris; 10 mmol/L NaCl; 0.5% Tween-20; 5% skimmed milk). Mouse anti-GS antibody (BD Biosciences, Billerica, ME, USA) was diluted to 1/2000 in TBST-Milk and incubated for 1 hour. The membranes were washed with TBST solution for five times of 5 minutes and then incubated with horseradish peroxidase (HRP) conjugated secondary antibody (anti-mouse IgG, Jackson ImmunoResearch, West Grove, PA, USA). After further washes, the membranes were exposed to an antibody detection reagent (Clarity Western ECL substrate, Bio-Rad Laboratories) and imaged using the ChemiDoc imaging system using the "chemiluminescent blot" setting (Bio-Rad Laboratories).

The GS expression was normalized by total protein staining as described by Ladner et al.[21] Briefly, 0.5% trichloroethanol was added to all SDS-PAGE gels. After electrophoresis, gels were exposed to UV light for 45 seconds for activation, using the stain free gel setting of the ChemiDoc imaging system. Immediately after transfer, the nitrocellulose membrane was imaged for the total protein using the "stain free blot" setting of the ChemiDoc imaging system. Finally, we quantified the total protein as well as the GS protein using the Image Lab software (Bio-Rad Laboratories).

2.9 | Glutamine synthetase activity

Glutamine synthetase activity was determined in gastrocnemius muscle based on the formation of a γ-glutamylhydroxamate ferric chloride complex. Briefly, after protein quantification using Bio-Rad DC protein assay kit (Bio-Rad Laboratories), muscle lysates (30 μL) were incubated with reaction mix (100 mmol/L Imidazole; 50 mmol/L sodium L-glutamate; 10 mmol/L β-mercaptoethanol; 20 mmol/L disodium ATP; 40 mmol/L magnesium chloride and 100 mmol/L hydroxylamine) for 1 hour at 37°C. The reaction was terminated by adding the stop solution (0.37 mol/L ferric chloride; 0.67 mol/L hydrochloric acid and 0.2 mol/L trichloroacetic acid). After 30 minutes at 4°C, absorbance was measured at 530 nm. Results are expressed as activity by μg of protein.[22]

2.10 | Brain water content

Brain water content was measured using the sensitive densitometry technique. After the animal was sacrificed, the frontal cortex was freshly dissected at 4°C and cut into 2-mm^3 pieces. Tissue pieces were placed in density gradient columns, and the equilibrium point was recorded after 2 minutes. Columns were made with different

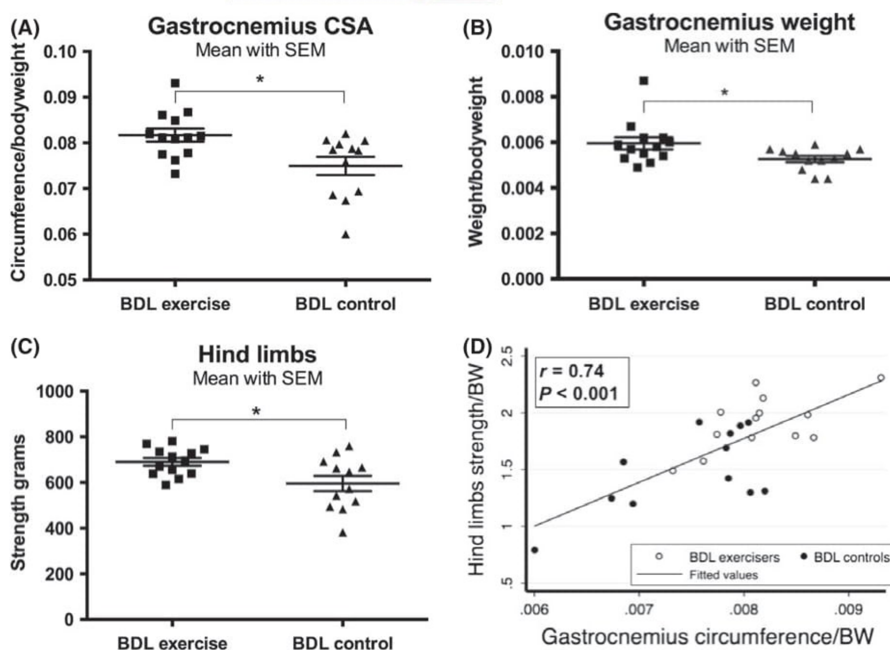

FIGURE 1 A, Gastrocnemius circumference by bodyweight, B, Gastrocnemius weight by bodyweight, C, Strength in hindlimbs all presented as mean ± SEM. Unpaired *t* test compared BDL-exercisers to BDL-controls, results shown as *$P < 0.05$. D, Pearson's correlation tested gastrocnemius circumference and hindlimb strength, both normalized to bodyweight (BW)

hindlimbs: 690 ± 17 g vs 596 ± 33 g ($P = 0.02$; Figure 1C). Hindlimb strength correlated with gastrocnemius weight; $r = 0.69$ ($P < 0.001$) and gastrocnemius circumference; $r = 0.74$ ($P < 0.001$; Figure 1D).

3.4 | Whole body composition

There was no significant difference in whole body composition parameters (lean mass, total water, free water and fat mass normalized with body weight) between the groups 5 weeks after surgery (Table 1).

3.5 | Blood samples

A reduction in plasma ammonia levels in BDL-exercisers vs BDL-controls was observed but did not reach significance: 106 ± 16 μmol/L vs 142 ± 13 μmol/L ($P = 0.10$; Figure 2A). Moreover, there was no significant difference in the liver biochemistry between the groups (Table 1).

3.6 | Glutamine synthetase

There was no significant difference in GS protein expression and GS activity between the groups (Table 1 and Figure 2B,C).

3.7 | Brain water content

There was no difference in brain water content between the groups (Table 1).

3.8 | The open field test

There was an increase in the total distance travelled between the two groups but this difference did not reach significance; distance travelled BDL-exercisers vs BDL-control: 54 ± 6.9 cm vs 35 ± 7.4 cm ($P = 0.07$). However, the exercisers explored and entered the centre more frequently than controls. Total time in centre was (median and IQR): 2.1 (0.9-4.2)% vs 0.3 (0.0-1.0)% ($P = 0.008$). Number of entries to the centre was (median and IQR): 3 (1-5.5) times vs 0.5 (0.0-1.8) times ($P = 0.03$).

4 | DISCUSSION

To the best of our knowledge, this is the first study to show that resistance training in BDL-rats diminishes the aggressive loss of both muscle size and strength ($P < 0.05$, Figure 1A,B). Our findings are in accordance with previous studies in other chronic disease states where strength and size of muscle improved with resistance training.[25,26] Plasma ammonia tended to be lower in the exercise group but did not reach significance ($P = 0.10$) and no improvement in behaviour or brain oedema was observed in BDL-exercisers vs BDL-controls. Whether an extended amount of time is required to observe a significant reducing effect on plasma ammonia and subsequently an improvement in HE remains to be further investigated.

Using EchoMRI, it was found that the overall lean body mass of the BDL-exercisers was unaffected. As seen in previous studies, specific exercise protocols affect certain muscles. Similar resistance training protocols have led to regional hypertrophy in gastrocnemius, flexor hallucis and digitorum longus muscles—all important climbing muscles.[27,28] Therefore, since only certain muscles may benefit from the exercise protocol, this may explain insignificant changes in lean body mass between the two groups. The resistance training protocol involves equipment which replicates the rodents natural climbing habits and therefore probably predisposes to affect the hindlimb relative to whole body. Moreover, fluid retention in cirrhosis often disturbs the estimates of lean mass. The lean body mass results should therefore be interpreted with caution compared to direct muscle measurements.[29] The gastrocnemius muscle does not

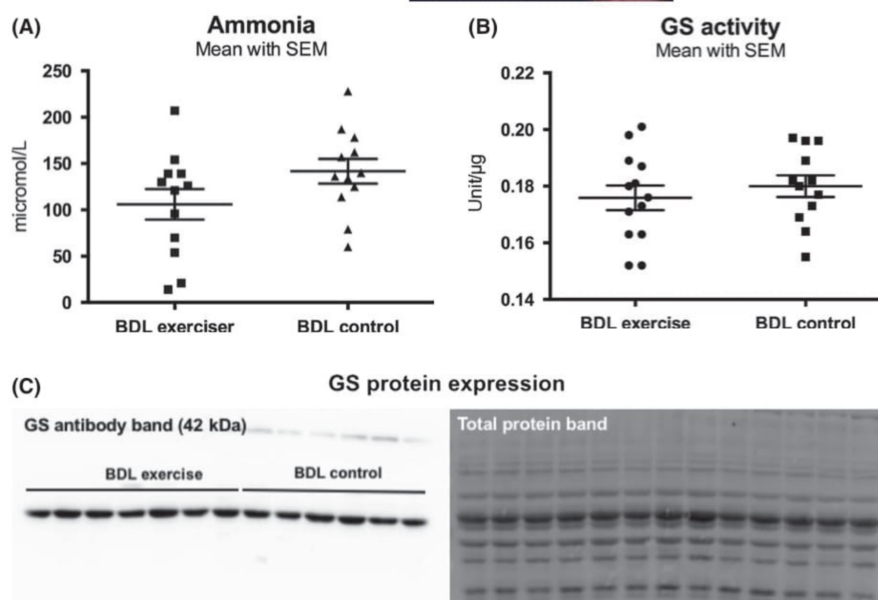

FIGURE 2 A, Ammonia levels. No difference was seen, $P = 0.10$. B, GS activity is presented as mean ± SEM activity by µg of protein. Unpaired *t* test compared BDL-exercisers to BDL-controls. C, Western plot showing GS protein expression in muscle in BDL-exercisers compared to BDL-controls. Total protein band is shown as quantification. Results in A and B are expressed as mean ± SEM of bands density of GS by total protein expression

directly contribute to the hindlimb grip strength. However, the extension of the tarsal joint caused by the muscle opposes the movement caused by the grip strength test and thereby contributes to the overall recorded strength.[18] Nonetheless, the clear correlations between gastrocnemius size (weight and circumference) and strength support that we optimized muscle fibre components and did not just increase muscle size by water retention.

Previous studies have shown that BDL-rats have a reduced muscle protein synthesis causing depletion of lean body mass including reduced gastrocnemius circumference and weight compared to healthy rats (SHAM-operated).[11] Also, in BDL-mice, the cross-sectional area of quadriceps is decreased with a reduced number of myofibres and disturbed contractile dysfunctions (reduced time to fatigue and force).[12] These muscle abnormalities imitate muscle depletion in patients with cirrhosis. Therefore, the BDL-model is a suitable model to test the effects of exercise on muscle and ammonia levels in chronic liver disease. This is the first study to demonstrate that BDL-rats are capable of performing a progressive resistance training programme. The protocol was well-tolerated and thus, the BDL-rat is a qualified animal model for future tests of the effects of resistance training in chronic liver disease.

The improved muscle size may support ammonia removal using branched-chain amino acids (BCAA) as substrates for the tricarboxylic acid (TCA) cycle, enhancing α-ketoglutarate and glutamate formation and ultimately stimulating glutamine synthesis. Optimizing skeletal muscle mass may therefore improve extra-hepatic ammonia clearance.[30] The gastrocnemius muscle changed favourably in the exercise group, and we observed a non-significant tendency towards a decrease in plasma ammonia in exercisers. We did not see an increase in GS protein expression and GS activity in muscle. Originally, it was found that 50% of the arterial ammonia is metabolized in muscle tissues in liver disease.[31] GS muscle activity is normally very low, but due to the large muscle volume a minor increase in GS could alter plasma ammonia. This may be statistically difficult to obtain. Additionally, muscle GS protein expression and activity are already elevated in BDL-rats compared to SHAMs.[32] Thus, this protocol may not induce a further increase in GS activity but by increasing muscle mass volume, it increases the capacity of ammonia removal by muscle. However, the increase in muscle mass following resistance training did not lead to a significant reduction in plasma ammonia, but a strong trend. An extended amount of time with muscle mass optimization may be required to see a significant effect on plasma ammonia in BDL-rats. Furthermore, there is also the possibility of limited TCA cycle substrates, as the levels of BCAA are known to be low in cirrhosis.[33]

Nevertheless, it is worth noting that muscle optimization occurred in spite of hyperammonaemia. In animal models, it has been shown that hyperammonaemia causes muscle depletion and contractile dysfunction.[14,34] Ammonia may upregulate myostatin expression[35] and myostatin has been shown to be elevated in BDL-models.[12] Myostatin deactivates the mammalian target of rapamycin (mTOR) resulting in decreased protein synthesis and increased autophagy, at least in part mediated through downstream molecules as p70S6K and 4E-BP1.[35,36] Several physiological factors are triggered by resistance training, but among other, weight training activates protein synthesis trough the above mentioned mTOR pathway.[37] This indicates exercise can counteract hyperammonaemia. Further investigations are required to describe the underlying pathways.

The two BDL groups maintained a similar weight on an isocaloric and isonitrogenous diet until week 4, when the BDL-exercisers had a significantly lower weight compared to BDL-controls. This is in accordance with other exercise studies and possibly caused by the elevated energy requirements from the intensive exercise programme.[38,39] The increased activity level with a liver-induced catabolic state may have required additional nutritional supplements to avoid further muscle proteolysis and ensure an optimal environment for optimizing muscle mass. Therefore, an increased calorie and

food intake in the exercise group probably could have improved the strength and muscle size further.

In this study, brain oedema was not affected by exercise. This is to be expected since the reduction in blood ammonia was not significant. It has been previously demonstrated that lowering plasma ammonia in BDL-rats leads to an improvement in brain oedema.[40] However, other factors are believed to be involved in the pathogenesis of brain oedema. However, we did see less anxiety and more exploring in BDL-exercisers vs non-exercisers using the Open Field Test; even though the BDL-rat is known to be less active.[41] This may point towards an increased brain function and an ameliorating effect of exercise on HE symptoms. However, this remains to be thoroughly investigated.

One major study limitation is the duration of the exercise protocol. The survival of the BDL-rat is restricted to approximately 5 weeks including 1 week of convalescence. This limits the total intervention time to 4 weeks. In addition, the required time following muscle mass optimization to see an effect on plasma ammonia may be limited. Chronic liver disease induced by carbon tetrachloride (CCl_4) could enable exercise to be tested for longer periods of time, but in these toxin models, TNF-α and IL-6 have been suggested as the main contributors to myopenia.[12] Variables such as carrying loads, duration and frequency of exercises are all adjustable and could strengthen the desired outcomes. Previous resistance training protocols for healthy and diseased rodents span from 1 to 26 weeks.[16] However, we believe that the most suitable protocol to investigate the liver-muscle-axis involves the BDL-model.

5 | CONCLUSION

Four weeks of exercise in BDL-rats optimized the overall skeletal muscle strength and the size of hindlimbs (gastrocnemius weight and circumference) compared to BDL-controls. Despite an increase in muscle mass, a significant decrease in blood ammonia was not observed but rather a trend. The BDL-rats were able to perform progressive resistance training despite of severe liver disease. We believe that this is an interesting model to test the pathways in the liver-muscle-axis influenced by resistance training.

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CONFLICT OF INTEREST

The authors do not have any disclosures to report.

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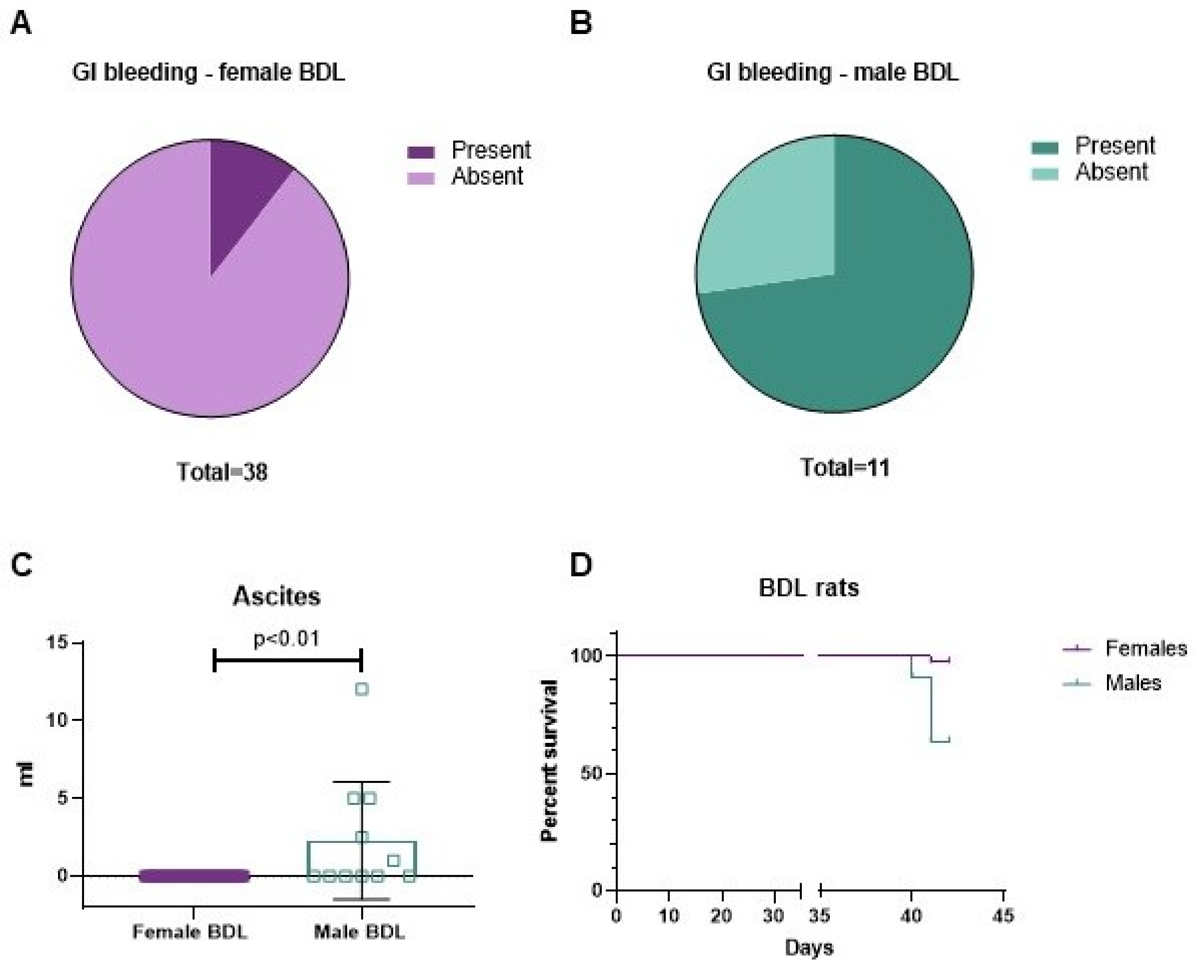
Annex 3: Complications of chronic liver disease in male and female BDL rats

Complications from the liver disease were assessed in male and female BDL rats 6 weeks after surgery. Abdominal fluid was collected and measured with a 10 ml syringe for ascites assessment during the euthanasia. Stomach and intestines were exposed, sectioned, and GI bleeding was considered present if apparent blood or bloody intestinal content (pink/purple) was found. Mortality assessment considered rats that had to be euthanized before completing 6 weeks after bile-duct ligation. Only rats that survived the first week of the model were included, excluding acute mortality from surgery complications. Euthanasia was recommended when rats showed weakness, paleness, weight loss, and decreased mobility. N=38 females and 11 males.

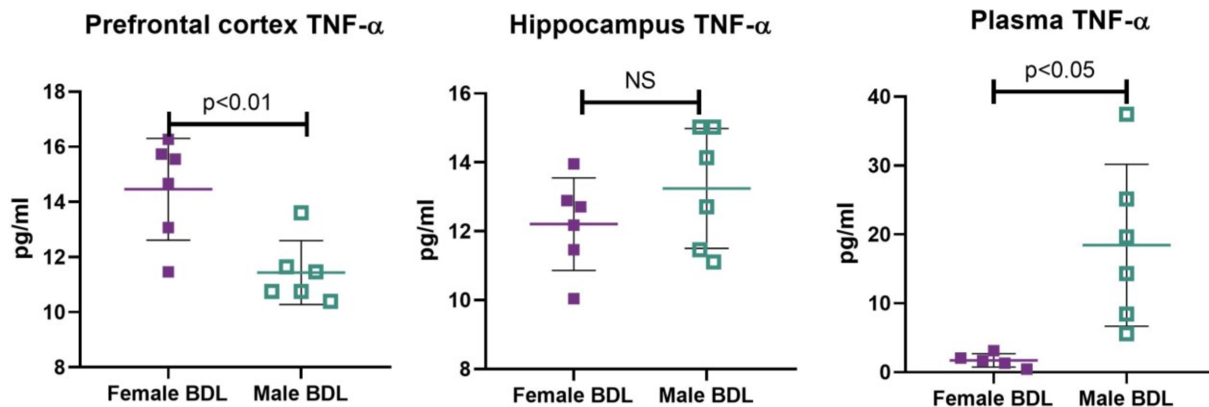


Complications of CLD in male and female BDL rats. After BDL surgery, female rats were protected against GI bleeding (p< 0.001)(A), ascites (p<0.01) (B), and mortality (p<0.001)(C). Chi-square test, parametric t-test, and Log-rank (Mantel-Cox) test. Numbers expressed as means ± SD or percentages; significance reached when p<0.05.

Annex 4: Inflammation in male and female BDL rats

Pro-inflammatory cytokines were measured in plasma and brain from 6 weeks male and females BDLs. Plasma was collected from the left ventricle in a heparinized syringe and centrifuged (4800 rpm) for 5 minutes during the euthanasia. Plasma was collected and snap-frozen until analysis. The brain was carefully dissected and snap-frozen until analysis.

TNF-α was assessed using an ELISA kit (88-7340, Thermo Fisher Scientific - USA). N= 6 animals per group. Brain tissue was lysed with RIPA buffer and, the protein was quantified by the Bio-Rad DC protein assay (see article 1 for more details on brain lysis). TNF-α was assessed using 100 µl of plasma or 100 µl of brain lysate (250 µg), in duplicates, according to manufacturer instructions.



Inflammation in male and female BDL rats. Six weeks after BDL surgery, female rats had lower brain frontal cortex ($p<0.05$) (A) but not hippocampus (B), and lower plasma TNF-α ($p<0.01$) (C) than males. Parametric t-test. Numbers expressed as means ± SD; significance reached when $p<0.05$.

CURRICULUM VITAE

Degrees

2017 Doctorate, Biomedical Sciences, Experimental medecine, Centre hospitalier de l'université de Montréal

Supervisors: Christopher Rose

2015 - 2016 Master's degree, Biomedical Sciences, Experimental medicine, Centre hospitalier de l'université de Montréal

Supervisors: Rose, Christopher

2009 - 2014 Bachelor's in Biological Sciences, University of the State of Sao Paulo – UNESP

Scientific activity

Publications (7)

1. Mariana M. Oliveira, Alexis Monnet-Aimard, Cristina R. Bosoi, Melanie Tremblay, Christopher F Rose (2021). Sex is associated with differences in oxidative stress and susceptibility to severe hepatic encephalopathy in bile-duct ligated rats. World J. Gastroenterol. Submitted.

2. Mariana M. Oliveira, Ole-Martin Fuskevag, Melanie Tremblay, Christopher F Rose (2021). Glutamine synthetase in endothelial cells of the blood-brain barrier. Journal of Neurochemistry. In preparation.

3. Rafael Ochoa-Sanchez, R., **Mariana M. Oliveira**, Melanie Tremblay, Grégory Petrazzo, Asha Pant, Cristina R Bosoi, Mylene Perreault, William Querbes, Caroline B Kurtz , Christopher F Rose (2021). Genetically engineered E. coli Nissle attenuates hyperammonemia and prevents memory impairment in bile-duct ligated rats. Liver Int. 41(5):1020-1032.

4. Clément, M.-A., Bosoi, C. R., **Oliveira, M. M**., Tremblay, M., Bémeur, C. and Rose, C. F. (2021) Bile-duct ligation renders the brain susceptible to hypotension-induced neuronal degeneration: Implications of ammonia J Neurochem 157(3):561-573.

5. Aamann, L., Ochoa-Sanchez, R., **Oliveira, M**., Tremblay, M., Bémeur, C., Dam, G., Vilstrup, H., Aagaard, N. K. and Rose, C. (2019) Progressive resistance training prevents loss of muscle mass and strength in bile duct ligated rats. Liver int. 39:676-683.

6. Matoori, S., Bao, Y., Schmidt, A., Fischer, E. J., Ochoa-Sanchez, R., Tremblay, M., **Oliveira, M. M**., Rose, C. F. and Leroux, J.-C. (2019) An Investigation of PS-b-PEO Polymersomes for the Oral Treatment and Diagnosis of Hyperammonemia. Small 15:e1902347.

7. Bosoi, C., **Oliveira, M**., Ochoa-Sanchez, R., Tremblay, M., TenHave, G., Deutz, N., Rose, C. F. and Bemeur, C. (2017) The bile-duct ligated rat: a relevant model to study muscle mass loss in cirrhosis. Metab Brain Dis. 32(2):513-518.

Oral presentations (4)

3 International conferences (ISHEN 2017, 2019, 2021)

1 National conference (Canadian liver meeting 2018)

Poster presentations (18)

10 International conferences (AASLD 2017, 2018, 2019, 2021; EASL 2017, 2021; ISHEN 2017, 2019 and ISN 2019)

4 National conference (Canadian liver meeting 2017, 2019, 2020, 2021)

4 Local conferences (CRCHUM congress 2018, 2021; Innove-action 2018; Neuro-metabolic club 2017)

Scholarships and prizes (16)

2021 EASL Meeting - free registration fee

2019 ISHEN Meeting - Travel Award

2019 ISHEN Meeting - Juan Cordoba Memorial Award for Best Junior Investigator

2019 Canadian Liver Meeting - Poster of Distinction

2018-2021 FRSQ - Bourse de recherche au doctorat

2018 - 2019 CRCHUM - Bourse d'excellence en recherche

2018 Université de Montréal - Bourse d’appui à la diffusion des résultats de recherche

2017-2018 – CRCHUM - Bourse de formation en recherche

2017-2018 Université de Montréal - Bourse d’exemption des droits de scolarité supplémentaires pour les étudiants internationaux

2017-2018 MITACS - MITACS Globalink Graduate Fellowship

2017 CRCHUM - Bourse de Voyage DÉFI-CRCHUM

2017 ISHEN Meeting - Best oral presentation

2017 ISHEN Meeting - Young investigator travel award

2017 EASL Meeting - Young Investigator bursary

2017 Université de Montréal - Bourse de cheminement aux études supérieures de la Faculté de médecine

2016-2017 Université de Montréal - Bourse du programme de sciences biomédicales