Ammonia: A novel target for the treatment of Non-Alcoholic Steatohepatitis

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SUMMARY

Non-alcoholic fatty liver disease (NAFLD) is a spectrum of liver diseases ranging from steatosis, through non-alcoholic steatohepatitis (NASH) to cirrhosis. The development of fibrosis is the most important factor contributing to NASH-associated morbidity and mortality. Hepatic stellate cells (HSCs) are responsible for extracellular matrix deposition in conditions of frank hepatocellular injury and are key cells involved in the development of fibrosis. In experimental models and patients with NASH, urea cycle enzyme gene and protein expression is reduced resulting in functional reduction in the *in vivo* capacity for ureagenesis and subsequent hyperammonemia at a pre-cirrhotic stage. Ammonia has been shown to activate HSCs *in vivo* and *in vitro*. Hyperammonemia in the context of NASH may therefore favour the progression of fibrosis and the disease. We therefore hypothesise that ammonia is a potential target for prevention of fibrosis progression of patients with NASH.

INTRODUCTION

The WHO estimates over 650 million people globally and more than 29 million Europeans have chronic liver disease [1]. Alarming increases in obesity rates and in tandem, increases in rates of non-alcoholic fatty liver disease (NAFLD), plus an ageing population mean that liver disease will become an even greater health concern over the next decade [2]. NAFLD is a spectrum of liver disease ranging from steatosis, through non-alcoholic steatohepatitis (NASH) to cirrhosis, and possible hepatocellular carcinoma. The prognosis of patients with simple steatosis appears good whereas NASH often has a progressive course with increased liver-related morbidity and mortality [3-5]. Given the increasing population prevalence of NAFLD and NASH, the ability to treat NASH would be a significant clinical breakthrough. Treatment options for NASH are limited to life-style modifications with no approved targeted drug therapy available. This article focuses on recent studies that provide the basis for proposing targeting of ammonia as a new therapeutic approach for NASH.

BACKGROUND

NAFLD is a condition characterized by fatty acid uptake, *de novo* fatty acid synthesis and reduced beta-oxidation leading to increased liver fat in patients that are not consuming excessive alcohol. The pathophysiological diagnosis of NASH refers to the presence of histological inflammation, pericellular fibrosis and liver injury super imposed on vesicular fat laden hepatocytes characteristic of NAFLD [6]. Recently, NASH has been defined as a “multiple parallel hits” disease that can progress to liver fibrosis, which develops primarily in the pericentral areas, surrounding groups of hepatocytes and thickening of the space of Disse with eventual nodule formation [7]. The development of advanced fibrosis is the

most important factor contributing to NASH-associated morbidity and mortality [8]. The main cell type responsible for extracellular matrix deposition are hepatic stellate cells (HSC), that undergo activation in conditions of frank hepatocellular injury, enabling them to participate in the wound healing process [9]. HSCs are key in maintaining architectural integrity and in the development of fibrosis, portal hypertension and liver cancer [10-12] and reduction in their activation is a target for therapy.

The Urea cycle, as a complete process running exclusively in the liver, has evolved in mammals to ultimately remove ammonia through conversion of excess amino-nitrogen to urea. The cycle comprises 5 enzymes, the first and the last of which reside in the mitochondria - cabamoylphosphate synthetase (CPS) and ornithine transcarbamoylase (OTC) [13]. In liver failure patients, ammonia accumulates through reduction in the function of the urea cycle due to a reduction in liver cell mass, producing numerous deleterious effects ranging from hepatic encephalopathy to neutrophil dysfunction and loss of muscle mass [14-17]. Genetic OTC deficiency is the most common type of inherited urea cycle enzyme deficiency disease and is manifest by catastrophic hyperammonemia and hepatic encephalopathy. In the adult form of OTC deficiency, milder degrees of hyperammonemia are manifest [18].

In NASH, many lines of investigation indicate that mitochondria are dysfunctional [19] and it is, therefore, possible that the resultant mitochondrial injury may affect CPS and OTC function, and in turn, result in compromised urea synthesis capacity and ultimately hyperammonemia. As mitochondria are mandatory for the production of cellular energy through β-oxidation of fatty acids, disruption in their function is often accompanied by an

enhanced production of reactive oxygen species (ROS), which mediates a pro-inflammatory microenvironment, promoting further liver injury.

Emerging data suggests a link between NASH, reduction in gene expression and function of urea cycle enzymes, resulting in hyperammonemia and progression of liver injury and fibrosis. This supports a rationale for targeting ammonia as a potential treatment for NASH. The mechanism is likely to be specific for liver disease in which triglycerides accumulate in hepatocytes and the liver becomes steatotic thereby reducing the function of urea synthesis leading to hyperammonemia at a pre-cirrhotic stage. Long-chain fatty acids promote perturbations in urea cycle enzyme gene expression resulting in hyperammonemia [20], whereas hepatic triglyceride accumulation (as is the case in NASH) is known to inhibit ureagenesis and increase ammonia concentrations around perivenous hepatocytes [21]. Hyperammonemia is associated with alteration of several genes including the Toll-like receptor pathway and increased hepatocyte apoptosis [22]. In urea cycle enzyme knock out mice, induction of chronic liver injury leads to increased oxidative stress, impaired fatty acid β-oxidation and enhanced liver injury compared to wild type mice [23] and likewise, livers from patients with urea cycle disorders, including OTC deficiency, demonstrate extensive hepatic fibrosis and cirrhosis [24].

OBSERVATIONS LEADING UP TO THE HYPOTHESES

Evidence that NASH results in hyperammonemia and a reversible reduction in activity and function of urea cycle enzymes

1. We have previously shown experimentally that diet-induced NASH in rats reduces CPS and particularly OTC gene and protein expression resulting in functional reduction in the *in*

vivo capacity for ureagenesis [25]. This impairs nitrogen homeostasis and results in hyperammonemia at a non-cirrhotic stage, Figure 1A and B. A follow-on study demonstrated that these changes were reversible upon recovery from NASH, and that reversal of NASH by reversion of the fat diet to normal chow, restored CPS and OTC gene and protein expression [26].

2. In another experimental model of NASH (methionine-choline deficient (MCD) mice), we demonstrated reduced OTC gene expression and showed that reducing the level of bacterial translocation through mesoporous carbons [27], prevents NASH and restores OTC gene expression. This suggests that inflammation in NASH contributes to depression of OTC gene expression and that functionality of OTC can be restored [26], Figure 2.

3. Importantly, in humans, NASH is also associated with reduced gene and protein expression of urea cycle enzymes. Data from human microarray shows that all the key urea cycle enzymes are down-regulated in the livers of patients with NASH, with OTC showing a >2 fold reduction compared to normal liver (Prof Ramon Bataller – personal communication). Furthermore, in NAFLD patients who had a liver biopsy obtained during bariatric surgery, those with NASH and fibrosis had significantly lower OTC gene expression than patients with steatosis alone [26], Figure 3A and increased ammonia concentrations in liver tissue, Figure 3B.

4. The reduced urea cycle enzyme expression in patients with NASH gives rise to a reduced ammonia clearance. In patients with biopsy-proven, non-cirrhotic NASH. Felipo et al. found higher plasma ammonia levels compared to patients with simple steatosis and

age-matched controls [28]. Additionally, these non-cirrhotic NASH patients showed evidence of neuropsychiatric disturbances that are commonly associated with hyperammonemia in the context of cirrhosis.

Evidence that ammonia produces functional and morphological changes in human HSCs

Pathological levels of ammonia are known to induce metabolic effects and cell swelling in astrocytes in the brain, which is central in the pathogenesis of hepatic encephalopathy [29]. Several studies have indicated that astrocytes and HSCs share a similar mesodermal origin and express similar cell markers of activation [30, 31]. We have recently demonstrated that pathological ammonia concentrations (50-300uM) produce changes in human HSC behaviour including significant alterations in cellular morphology (cytoplasmic vacuolisation, ER enlargement), reactive oxygen species production and induction of further HSC activation. Removal of ammonia from the cultures restored HSC morphology and function towards normality indicating that the ammonia-induced changes in HSCs are reversible [32], Figure 4.

Evidence that reduction of ammonia concentration in vivo reduces stellate cell activation and reduces portal hypertension

We undertook an *in vivo* study to establish if the ammonia scavenging drug ornithine phenylacetate (OP) was able to prevent the activation of HSC. Four-week bile duct ligated rats with advanced fibrosis and hyperammonemia were treated with OP and we observed reduced plasma ammonia, abrogated tissue markers of HSC activation and significantly

reduced portal pressure indicating that targeting ammonia *in vivo* reduces HSC activation and may also be beneficial for treatment of portal hypertension [32], Figure 5.

THE HYPOTHESIS

The data presented above shows that NASH produces a reversible reduction in the expression and function of urea cycle enzymes resulting in decreased ammonia elimination and ultimately hyperammonemia at a non-cirrhotic stage. Ammonia produces morphological changes and further activation of HSCs, which, in the context of NASH, by means of their pro-fibrotic and pro-inflammatory potential, may favour the progression of NASH. It follows that ammonia scavenging may represent a therapeutic target in NASH, Figure 6.

Hypothesis:

1. Administration of an ammonia-lowering drug in NASH reduces disease progression through non-urea ammonia scavenging and thereby reduction in activation of HSCs.

DISCUSSION

Our hypothesis provides the rationale for targeting ammonia as a potential treatment for NASH. We suggest that hyperammonemia is present in NASH at the non-cirrhotic stage and that ammonia is an important molecule involved in the progression towards development of fibrosis. We believe this mechanism is specific for steatotic liver diseases and ammonia scavenging could therefore also be an anti-fibrotic therapy in e.g. alcoholic steatohepatitis. In contrast, in patients with decompensated cirrhosis ammonia lowering is

a strategy for the treatment of hepatic encephalopathy. Extra cerebral effects of ammonia have not been fully appreciated [14, 33]. More recently, pathophysiological concentrations of ammonia were shown to result in neutrophil dysfunction both in vivo and in vitro [16]. Also, Dasarathy et al. have shown that ammonia is important in the muscle metabolism and may be a target to treat sarcopenia [34]. OP has been proven to reduce ammonia levels in animals with acute-on-chronic and acute liver failure [35, 36], and our findings indicate that this may also reduce activation of HSCs [32]. Preliminary data from our own laboratories have shown similar results in a NASH animal model [37]. Also in humans, OP is considered safe and beneficial as an ammonia scavenger to treat hyperammonemia in healthy subjects and patients with cirrhosis [38]. Therefore, the potential for early translation to test our hypothesis in NASH patients is not far away, especially as other ammonia lowering agents e.g. lactulose are already used as standard-of-care in the prevention and treatment of hyperammonemia and hepatic encephalopathy in cirrhotic patients.

ACKNOWLEDGEMENTS

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FIGURE CAPTIONS

Figure 1. Capacity of urea nitrogen synthesis (CUNS) (A) and liver ammonia concentrations (B) in control and NASH animals.

Changes in the *in vivo* capacity of urea nitrogen synthesis (CUNS) in animals fed a standard diet (controls) or a high-fat high-cholesterol (HFC) diet for 4 (early NASH) and 16 (advanced NASH) weeks. Bars represent the mean and SEM. * P=0.01 compared to controls (A). Ammonia concentrations in liver tissue from animals fed a standard diet (controls) or a HFC diet for 16 weeks assessed using Nessler's reagent [39]. In controls, ammonia is present in red blood cells only (pale-yellow, black arrows). In HFC-fed animals, ammonia is diffusively scattered in the hepatic parenchyma and tends to accumulate in the walls of vessels (black arrows) (B).

Footnote: Figure 1A: Reprinted from Am J Physiol Gastrointest Liver Physiol, 307, Thomsen KL, Grønbæk H, Glavind E, Hebbard L, Jessen N, Clouston A, George J, Vilstrup H. Experimental non-alcoholic steatohepatitis compromises ureagenesis, an essential hepatic metabolic function, G295-G301, Copyright (2014), with permission from the American Physiological Society.

Figure 2. H&E staining of liver tissue and ornithine transcarbamylase mRNA levels in controls and methionine-choline deficient mice with and without carbon treatment.

Representative H&E stain of liver tissue and ornithine transcarbamylase (OTC) mRNA levels from controls and from methionine-choline deficient (MCD) mice with and without treatment with mesoporous carbons (Yaq-001). Results from MCD mice are presented as relative levels compared to controls. Bars represent the mean and SD. ** P<0.01.

Figure 3. Ornithine transcarbamylase mRNA levels in patients with steatosis and NASH (A) and liver ammonia concentrations in healthy controls and NASH patients (B).

Ornithine transcarbamylase (OTC) mRNA levels in the liver from patients with simple steatosis and patients with NASH. Results are presented as relative levels compared to GAPDH. The solid horizontal lines indicate the mean values and the bars SD. The OTC mRNA levels are decreased in patients with NASH compared with simple steatosis (P=0.05) (A). Ammonia concentrations in liver tissue from healthy controls and in patients with NASH assessed using Nessler's reagent [39]. Black arrows point to ammonia. In NASH patients, the ammonia tends to accumulate in the cytoplasm of the hepatocytes (pale yellow) on occasion in very high concentrations (black spot). L indicates lipid droplets (B).

Figure 4. Ammonia causes alterations in cellular morphology, reactive oxygen species (ROS) production and induction of human stellate cell (HSC) activation.

(A) Transmission Electron Microscopy shows that ammonia causes dramatic morphological changes in a dose-dependent manner, with appearance of cytoplasmic vacuolisation and ER enlargement. (B) Mean fluorescence intensity (MFI) of ROS signal is normalized according to the number of cells and expressed as percentage of control. Bar graphs show means of three independent values ± SD. *** P<0.001 vs. corresponding values of SFM. (C) Ammonia affects protein expression of the HSC activation markers α-SMA, myosin IIa and IIb, PDGFRβ and vinculin.

Figure 5. Plasma ammonia, tissue markers of human stellate cell (HSC) activation and portal pressure in bile duct ligated (BDL) rats following ornithine phenylacetate (OP) treatment.

(A) Plasma levels of ammonia are significant upregulated in BDL and amino acids (AAs)-fed BDL animals in comparison to sham-operated rats (* P<0.05 and ** P<0.01 vs. sham). OP treatment reduces significantly ammonia levels in BDL-AAs-fed animals in comparison to BDL animals (** P<0.01). (B) Hyperammonemia treatment in BDL-induced fibrosis shows an additional significant increase in myosin IIb, collagen type I and PDGFRβ protein expression in comparison to BDL-induced fibrosis. In contrast, treatment with OP abrogates the strong effect of AAs-fed BDL on all HSC-related activation markers. (C) Portal pressure is increased in BDL rats compared to sham (*** P<0.001). Administration of OP results in a significant lowering of portal pressure compared to BDL saline-treated rats (* P<0.01).

Footnote: Figure 4 and 5: Reprinted from J Hepatol, Vol 64, Jalan R, De Chiara F, Balasubramaniyan V, Andreola F, Khetan V, Malago M, Pinzani M, Mookerjee RP, Rombouts K. Ammonia produces pathological changes in human hepatic stellate cells and is a target for therapy of portal hypertension, 823-833, Copyright (2016), with permission from Elsevier.

Figure 6. Schematic showing NASH induces hyperammonemia resulting in activation of human hepatic stellate cells (HSCs)

NASH produces a reversible reduction in the expression and function of urea cycle enzymes, resulting in a functional reduction in *in vivo* capacity for ureagenesis. The resulting hyperammonemia activates HSCs generating both morphological changes and pro-fibrotic and pro-inflammatory factors that may favour the progression of NASH. It follows that ammonia scavenging may represent a therapeutic target in NASH.

Figure 1A

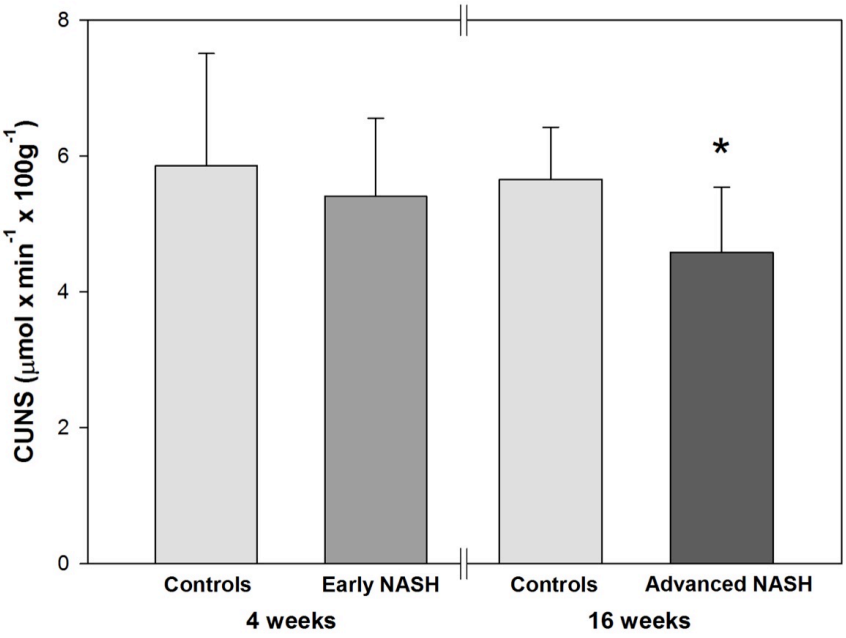

8
6
4
2
0
CUNS (μmol x min -1 x 100g -1)
*
Controls
Early NASH
Controls
Advanced NASH
4 weeks
16 weeks

Figure 1B

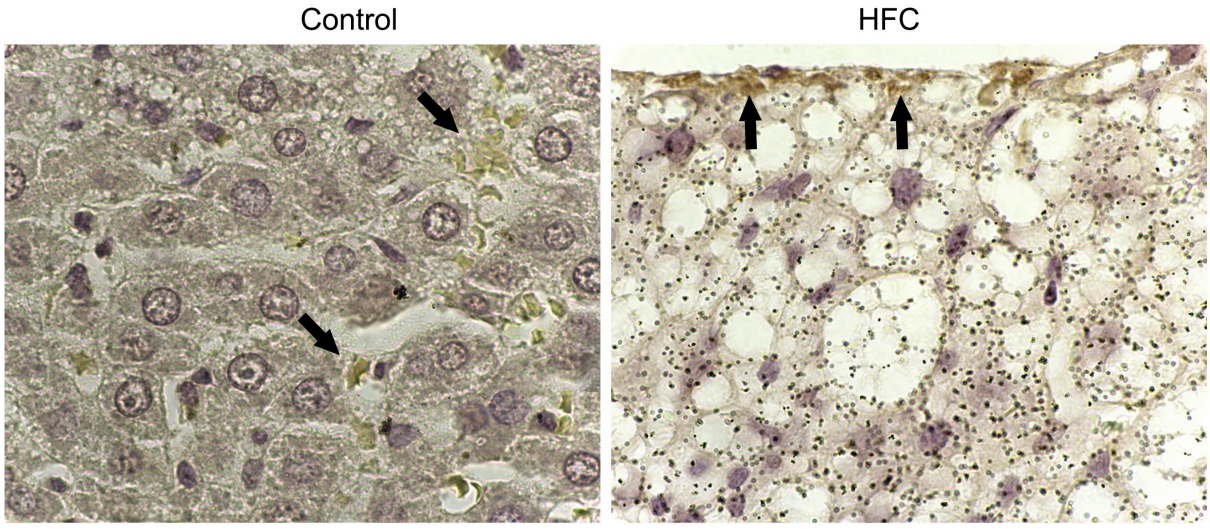
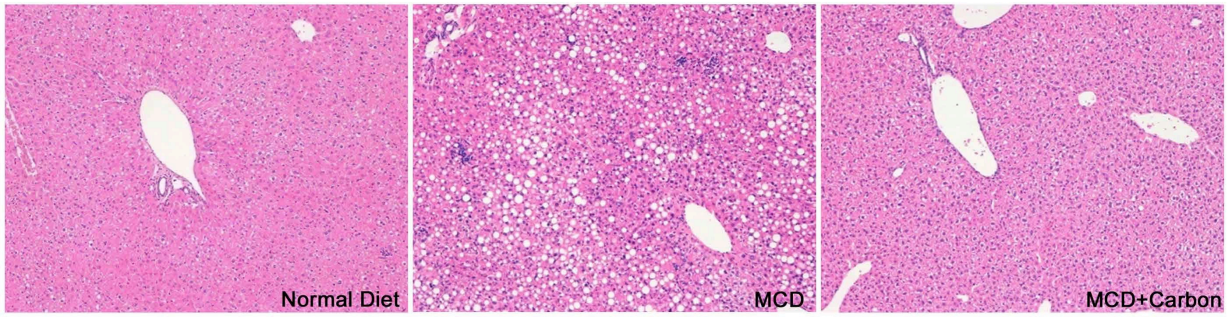


Figure 2


Normal Diet
MCD
MCD+Carbon

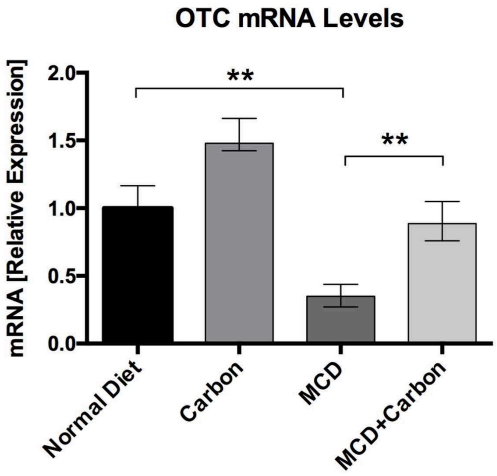

OTC mRNA Levels
mRNA [Relative Expression]
2.0
1.5
1.0
0.5
0.0
**
**
Normal Diet
Carbon
MCD
MCD+Carbon

Figure 3A

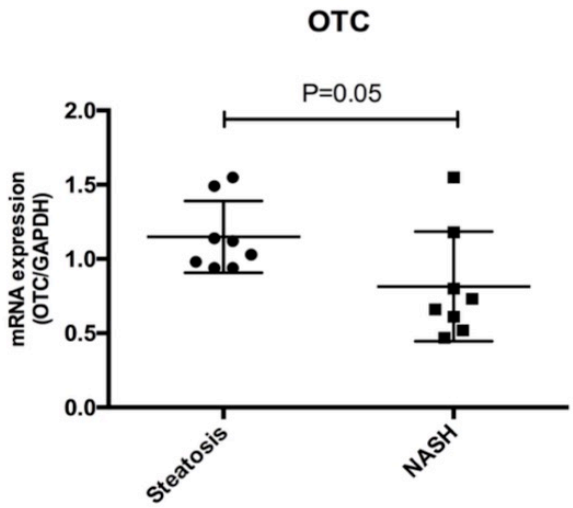

OTC
P=0.05
mRNA expression (OTC/GAPDH)
2.0
1.5
1.0
0.5
0.0
Steatosis
NASH

Figure 3B

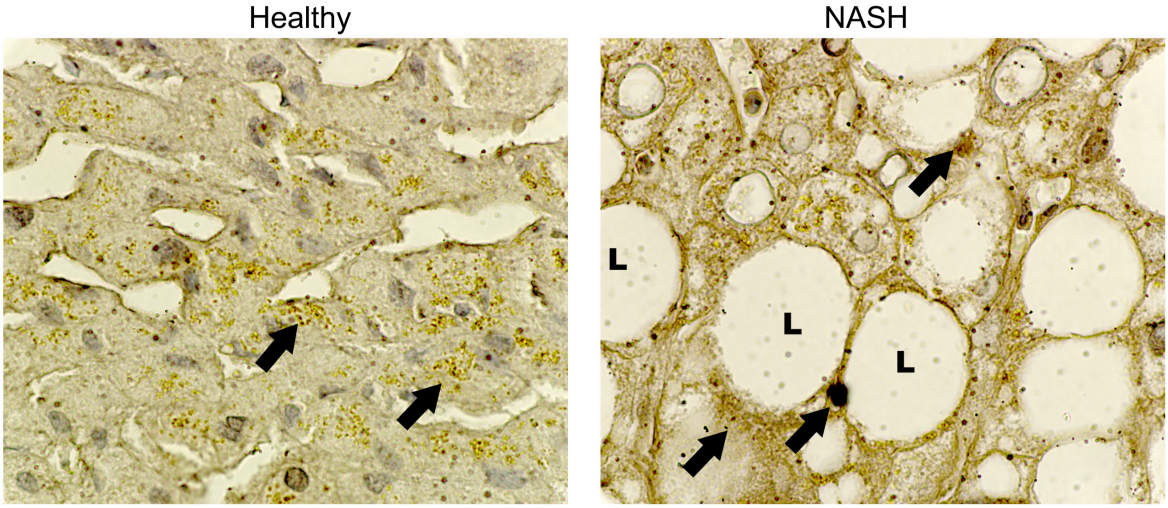


Figure 4

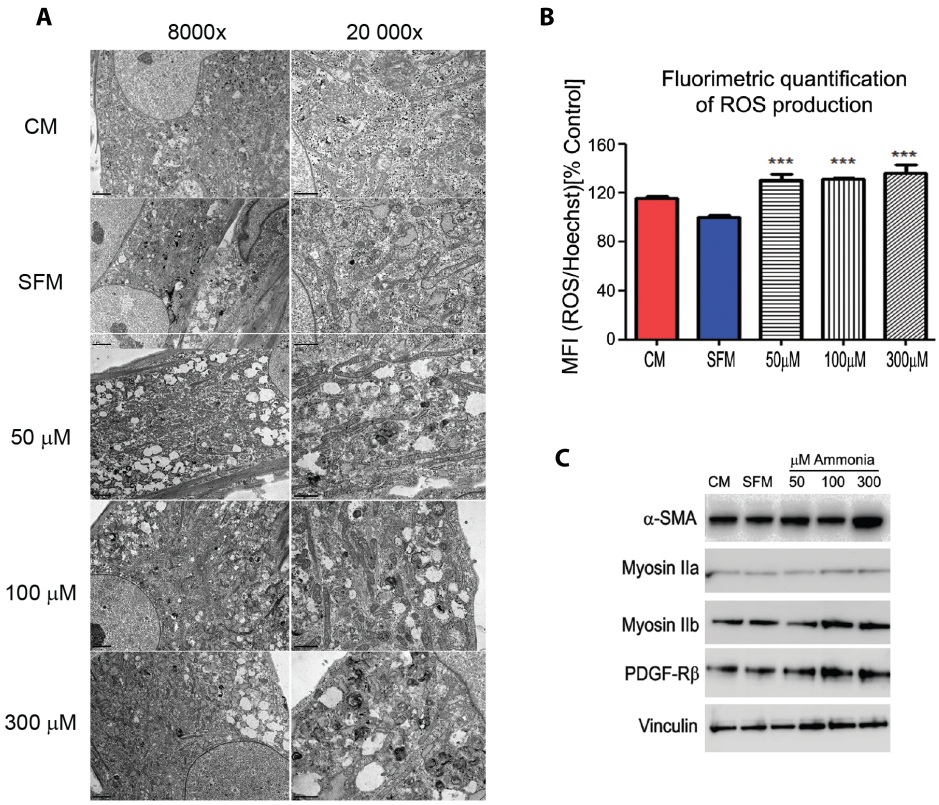

A
8000x
20 000x
CM
SFM
50 μM
100 μM
300 μM
B
Fluorimetric quantification of ROS production
MFI (ROS/Hoechst)[% Control]
160
120
80
40
0
***
***
***
CM
SFM
50μM
100μM
300μM
C
μM Ammonia
CM
SFM
50
100
300
α-SMA
Myosin IIa
Myosin IIb
PDGF-Rβ
Vinculin

Figure 5

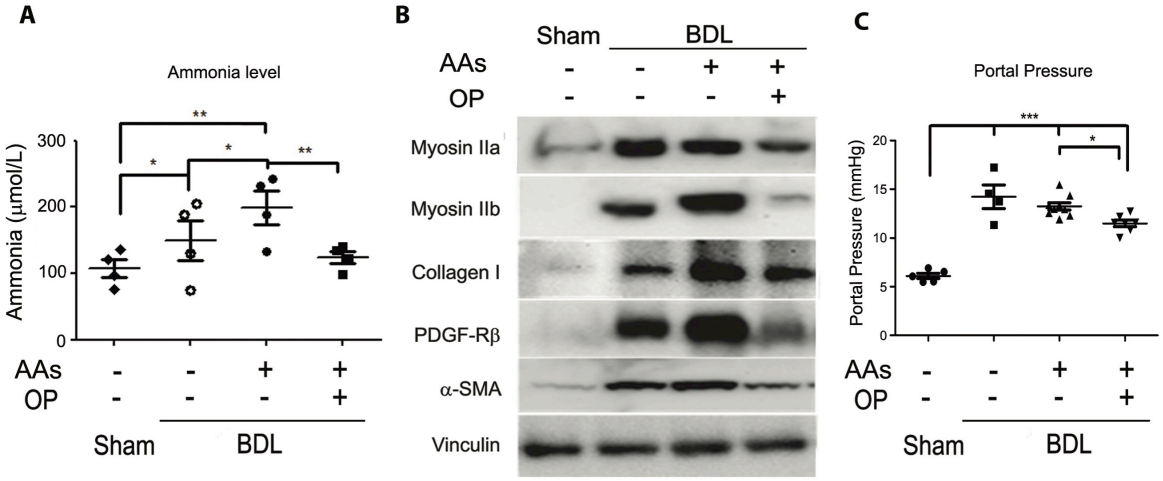
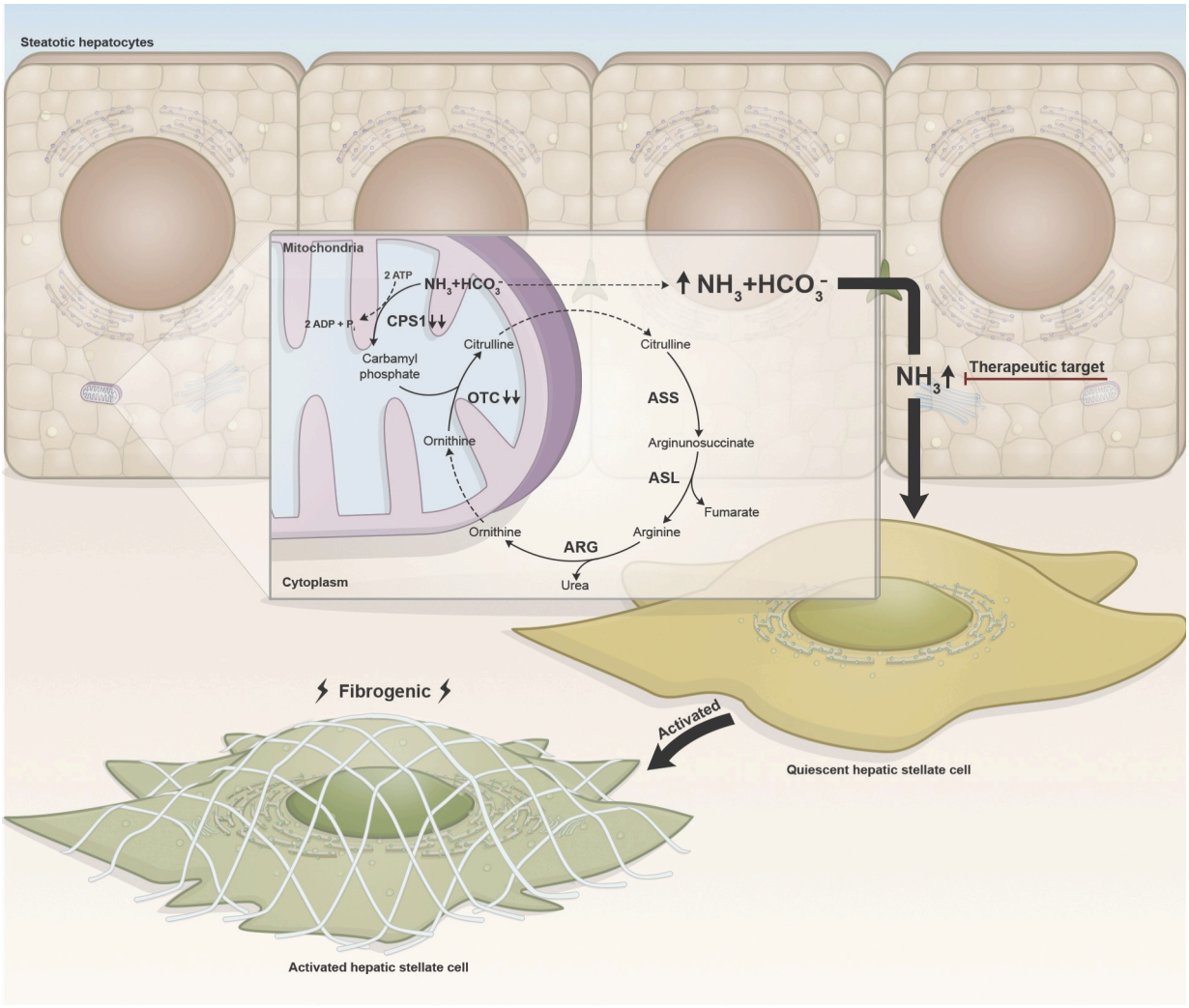

A
Ammonia level
Ammonia (μmol/L)
300
200
100
0
**
*
*
**
AAs - - + +
OP - - - +
Sham
BDL
B
Sham
BDL
AAs - - + +
OP - - - +
Myosin IIa
Myosin IIb
Collagen I
PDGF-Rβ
α-SMA
Vinculin
C
Portal Pressure
Portal Pressure (mmHg)
20
15
10
5
0
***
*
AAs - - + +
OP - - - +
Sham
BDL

Figure 6


Steatotic hepatocytes
Mitochondria
2 ATP
$NH_3+HCO_3^-$
2 ADP + P_i
CPS1↓↓
Carbamyl phosphate
Citrulline
OTC↓↓
Ornithine
Cytoplasm
↑ $NH_3+HCO_3^-$
Citrulline
ASS
Arginunosuccinate
ASL
Fumarate
Arginine
ARG
Urea
Ornithine
NH_3↑
Therapeutic target
Fibrogenic
Activated
Quiescent hepatic stellate cell
Activated hepatic stellate cell