

Urea cycle dysregulation and arginine pathways in the pathogenesis of NAFLD and NASH (Review)

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Abstract. Non-alcoholic fatty liver disease (NAFLD) and its advanced form, non-alcoholic steatohepatitis (NASH), are now leading causes of chronic liver disease worldwide. Recent evidence highlights the central role of urea cycle and arginine metabolism dysregulation in NAFLD/NASH pathogenesis. Downregulation of key urea cycle enzymes, such as carbamoyl phosphate synthetase 1, ornithine transcarbamylase and argininosuccinate synthase, impairs ammonia detoxification, leading to hyperammonemia, mitochondrial dysfunction and oxidative stress. Epigenetic modifications, notably DNA methylation and histone changes, contribute to this metabolic reprogramming. Aberrant arginine metabolism, particularly the imbalance between urea and nitric oxide (NO) pathways, exacerbates liver inflammation and fibrosis through increased inducible nitric oxide synthase (NOS) activity in macrophages and hepatic stellate cells, driving excessive NO and polyamine production. In contrast, physiological NO signaling via endothelial NOS supports mitochondrial health and metabolic homeostasis. Clinically, urea cycle and arginine metabolites, including blood ammonia and urea cycle metabolites, serve as potential biomarkers for disease severity. Restoring urea cycle function and rebalancing arginine-NO metabolism through targeted

interventions, such as citrulline/arginine supplementation or enzyme modulation, are promising strategies.

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1. Introduction

In recent years, with the increasing global burden of obesity and metabolic syndrome, non-alcoholic fatty liver disease (NAFLD) has become one of the most common chronic liver diseases in adults. The global prevalence of NAFLD is estimated to be around 29.8%, reaching as high as 30-40% in regions such as Europe and North America (1,2). The pathological spectrum of NAFLD ranges from simple hepatic steatosis to non-alcoholic steatohepatitis (NASH), which is characterized by inflammation and varying degrees of fibrosis. In some patients, NASH can further progress to cirrhosis or even hepatocellular carcinoma, significantly increasing the risk of both liver-related and systemic complications. Epidemiological data show that patients with NAFLD have a markedly increased incidence of cardiovascular events and type 2 diabetes and approximately 1.5-6.5% of cases will progress to NASH within 10 years, accompanied by worsening fibrosis and an exponentially increasing risk of poor outcomes (3). Moreover, NASH is closely associated

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with chronic kidney disease, atherosclerosis and sleep apnea, suggesting that it is not merely an isolated liver disorder, but a complex manifestation of systemic multi-organ metabolic dysregulation, posing unprecedented challenges for public health and clinical management.

The urea cycle, which is unique to mammalian hepatocytes, is a central hub of nitrogen metabolism. It detoxifies excess ammonia (NH_3) by converting it into soluble urea, thereby enabling its safe excretion and maintaining nitrogen homeostasis. This cycle is accomplished through the cooperation of mitochondrial and cytosolic compartments and involves six key enzymes: N-acetyl-L-glutamate synthase (NAGS), carbamoyl phosphate synthetase 1 (CPS1), ornithine transcarbamylase (OTC), argininosuccinate synthase (ASS1), argininosuccinate lyase (ASL) and cytosolic arginase 1 (ARG1). Among these, NAGS-produced N-acetylglutamate is an essential allosteric activator of CPS1, ensuring precise regulation of cycle flux. The expression and activity of these enzymes are influenced by various physiological hormones (such as glucagon and glucocorticoids) and nutritional status and are further finely tuned by epigenetic mechanisms including DNA methylation and histone modifications (4,5). As well as detoxification, the urea cycle also plays important roles in amino acid biosynthesis, one-carbon metabolism and its crosstalk with the tricarboxylic acid (TCA) cycle (6). The tight coupling of its carbon skeleton to the energy metabolic network highlights the critical importance of coordinated nitrogen and carbon metabolism for hepatic homeostasis (7).

A study has shown that urea cycle flux is markedly impaired in the livers of NAFLD/NASH patients (8). Multiple transcriptomic and proteomic analyses have revealed down-regulation of key enzymes such as CPS1, OTC and ASS1, accompanied by elevated intrahepatic free ammonia concentrations and glutamine metabolic reprogramming. During the development of NASH, hepatic CPS1 and OTC protein levels and activities are found to decrease proportionally and these changes can be partially reversed by refeeding with a normal diet, suggesting a central role for epigenetic regulation in this process (9). Meanwhile, loss or dysfunction of ARG1 can exacerbate nitric oxide (NO) production imbalance in hepatocytes, triggering excessive oxidative stress and inflammatory cell apoptosis, which significantly promotes hepatic fibrogenesis. Conversely, maintaining the metabolic integrity of the arginine-citrulline-arginine cycle helps inhibit pathological NO synthesis and mitigate hepatocyte inflammatory injury (10). Additionally, metabolomics studies have identified characteristic alterations in urea cycle intermediates and arginine metabolic branches (including nitric oxide and polyamines) in the serum of NASH patients, underscoring a bidirectional feedback relationship between nitrogen metabolic imbalance and hepatic immunometabolic microenvironment remodeling (11).

2. Materials and methods

The present study was a narrative review based exclusively on published literature and no original data from our group are included. Nevertheless, by integrating basic mechanistic studies and clinical observations, it aimed to provide a coherent framework linking urea-cycle impairment and arginine/NO

metabolism to NAFLD/NASH pathogenesis, to the development of tissue and circulating biomarkers and to potential therapeutic strategies targeting these pathways.

A narrative literature search was conducted using the PubMed database (<https://pubmed.ncbi.nlm.nih.gov/>) from its inception to October 31, 2025. The search combined Medical Subject Headings and free-text terms related to non-alcoholic fatty liver disease and nitrogen/arginine metabolism. Representative search strings included combinations of the following terms: 'non-alcoholic fatty liver disease' OR 'non-alcoholic steatohepatitis' OR NAFLD OR NASH; and 'urea cycle' OR 'carbamoyl phosphate synthetase 1' OR CPS1 OR 'ornithine transcarbamylase' OR OTC OR 'argininosuccinate synthase' OR ASS1 OR 'argininosuccinate lyase' OR ASL OR arginase OR ARG1 OR ARG2 OR 'arginine metabolism' OR 'nitric oxide' OR iNOS OR 'hepatic stellate cell*' OR 'Kupffer cell*' OR 'hepatic macrophage*' OR 'hyperammonemia' OR 'ammonia.' These terms were combined with Boolean operators (AND/OR) to capture both mechanistic and clinical studies relevant to urea-cycle impairment and arginine metabolism in NAFLD/NASH. Eligible literature included peer-reviewed English-language primary studies and selected reviews on urea-cycle/arginine-NO/arginase biology in NAFLD/NASH (or related metabolic liver injury/fibrogenesis), spanning mechanistic work in cells/animals and translational evidence from human tissues, cohorts, enzyme assays and multi-omics. The present study excluded non-peer-reviewed or non-English articles, abstracts only, studies focused mainly on inherited urea-cycle disorders without clear NAFLD/NASH relevance and reports centered on non-metabolic liver diseases.

Eligible publications were peer-reviewed articles in English, including original basic research (cell culture, animal models and human tissue studies), clinical observational studies, interventional trials and high-quality narrative or systematic reviews that provided mechanistic or translational insights into urea-cycle and arginine metabolism in NAFLD/NASH. Exclusion criteria comprised case reports or small case series without mechanistic data; conference abstracts, editorials, or comments lacking primary results; studies focusing exclusively on inborn errors of the urea cycle or other liver diseases without clear relevance to NAFLD/NASH; and articles not providing specific information on urea-cycle enzymes, arginine/NO metabolism, or related biomarkers/therapeutic targets. When multiple reports from the same cohort or model were available, priority was given to the most comprehensive or recent publication.

3. Fundamentals of the urea cycle and arginine metabolism

Urea cycle. In mammals, the integration of nitrogen and carbon metabolism forms a crucial network that maintains homeostasis, with the urea cycle and L-arginine metabolism serving as central nodes. The urea cycle efficiently converts toxic NH_3 , produced by protein degradation and amino acid deamination, into non-toxic, water-soluble urea for safe excretion and detoxification. Meanwhile, arginine, a semi-essential amino acid, not only participates in protein synthesis but also gives rise to multiple functional molecules via various enzyme branches, including nitric oxide NO, polyamines, creatine and citrulline. These form complex metabolic pathways

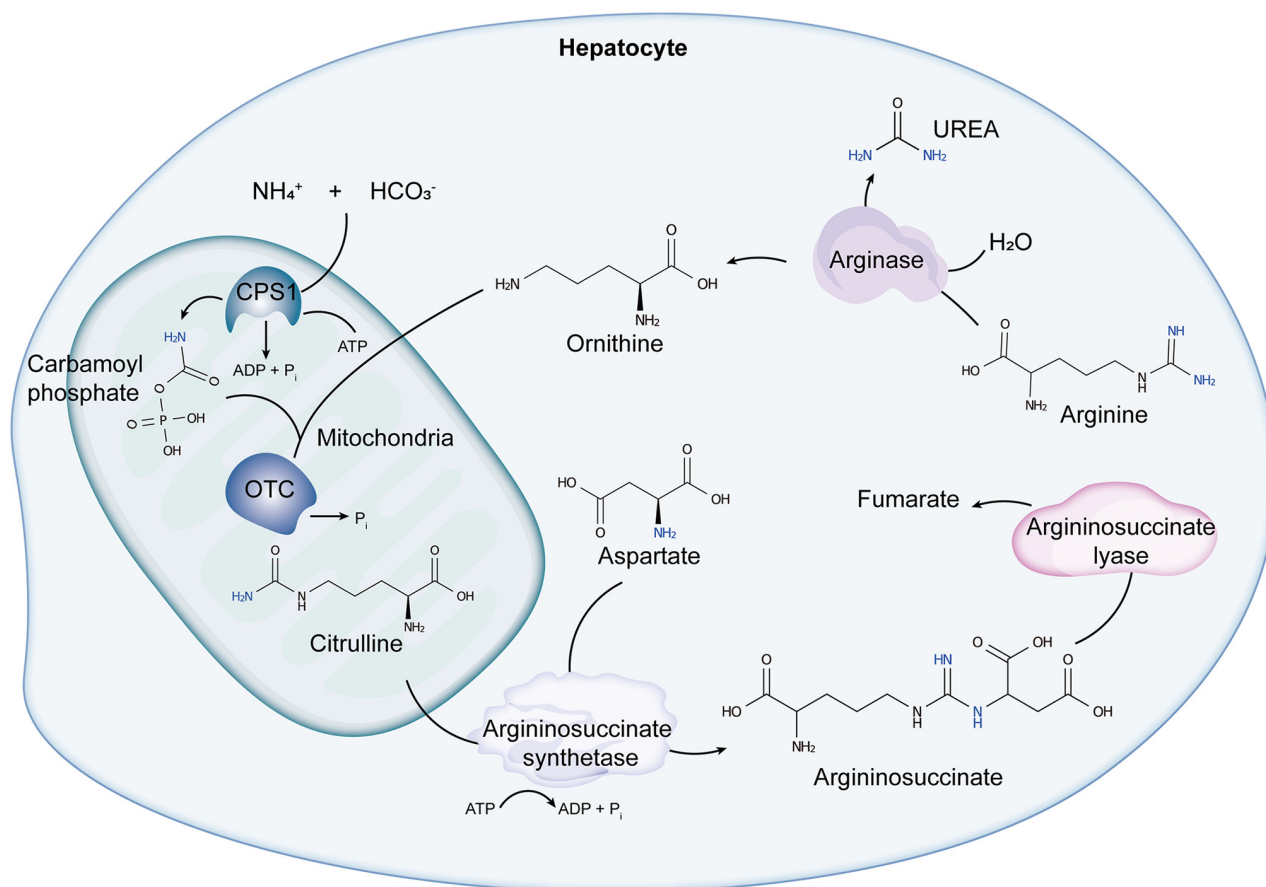


Figure 1. Simplified schematic of the hepatocyte urea cycle and L-arginine network. In mitochondria, NAGS, CPS1 and OTC convert NH_3 to citrulline, which in the cytosol is processed by ASS1 and ASL into arginine and fumarate. ARG1 then yields urea and regenerates ornithine. All figures were generated using Microsoft PowerPoint (Microsoft Corporation). NAGS, N-acetyl-L-glutamate synthase; CPS1, carbamoyl phosphate synthetase 1; OTC, ornithine transcarbamylase; ASS1, argininosuccinate synthase; ASL, argininosuccinate lyase; ARG1, cytosolic arginase 1.

with increasingly recognized physiological and pathological significance (7,12-14).

In hepatocytes, the urea cycle is divided into mitochondrial and cytosolic phases, coordinated by six core enzymes (15,16). It initiates in the mitochondrial matrix, where NAGS catalyzes the formation of N-acetylglutamate (NAG) from glutamate and acetyl-CoA. NAG serves as an essential allosteric activator of CPS1, driving the formation of carbamoyl phosphate from ammonia and bicarbonate in the presence of two ATP molecules. OTC then combines carbamoyl phosphate and ornithine to produce citrulline. After citrulline is transported to the cytosol, ASS1 and ASL sequentially convert it into arginine and fumarate. Finally, ARG1 hydrolyzes arginine into ornithine and urea, thus completing the nitrogen metabolic cycle. This process consumes four phosphate bonds ($3 \text{ ATP} \rightarrow 2 \text{ ADP} + \text{AMP}$) per mole of urea produced and is tightly coupled to the TCA cycle and other metabolic pathways to maintain energy and material balance (7) (Fig. 1).

Regulation of the urea cycle. Efficient operation of the urea cycle in hepatocytes relies on precise multi-level molecular regulation, including gene transcription, epigenetic modifications and post-translational modifications, ensuring that ammonia detoxification flux remains synchronized with metabolic demands. NAGS, which catalyzes NAG synthesis as the cycle's entry point, is tightly regulated at the transcriptional

level by various factors. For example, phosphorylation of Sp1 and CREB in response to increased intracellular cAMP enhances their binding to the NAGS promoter, while liver-specific transcription factors hepatocyte nuclear factor 1 and nuclear factor Y respond to glucagon and glucocorticoid signals by synergizing with enhancer elements to upregulate NAGS expression, adapting to changes in nitrogen excretion under fasting or stress (17,18). Additionally, DNA methylation and histone acetylation play key roles; methylation at CpG sites in the NAGS promoter correlates negatively with its mRNA expression and histone deacetylase 1-mediated deacetylation of H3K9ac represses NAGS transcription (19-22).

As the rate-limiting enzyme of the urea cycle, CPS1 activity and expression depend on NAG allosteric activation and are also finely regulated by post-translational modifications. In the mitochondrial matrix, the NAD^+ -dependent deacetylase SIRT5 can deacetylate CPS1 at K55 and K287, markedly enhancing its catalytic efficiency; fasting or high-protein diets promote mitochondrial NAD^+ accumulation, increasing SIRT5 activity and accelerating ammonia detoxification. Sirt5-knockout mice exhibit hyperammonemia and impaired urea production when fasting, underscoring the core role of the SIRT5-CPS1 axis in ammonia metabolism adaptation (23-26). Furthermore, SIRT5 also mediates desuccinylation and demalonylation of CPS1, constructing a multi-dimensional regulatory network responsive to energy state fluctuations (24,26). Additionally,

O-GlcNAc glycosylation of CPS1 rises in hyperglycemia or aging, blocking NAG binding and suppressing enzyme activity; caloric restriction or low-carbohydrate diets reduce this modification and restore function (23,27). Oxidative stress induces peroxynitrite (ONOO⁻)-mediated tyrosine nitration of CPS1, disrupting allosteric sites and further reducing activity, a mechanism validated in drug-induced liver injury, implicating protein nitration as a key node in ammonia metabolism imbalance under oxidative/nitrosative stress (23,28). Phosphoproteomic studies also show that CPS1 can be phosphorylated at serine/threonine sites by kinases such as PKA and PKC, potentially altering mitochondrial localization or protein interactions to couple energy and urea cycle signaling (27,29).

The second enzyme in the cycle, OTC, is similarly subject to multi-level regulation. Proteomics of Sirt5-knockout mice reveals hyper-succinylation of OTC, indicating SIRT5's role in maintaining OTC activity and ammonia flux (23). On the epigenetic level, DNA hypermethylation of the OTC promoter in NAFLD/NASH patients is closely associated with transcriptional downregulation, directly impairing hepatocyte detoxification capacity and providing a potential therapeutic target (30,31).

ASS1 catalyzes the critical step converting citrulline to arginine and is regulated by cell proliferation and stress signals. Tumor suppressor p53 can bind directly to the ASS1 promoter to induce transcription, thus protecting cell survival in DNA damage or metabolic stress by suppressing excessive Akt pathway activation; ASS1-deficient cells are more sensitive to radiotherapy and chemotherapy, confirming its dual role as a p53 target gene (32,33). Moreover, activation of the AMPK pathway promotes ASS1 mRNA expression, enhancing the citrulline-arginine cycle to meet the demand for arginine and downstream NO production under energy deprivation (34,35).

ASL is not only key to arginine synthesis but also influences downstream NO production, affecting vascular homeostasis and immune responses. ASL transcription is jointly regulated by hypoxia, insulin and glucocorticoid signals (13). In malnutrition models, glucose and cAMP pathways suppress ASL mRNA levels, while glucocorticoids upregulate ASL expression via GRE elements, partially mediated by increased PGC-1 α and FoxO, ensuring a continuous supply of urea cycle intermediates for acute and chronic metabolic stress adaptation (36-39).

Finally, ARG1 catalyzes the hydrolysis of arginine to ornithine and urea, completing nitrogen excretion. Transcription of ARG1 is directly regulated by the hepatic glucocorticoid receptor (GR): *In situ* experiments and liver-specific GR knockout models show that GR activity determines ARG1 mRNA and protein expression. GR agonists (such as dexamethasone) robustly induce ARG1 transcription, boosting urea synthesis and preventing hyperammonemia; conversely, GR-deficient mice fail to induce ARG1 even with dexamethasone, resulting in ammonia metabolism disorders and neuromuscular impairment (40). Additionally, caloric restriction significantly upregulates ARG1, highlighting its adaptive role in aging and nutrient limitation. Experiments in mice of different ages show that fasting or caloric restriction elevates hepatic ARG1 mRNA and enzyme activity, delaying age-related ammonia metabolism decline (41). In macrophage

M2 polarization, IL-4 activates STAT6 and C/EBP β , collaborating with RAR/RXR heterodimers to recruit chromatin remodeling complexes and the transcription elongation factor TFIIS, massively upregulating ARG1 and promoting tissue repair and anti-inflammatory responses (42-46). Moreover, hypoxia-inducible factor HIF-1 α has been shown to down-regulate ARG1 in hyperuricemia-induced liver injury, linking ARG1 to hepatic inflammation, oxidative stress and apoptosis and highlighting its importance as a regulatory target in metabolic and inflammatory diseases (47).

Arginine metabolism and its spatial heterogeneity. Arginine is not only an intermediate of the urea cycle but also a hub for multiple downstream metabolic pathways (9). In the cytosol, ASS1 and ASL form the citrulline-arginine cycle to regenerate arginine from citrulline, which is indispensable for systemic arginine balance, protein synthesis and NO production (48). Arginine metabolism branches through various enzyme systems: Generation of NO and citrulline via nitric oxide synthase (NOS), with NO serving as a signaling molecule in hemodynamics, immune defense and cellular signaling; polyamine production via ornithine decarboxylase (ODC), involved in cell proliferation and differentiation; creatine biosynthesis via arginine:glycine amidinotransferase, supporting energy supply for muscle and nervous tissue; formation of methylated arginine derivatives, such as asymmetric dimethylarginine (ADMA) and SDMA, affecting endothelial function and cardiovascular risk assessment (7). Overall, the complex branches of arginine metabolism illustrate the intersection of nitrogen metabolism with broad biological processes and its physiological/pathological significance continues to expand with ongoing research (7,14,49).

In the conversion of arginine to NO, three main NOS isoforms, neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS), regulate NO production in a synergistic or antagonistic manner (49-51). All NOS enzymes require NADPH, molecular oxygen and tetrahydrobiopterin as cofactors, using an electron transfer chain to oxidize arginine to N ω -hydroxyarginine and subsequently to NO and citrulline (52,53). The isoforms differ in calcium/calmodulin sensitivity, gene regulation, tissue distribution and functional effects: nNOS is mainly found in the nervous system (neurotransmission, plasticity), eNOS is restricted to vascular endothelium (maintaining vascular tone and blood flow) and iNOS is strongly induced during inflammation or infection, producing high NO concentrations for antimicrobial and signaling purposes (51,54,55).

Competing with the NOS pathway, arginase hydrolyzes arginine to ornithine and urea (56). Mammals have two arginase isoforms: Hepatic ARG1 (cytosolic, terminal step in the urea cycle) and mitochondrial ARG2 (found in kidney, immune cells and other tissues, involved in local ornithine and polyamine synthesis) (57). Studies confirm that arginase activity is essential not only for urea production but also for competing with NOS for arginine, thereby regulating NO levels and playing complex roles in immunity, fibrosis and metabolic disease (58). For example, upregulated Arg1 expression in activated hepatic stellate cells (HSCs) promotes collagen synthesis and fibrosis progression, while Arg1 inhibition alleviates fibrosis, indicating therapeutic potential in tissue repair and

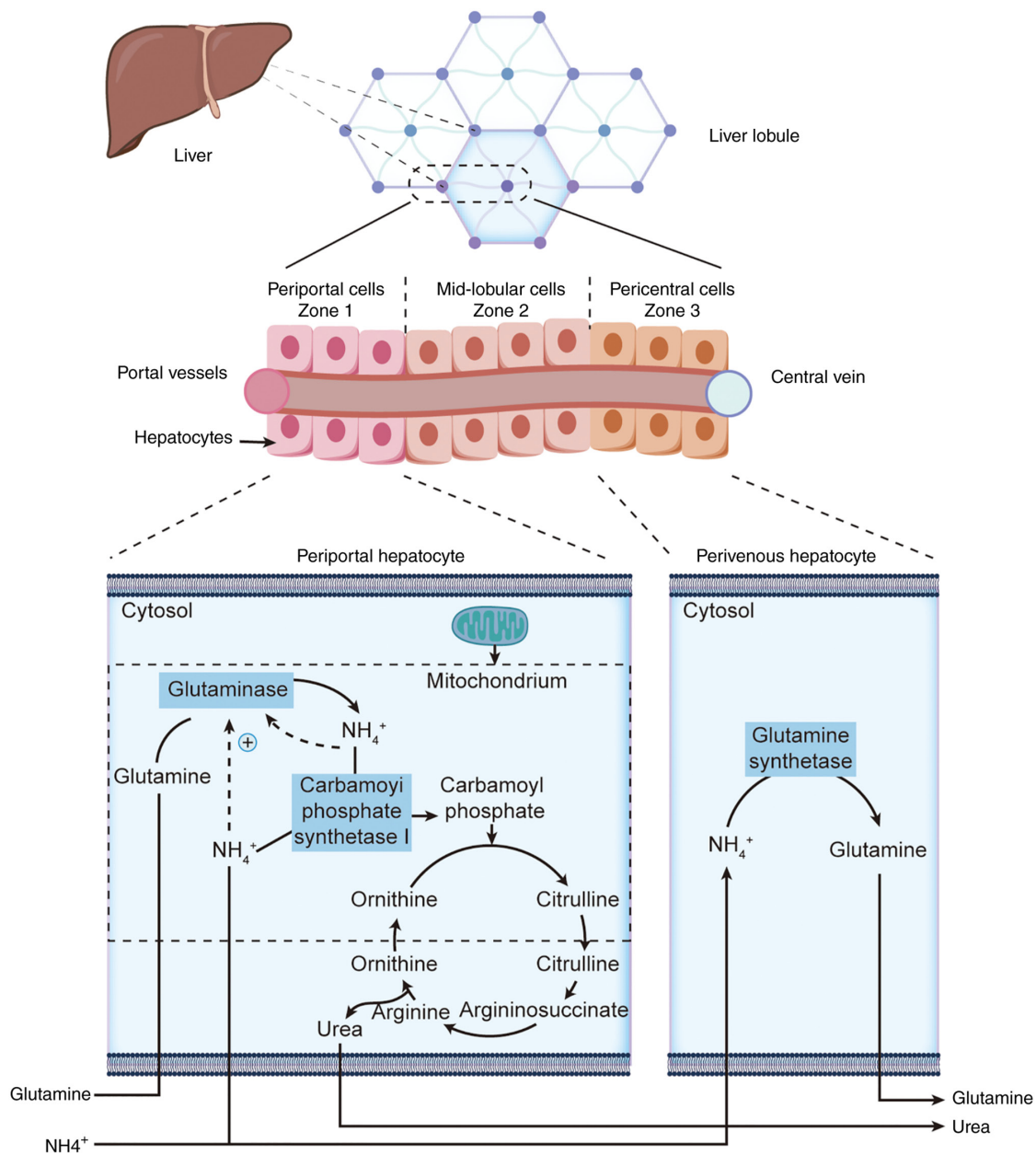


Figure 2. Spatial and cell-type heterogeneity of hepatic nitrogen metabolism. Hepatocytes are divided into periportal (zone 1), midzonal (zone 2) and pericentral (zone 3) regions along oxygen and nutrient gradients. Zone 1 cells express high levels of urea cycle enzymes to convert portal-vein-derived NH_3 into urea, while residual ammonia reaching zone 3 is captured by pericentral glutamine synthetase to form glutamine. All figures were generated using Microsoft PowerPoint (Microsoft Corporation).

pathology (59,60). Taken together, these enzymatic branches highlight that arginine metabolism is not only biochemically diversified but also poised to be differentially engaged across tissues and cell types.

As well as this enzymatic complexity at the whole-organ level, arginine metabolism is further shaped by pronounced spatial organization within tissues, particularly in the liver. Within the highly structured liver, urea cycle and arginine metabolism activity exhibit significant spatial and functional heterogeneity that operates at two interrelated levels: metabolic zonation among hepatocytes and differences between parenchymal and non-parenchymal cell types (61,62). The classical Couinaud segmentation divides hepatocytes into periportal (zone 1), midzonal (zone 2) and pericentral (zone 3) regions based on blood supply gradients (63,64). In the periportal

region, blood is rich in oxygen and amino acids and urea cycle enzymes are highly expressed to detoxify most ammonia and generate urea (65,66); minimal ammonia escaping to the pericentral region is converted to glutamine by glutamine synthetase at extremely low concentrations, ensuring blood ammonia levels remain safe, demonstrating fine spatial division of labor within hepatic lobules (67-70) (Fig. 2). Complementing this hepatocyte zonation, non-parenchymal cells provide additional layers of heterogeneity to hepatic nitrogen and immunometabolic cross-talk. Kupffer cells, the most abundant resident macrophage population, display strong phagocytic and secretory capacities and, depending on their M1/M2 polarization state, express iNOS or Arg1, biasing local NO or ornithine metabolism and influencing global inflammation and repair; their plasticity and microenvironmental

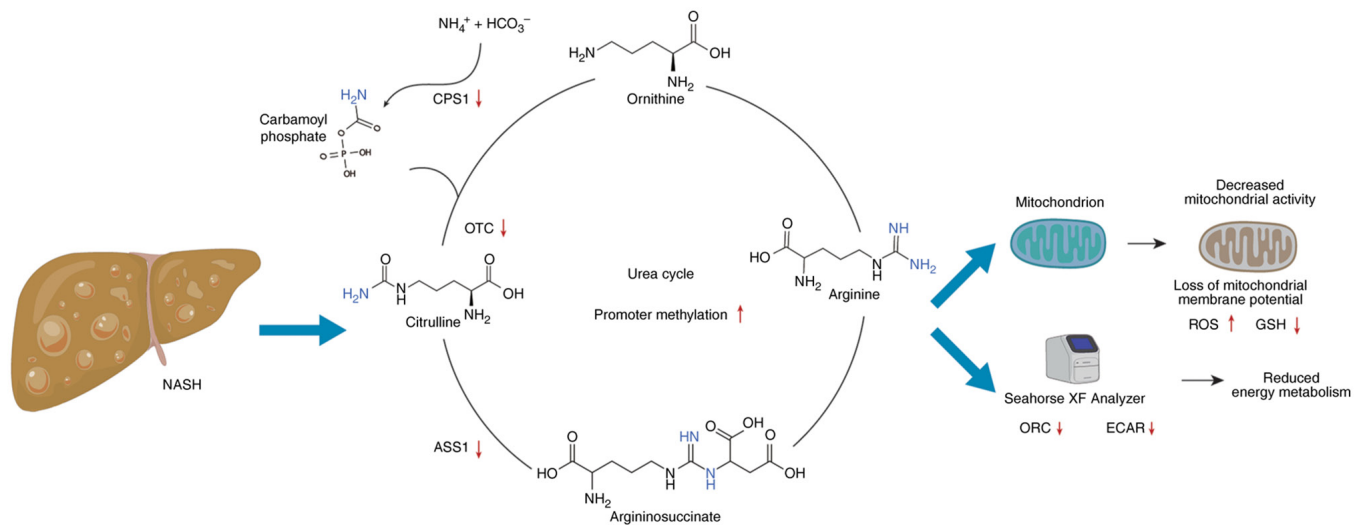


Figure 3. Epigenetic suppression of the hepatocyte urea cycle under metabolic stress: in highfat, highcholesterol diet-induced NASH rats, CpG hypermethylation of CPS1 and OTC promoters leads to a marked drop in their mRNA/protein levels, reduced enzymatic activity and intrahepatic ammonia buildup—changes that are partially reversed upon return to a normal diet. Likewise, human NAFLD (steatosis and NASH) liver samples show a 30–40% decrease in CPS1, OTC and ASS1 transcripts correlating with impaired urea production and highfructose, highfat mouse hepatocytes display similar promoter hypermethylation and enzyme downregulation. All figures were generated using Microsoft PowerPoint (Microsoft Corporation). NASH, non-alcoholic steatohepatitis; CPS1, carbamoyl phosphate synthetase 1; OTC, ornithine transcarbamylase; NAFLD, non-alcoholic fatty liver disease; ASS1, argininosuccinate synthase; ECAR, extracellular acidification rate.

dependence make them core players in NAFLD/NASH progression (71–74). HSCs, located in the space of Disse, store vitamin A in quiescence and upon activation differentiate into myofibroblast-like cells with high Arg1 expression, promoting polyamine and collagen production to drive fibrosis; hepatic sinusoidal endothelial cells utilize eNOS-derived NO to regulate blood flow and the local microenvironment, which is critical for oxygen gradient and metabolic zonation (55,60).

4. Metabolic reprogramming in hepatocytes

Under physiological homeostasis, hepatocytes maintain systemic nitrogen balance and efficient ammonia detoxification via a robust nitrogen metabolic network (75,76). Ammonia, primarily derived from protein and amino acid catabolism, must be tightly regulated intracellularly; otherwise, it can strongly inhibit mitochondrial function and oxidative phosphorylation (77). In hepatocytes, the urea cycle remains the only pathway in mammals capable of efficiently converting ammonia into urea, with its flux sufficient to detoxify tens of grams of ammonia daily and ensure blood ammonia concentrations are kept within a safe range ($<50 \mu\text{mol/l}$) (13,75).

Alterations of the urea cycle in hepatocytes under diabetic conditions and their implications. Under high-fat and/or high-glucose conditions, the urea cycle in hepatocytes often undergoes functional failure (30) (Fig. 3). Animal and clinical studies have demonstrated that in high-fat, high-cholesterol diet-induced NASH rat models, the gene and protein expression of OTC and CPS1 is markedly downregulated, accompanied by hypermethylation in their promoter regions, decreased enzymatic activity and intrahepatic ammonia accumulation. Restoration of a normal diet partially reverses these epigenetic alterations, indicating a critical role for DNA methylation in the plastic regulation of the urea cycle under

pathological states (30). In human patients with NAFLD, liver tissue analyses from 20 cases of steatosis and 15 cases of NASH also confirmed that mRNA levels of CPS1, OTC, ASS1 and other urea cycle-related enzymes are decreased by an average of 30–40%, with urea production capacity significantly correlating with gene expression. This suggests that lipid accumulation itself suppresses hepatic ammonia detoxification via transcriptional and epigenetic mechanisms (8). In addition, in high-fructose, high-fat-induced steatosis mouse hepatocytes, hypermethylation of urea cycle enzyme gene promoters correlates with low expression, further exacerbating the risk of cell injury under hyperammonemic conditions (30). A further epigenetic study indicated that the low expression of urea cycle enzymes associated with NAFLD is not an isolated event but rather part of a global remodeling of the hepatocellular DNA methylation landscape (78). In a NASH-like liver injury model induced in C57BL/6J and DBA/2J mice by a lipogenic methyl-deficient diet, the liver exhibits not only marked steatosis but also global and repetitive element DNA hypomethylation, altered trimethylation of histone H3K9 and H3K27 and changes in H4K20 trimethylation, together with pronounced alterations in the protein expression of the maintenance DNA methyltransferase DNMT1 and the *de novo* methyltransferase DNMT3A, suggesting that DNA methyltransferases themselves are highly sensitive to nutritional and metabolic status during the development of fatty liver injury (78). In human NAFLD liver biopsies, methylation levels at specific CpG sites within the mitochondrial MT ND6 gene are higher in patients with NASH than in those with simple steatosis and the ratio of methylated to unmethylated DNA is positively correlated with the NAFLD activity score and histological severity, whereas MT ND6 mRNA and protein expression are reduced; DNMT1 is markedly upregulated in NASH and subcellular abnormalities such as disrupted mitochondrial cristae and increased numbers of

peroxisomes can be observed (79). Taken together with the global DNA methylation and histone modification abnormalities described in the mouse model, these findings suggest that as disease progresses from simple steatosis to NASH, the liver undergoes methylation reprogramming aligned with metabolic stress at both the nuclear and mitochondrial genomic levels (78,79). These lines of evidence collectively support the concept that NAFLD-related nutritional imbalances, such as methyl donor deficiency and environmental exposures such as reduced physical activity, can alter one-carbon metabolism and DNMT expression or activity, thereby creating a hepatic epigenetic milieu that is prone to aberrant CpG methylation in the promoters of key metabolic genes and in turn provides a permissive background for transcriptional repression of urea cycle-related genes (78,79). However, to the best of the authors' knowledge, no previous study has directly analyzed whether epigenetic alterations at urea cycle enzyme genes in NAFLD/NASH liver tissue directly drive their reduced expression and the associated impairment of ammonia detoxification. The most relevant to this possibility comes from a epigenetic study of CPS1 (22), the rate-limiting enzyme of the urea cycle. Analyses of human hepatocellular carcinoma cell lines and tumor tissues have shown that two CpG dinucleotides in the proximal CPS1 promoter and a CpG-rich region within the first intron are hypermethylated, coinciding with marked downregulation of CPS1 mRNA and loss of CPS1 protein in tumor specimens and HCC cells; treatment of HCC cells with the demethylating agent 5-aza-2'-deoxycytidine reduces methylation at these CpG sites, markedly restores CPS1 transcription and increases the activity of reporter constructs containing the CPS1 promoter in parallel with CpG demethylation, demonstrating that DNA methylation at specific CpG sites directly mediates CPS1 transcriptional silencing in HCC (22). By measuring expression of the liver-specific transcription factor HNF3 β and the hepatocyte marker gene AAT, this study also ruled out the possibility that CPS1 downregulation is mainly attributable to hepatocyte dedifferentiation and a global collapse of liver-specific transcriptional programs and in its discussion, drawing on prior evidence, it emphasized that promoter CpG hypermethylation often silences genes by blocking transcription factor access, thereby providing a rationale for viewing CPS1 promoter CpG hypermethylation as a potential mechanism regulating urea cycle enzyme expression rather than definitive evidence in NAFLD/NASH (22). Thus, integrating the evidence for altered DNMT1 and DNMT3A expression in NAFLD animal models, abnormal MT ND6 methylation in livers from patients with NAFLD and CPS1 promoter and gene body CpG hypermethylation in HCC, it can be hypothesized that under chronic nutritional imbalance, metabolic overload and inflammatory stress, hepatocytes may undergo DNMT-mediated CpG methylation reprogramming to target genes encoding urea cycle enzymes, maintaining low expression of CPS1 and other key enzymes and ultimately weakening hepatic ammonia detoxification capacity; however, this interpretation remains inferential and requires direct validation in NAFLD/NASH (22,78,79).

Decreased urea cycle enzyme activity in the livers of patients with NAFLD is positively correlated with intrahepatic ammonia concentrations. Excess ammonia can induce HSCs activation and fibrogenesis, providing a kinetic basis for the

transition from simple steatosis to inflammatory fibrosis (80). At the cellular level, *in vitro* studies have revealed that when ammonia exceeds 100 $\mu\text{mol/l}$, it interacts with key enzymes of the mitochondrial electron transport chain, inhibiting NADH:ubiquinone oxidoreductase (complex I) and cytochrome c oxidase (complex IV), thereby disrupting proton pump function in the respiratory chain (77). Experiments with isolated hepatic mitochondria show that NH_4^+ concentrations of 1-20 mM significantly inhibit dehydrogenase activity, leading to loss of mitochondrial membrane potential, opening of permeability transition pores, excess ROS generation, lipid peroxidation, glutathione (GSH) depletion and a precipitous drop in ATP synthesis: Hallmarks of an energy crisis (81).

Further metabolic flux analysis shows that under excess ammonia, glutamate dehydrogenase 2 reversibly aminates α -ketoglutarate (α -KG), rapidly depleting TCA cycle α -KG, promoting glutamate and glutamine synthesis and redirecting substrate flux to non-oxidative branches: Ultimately impairing sustained TCA cycle energy supply and metabolic homeostasis (77). This not only slows regeneration of critical cycle intermediates, but also leads to diminished proton-motive force in the mitochondrial matrix, hindering normal oxidative phosphorylation. Meanwhile, ammonia exerts bidirectional effects on glucose metabolism: Seahorse and metabolomics experiments demonstrate that in cell models exposed to high ammonia, both mitochondrial respiration (oxygen consumption rate) and glycolytic function (extracellular acidification rate) sharply decline, indicating that ammonia can rapidly suppress the two major cellular energy pathways, further aggravating energy deficiency (77).

Arginine-mediated nitric oxide cycle in hepatocytes. As an intermediate of the urea cycle, arginine is also the sole substrate for various NOS that generate NO. The metabolic fate of arginine within hepatocytes directly determines NO content and signal strength and the 'arginine-NO axis' finely regulates mitochondrial function, TCA cycle activity and glucose/lipid metabolism. Basal NO in hepatocytes primarily derives from endogenous eNOS activity. This nanomolar-level NO activates soluble guanylate cyclase (sGC) to catalyze GTP to cyclic GMP (cGMP), subsequently activating cGMP-dependent protein kinase (PKG) and downstream coactivator PGC-1 α , thereby promoting mitochondrial biogenesis and respiratory chain gene expression, increasing mitochondrial density and oxidative phosphorylation capacity (82,83). The sGC/cGMP pathway also inhibits acetyl-CoA carboxylase (ACC), lowers malonyl-CoA, relieves carnitine palmitoyltransferase-1 (CPT-1) inhibition, enhances fatty acid transport into mitochondria and β -oxidation, ultimately suppressing lipogenesis and boosting fatty acid utilization (84).

Low NO exerts biphasic effects on the TCA cycle: It mildly inhibits complex IV activity, transiently lowers membrane potential, triggers ROS as signaling molecules and activates AMPK and PGC-1 α -mediated mitochondrial biogenesis. At moderate NO, the pyruvate dehydrogenase (PDH) complex and mitochondrial aconitase are reversibly inhibited, reducing pyruvate flux into the TCA cycle and promoting glutamine-dependent anaplerosis, thereby sustaining α -KG and ATP homeostasis (85). When NO rises further, PDH and aconitase inhibition intensifies, causing mitochondrial

citrate accumulation that not only feeds back to inhibit glycolytic enzymes (pyruvate kinase), but also suppresses CPT-1, reducing fatty acid oxidation and shifting cell metabolism toward glycolysis and glutamine pathways to adapt to compromised oxidative phosphorylation (85).

In glucose metabolism, NO significantly inhibits hepatic glycogen synthesis and gluconeogenesis: experiments show that in primary rat hepatocytes treated with NO donor S-nitroso-N-acetylcysteine, glycogen synthase activity and gluconeogenic enzymes (pyruvate carboxylase, phosphoenolpyruvate carboxykinase) are markedly reduced, with intracellular glucose-6-phosphate and UDP-glucose accumulating; indicating that NO suppresses glycogen synthase conversion to the active form and limits glucose storage and gluconeogenic substrate availability (86). eNOS-derived NO via cGMP-PKG also downregulates G6Pase, reducing hepatic glucose output and thereby synergistically modulating systemic glucose balance (85).

In lipid metabolism, low NO via sGC/cGMP promotes fatty acid oxidation, downregulates ACC, activates AMPK, increases CPT-1 activity and enhances mitochondrial fatty acid uptake and degradation (84,85). However, in inflammatory or endotoxin-stimulated settings, iNOS becomes highly expressed in hepatocytes, producing micromolar to millimolar NO that rapidly reacts with superoxide to form ONOO⁻. ONOO⁻ causes nitration and oxidation of mitochondrial complexes I and III and various metabolic enzymes (PDH, CPT-1), resulting in loss of membrane potential, S-nitrosylation and persistent inhibition of complex I, excessive ROS production, GSH depletion, energy crisis and activation of apoptosis signals (87-90).

Thus, the competition between arginase and iNOS for arginine in hepatocytes is pivotal: Arg2 knockout mice on a high-fat diet exhibit worse hepatic lipid accumulation, upregulated iNOS and exacerbated NO/ONOO⁻-mediated oxidative damage, indicating that enhancing Arg2 activity can mitigate hepatic inflammation and steatosis (91). Conversely, nonselective NOS inhibitor L-NAME treatment in obese rats aggravates mitochondrial dysfunction, triglyceride accumulation and proinflammatory cytokine levels, further validating the bidirectional role of NO homeostasis in hepatic metabolic adaptation (92).

In summary, the arginine-NO axis in hepatocytes regulates mitochondrial biogenesis, TCA flux, glucose-lipid metabolic balance and redox state in a concentration-dependent manner. Its precise equilibrium is vital for energy homeostasis and stress resilience. During NAFLD-to-NASH progression, inflammation and/or DNA methylation-driven downregulation of urea cycle enzymes and impaired Arg2 activity shift arginine metabolism toward iNOS, exacerbating NO-ONOO⁻-induced mitochondrial and metabolic injury.

Metabolic cross-talk between the hepatic urea cycle and glucose-lipid metabolism in NAFLD/NASH. Under physiological conditions, amino acids enter the tricarboxylic acid TCA cycle via transamination to generate oxaloacetate and aspartate, the latter serving as a substrate for the urea cycle. At the same time, fumarate generated by the urea cycle can re-enter the TCA cycle, providing carbon backbones and reducing equivalents for gluconeogenesis and fatty acid synthesis, thereby tightly coupling nitrogen metabolism with

hepatic glucose and lipid metabolism (93). In obesity-related NAFLD and NASH, hepatic fatty acid influx is increased while fatty acid oxidation and very low-density lipoprotein export are constrained, leading to excessive lipid accumulation in hepatocytes and establishing a background of metabolic imbalance together with insulin resistance and disordered glucose metabolism. On this basis, expression and activity of key urea cycle enzymes are downregulated, ureagenesis capacity is diminished and hyperammonemia develops (8,30).

Transcriptomic and proteomic analyses of liver tissue from patients with NAFLD show a coordinated downregulation of multiple nitrogen-conversion pathways responsible for urea production and ammonia clearance, whereas alternative pathways such as glutamine synthetase are relatively preserved or even upregulated. This indicates a redistribution of nitrogen flux away from the urea cycle toward alternative ammonia-detoxifying routes, a reprogramming pattern that correlates with intrahepatic fat content and disease stage (8,30). Human stable isotope tracer and metabolic flux studies further demonstrate that hepatic TCA cycle and gluconeogenic fluxes are globally elevated in NAFLD and positively correlated with intrahepatic triglyceride content, suggesting that in fatty liver the mitochondrial carbon flux is preferentially directed toward the TCA cycle and gluconeogenesis (94). Against a background of impaired urea cycle enzyme expression and function, this enhanced carbon flux cannot be adequately matched to nitrogen metabolism, favoring accumulation of ammonia and related metabolites in the liver and systemically (8,30,80).

Glutamine is not only an alternative product for ammonia detoxification but also a major energy and carbon source for activated hepatic stellate cells. In NASH models and liver tissue from NASH patients, glutamine metabolism and glutaminolysis are enhanced, sustaining oxidative metabolism in activated stellate cells and driving scar formation and matrix deposition, thereby functionally linking suppression of the urea cycle and glutamine metabolic reprogramming directly to the fibrotic phenotype (80,93,95). In normal mice and isolated perfused rat livers, acute glucagon stimulation rapidly enhances amino acid clearance and urea production, reflecting physiological hepatic glucagon sensitivity at the level of amino acid metabolism. By contrast, in fatty liver models induced by high-fat diet or in ob/ob obese mice, the same glucagon stimulus produces a markedly attenuated increase in amino acid catabolism and ureagenesis, manifesting as impaired amino acid clearance and blunted ureagenic responses, consistent with a state of glucagon resistance primarily affecting amino acid metabolism in fatty liver (5). In obese adult mice with hepatic steatosis, intravenous glucagon elicits a relatively preserved glycemic response, whereas the increments in amino acid clearance and urea production are diminished, providing clinical evidence for glucagon resistance predominantly at the level of amino acid turnover. This selective resistance allows glucagon-induced gluconeogenesis to be maintained while impairing the capacity of the urea cycle to dispose of amino acids and excess nitrogen, thereby aggravating hyperammonemia and amino acid overload (96). Taken together, in NAFLD/NASH, downregulation of urea cycle enzymes, redistribution of nitrogen toward the glutamine pathway, increased TCA and gluconeogenic fluxes and glucagon resistance specifically affecting amino acid metabolism collectively

form a self-reinforcing metabolic circuit in which disordered nitrogen handling and glucose-lipid metabolic reprogramming exacerbate each other and drive the persistent progression of inflammation, fibrosis and insulin resistance (30,80,95).

Interplay between the hepatic urea cycle and one-carbon metabolism in NAFLD/NASH. In hepatocytes, nitrogen derived from amino acid catabolism is primarily cleared via the urea cycle, whereas one-carbon metabolism channels nutrients and metabolic substrates such as serine into one-carbon units and reducing equivalents for nucleotide synthesis, redox defense and the generation of methyl donors; consequently, under conditions of nutrient excess and fatty liver, nitrogen disposal capacity, methyl donor availability and the epigenetic landscape tend to be perturbed in a coordinated manner (97,98). One-carbon metabolism generates S-adenosylmethionine via the methionine cycle and S-adenosylmethionine functions as a universal methyl donor for DNA and histone methylation, as well as for phosphatidylethanolamine methylation by phosphatidylethanolamine N-methyltransferase to form phosphatidylcholine; these reactions influence membrane phospholipid composition, very-low-density lipoprotein assembly and hepatic lipid export and are closely linked to the development and progression of NAFLD (97,98). In a 52-week high-fat, high-cholesterol diet-induced NAFLD mouse model, hepatic methionine levels were depleted, whereas S-adenosylhomocysteine and homocysteine concentrations were increased; at the same time, serine was markedly depleted and glycine levels were reduced, accompanied by downregulation of glycine N-methyltransferase and an elevated phosphatidylcholine to phosphatidylethanolamine ratio, indicating system-wide remodeling of the methionine cycle and associated transmethylation and transsulfuration pathways (99). Among the genes examined in that study, only the Hmgcr promoter exhibited hypermethylation, whereas genes such as Fasn did not show similar changes, suggesting that DNA methylation alterations may be targeted to specific loci rather than occurring in a global, nonselective fashion (99). Methyl donor-deficient models further demonstrate that insufficient one-carbon flux can elicit NAFLD/NASH-like phenotypes accompanied by extensive epigenetic reprogramming: male C57BL/6J and DBA/2J mice fed a lipogenic methyl-deficient diet for 6-18 weeks developed liver injury resembling human NASH, together with global and repetitive element DNA hypomethylation, reduced DNMT1 expression, altered DNMT3A expression and broad remodeling of histone modification profiles, including changes in H3K9me3, H3K27me3 and H4K20me3 with strain-dependent patterns (78). In parallel, urea cycle impairment is a reproducible feature of NAFLD/NASH: in Wistar rats fed a high-fat, high-cholesterol diet for 10 months to induce NASH and subsequently switched to a normal chow diet and in liver biopsies from patients with NAFLD or NASH, hepatic OTC and CPS1 gene and protein expression and OTC enzymatic activity were reduced, accompanied by elevated ammonia concentrations and hypermethylation of OTC and CPS1 promoter regions; in the rat model, these changes were at least partially reversible upon dietary normalization, underscoring the plasticity of the urea cycle in response to nutritional and metabolic cues (30). Taken together, these data support a model in which disturbed

one-carbon metabolism, by altering methyl donor supply and the network of methylation-related enzymes, modulates the transcriptional regulation of key urea cycle genes, while impaired urea cycle activity and consequent ammonia accumulation feedback to exacerbate hepatocellular metabolic stress and disrupt amino acid and one-carbon substrate homeostasis, thereby establishing a feed-forward pathological circuit between epigenetic remodeling and nitrogen disposal in NAFLD/NASH (30,97,100).

5. Metabolic reprogramming of the urea cycle and arginine metabolism in HSCs

Against a background of hepatic injury and lipid deposition, the transition of HSCs from quiescent to activated states is accompanied by global remodeling of arginine metabolism and related enzyme systems. This metabolic reprogramming not only influences the proliferation, migration and matrix synthesis of HSCs themselves, but also, through metabolic cross-talk with hepatocytes, collectively drives fibrogenesis. The dynamic balance between the urea cycle and NO cycle thus emerges as a central regulatory hub in HSCs activation and the progression of NASH/NAFLD (30,101,102).

In the quiescent state, HSCs reside in the perisinusoidal space of hepatic lobules, where their main functions include storage of vitamin A and secretion of matrix metalloproteinases. At this time, amino acid metabolism is low and energy is mainly derived from lipid oxidation driven by intracellular lipid droplets; the expression of ARG1/ARG2 is nearly undetectable, iNOS is not expressed among NOS family members and eNOS is mainly localized to microvascular endothelial cells, providing limited NO to HSCs themselves (60,103). During this stage, arginine is used mainly for basal metabolism, with little conversion to NO or ornithine derivatives (104); intracellular ornithine is primarily derived from urea cycle intermediates secreted by hepatocytes or dietary amino acids. Meanwhile, ornithine decarboxylase 1 activity is low and polyamine synthesis is maintained at minimal levels, preventing uncontrolled proliferation and excess matrix production (105). It is in this stable environment that HSCs are highly sensitive to external injury signals such as TGF- β and PDGF and upon stimulation rapidly rewire their metabolic network, priming for activation.

Urea cycle remodeling in HSCs under pathological conditions. When hepatic inflammation and apoptosis are triggered by high-fat, high-glucose, or toxic insults, HSCs are mobilized as principal fibrogenic effectors. Their morphology shifts from stellate to flattened and α -smooth muscle actin (α -SMA), type I collagen and fibronectin expression are markedly upregulated (106,107). Accompanying this phenotypic transformation, cellular energy metabolism switches from mitochondrial lipid oxidation to a pattern reliant on both aerobic glycolysis and glutamine metabolism, rapidly supplying ATP and intermediate metabolites (108,109). In this process, ODC1 is markedly upregulated in activated HSCs, catalyzing the decarboxylation of ornithine to produce putrescine; subsequent actions of spermidine synthase and spermine synthase convert putrescine into spermidine and spermine, meeting high demands for nucleic acid stability

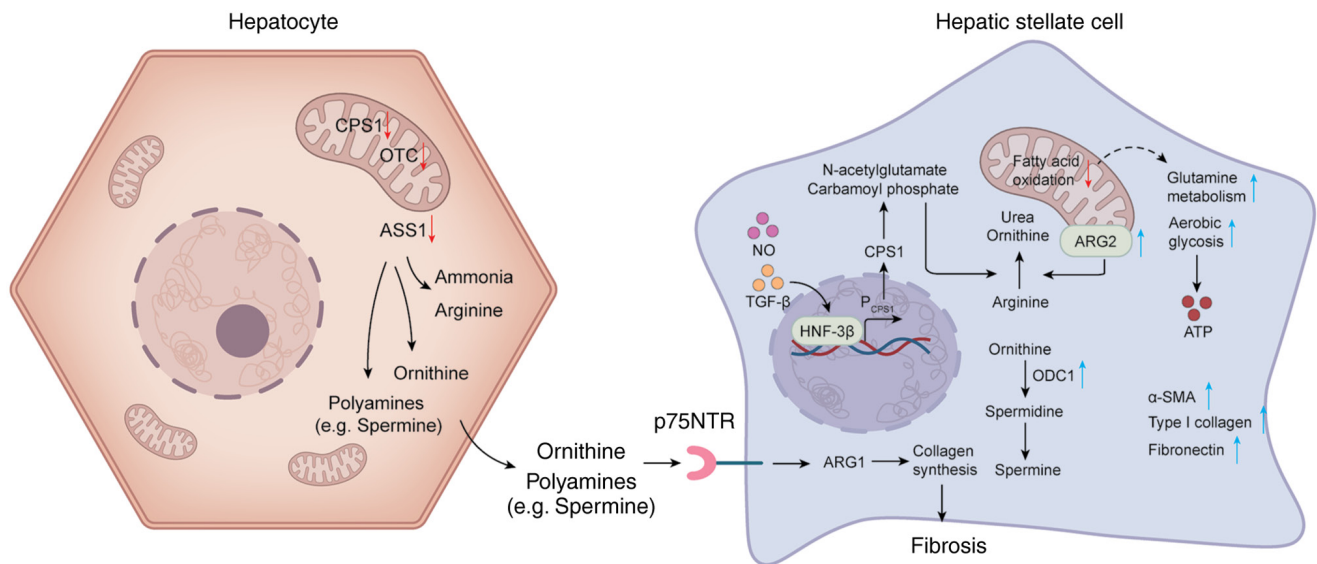


Figure 4. Metabolic reprogramming of hepatic stellate cells and ornithine-polyamine flux during hepatic inflammation and NAFLD/NASH. During hepatic inflammation, hepatic stellate cells are mobilized, their morphology shifts from a stellate to a flattened shape and the expression of α -SMA, type I collagen and fibronectin is markedly upregulated. Cellular energy metabolism is reprogrammed from mitochondrial fatty acid oxidation toward a phenotype predominantly reliant on aerobic glycolysis and glutamine metabolism, providing rapid ATP and biosynthetic intermediates. In activated HSCs, ornithine ODC1 is strongly upregulated, catalyzing the decarboxylation of ornithine and its further conversion into the polyamines spermidine and spermine. Mitochondrial ARG2 is upregulated and hydrolyzes arginine to ornithine and urea. Regulation by NO and TGF- β increases the affinity of the HNF-3 β binding site within the CPS1 promoter, maintaining CPS1 at a low-to-intermediate expression level, thereby sustaining the production of carbamoyl phosphate and NAG and providing essential intermediates for ARG2-driven ornithine generation. In the pathological context of NAFLD/NASH, hepatic expression of CPS1, OTC and ASS1 is reduced, leading to excessive accumulation of ammonia and arginine. Hepatocyte-derived ornithine and polyamines (such as spermine) can act on HSCs via receptors including p75NTR and, through ARG1-mediated pathways, further promote collagen synthesis, thereby establishing a vicious cycle of local fibrogenesis. All figures were generated using Microsoft PowerPoint (Microsoft Corporation). NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; α -SMA, α -smooth muscle actin; HSCs, hepatic stellate cells; ODC1, ornithine decarboxylase 1; ARG, cytosolic arginase; NO, nitric oxide; CPS1, carbamoyl phosphate synthetase 1; OTC, ornithine transcarbamylase; ASS1, argininosuccinate synthase.

and protein modification during proliferation and matrix synthesis (110,111) (Fig. 4).

Simultaneously, ARG2 is significantly upregulated in mitochondria, hydrolyzing arginine to ornithine and urea, thus providing a constant substrate supply for ODC1; cytosolic ARG1 activity is also enhanced, supporting polyamine synthesis and metabolic intermediate production (60). Notably, although classic urea cycle enzymes such as CPS1 and OTC are generally downregulated in activated HSCs, atypical modular reassembly occurs within the cell: under the co-regulation of NO and TGF- β , the affinity of the HNF-3 β binding site in the CPS1 promoter increases, allowing CPS1 to be maintained at low to moderate expression, continuously generating carbamoyl phosphate and NAG and supplying essential intermediates for ARG2-driven ornithine production and the polyamine pathway (31,112-115). This atypical urea cycle configuration enables HSCs to flexibly shift between limited urea production and robust polyamine synthesis in response to extracellular ammonia and synthetic demands, achieving fine-tuned metabolic adaptation (112,114).

Under the pathological background of NASH/NAFLD, the expression of key enzymes such as CPS1, OTC and ASS1 in hepatocytes is significantly reduced due to high-fat, high-glucose and inflammatory environments, leading to excessive accumulation of ammonia and arginine in the periportal region (31). This surplus of ammonia and arginine further upregulates ARG2 and ODC1 in HSCs, enhancing ornithine and polyamine metabolism to meet the energy and matrix synthesis demands of activation (116). Meanwhile,

ornithine and polyamines (such as spermine) released by hepatocytes can act on HSCs via receptors like p75NTR and, through ARG1-mediated pathways, further promote collagen synthesis, creating a vicious cycle of localized fibrosis (60). In advanced fibrotic stages, accumulated polyamines not only enhance PARP and SMAD signaling in TGF- β -induced collagen gene transcription and matrix crosslinking, but may also suppress autophagy via excessive activation of mTOR, resulting in protein aggregate accumulation and mitochondrial damage, pushing fibrotic tissue toward irreversibility (116-119).

NO cycle remodeling in HSCs. In the quiescent state, HSCs express almost no iNOS and respond only weakly to NO signals secreted by neighboring Kupffer cells or hepatocytes; however, under inflammatory stimulation (such as TNF- α , IL-1 β , IL-6 and LPS), NOS2 gene expression and iNOS activity in HSCs are rapidly induced, with both mRNA and protein levels rising dramatically within hours, leading to a surge in cellular NO production to micromolar levels (120-122). High concentrations of NO can directly inhibit ODC1 activity, reducing polyamine synthesis and through S-nitrosylation, post-translationally modify CPS1 and ARG2, decreasing arginine consumption and channeling more substrate into the NO pathway to amplify inflammatory signaling (120,123). NO reacts with superoxide to form ONOO $^-$, which nitrates mitochondrial complexes I/III, the PDH complex and CPT-1, leading to loss of mitochondrial membrane potential, massive ROS generation, ATP depletion and consequently HSC apoptosis or progression to a pro-fibrotic phenotype (120,124).

By contrast, low to moderate NO signals can reversibly inhibit oxidative phosphorylation and activate the AMPK/PGC-1 α pathway, inducing compensatory aerobic glycolysis and TCA cycle remodeling, ensuring abundant ATP and NADPH for matrix synthesis (125,126). This biphasic effect makes NO a key determinant in HSC activation: even minor changes in concentration can create a tipping point between fibrosis progression and spontaneous resolution (125,126). In early activation, low NO mainly acts through cGMP/PKG-mediated AMPK phosphorylation to boost aerobic glycolysis and TCA cycle activity in HSCs, supporting proliferation and matrix generation; at moderate NO, reversible inhibition of PDH and aconitase shifts metabolism toward glutamine dependence and lactate fermentation, producing more matrix precursors and antioxidant NADPH (127,128).

However, sustained iNOS upregulation and extensive ONOO⁻ formation can cause complete mitochondrial failure: irreversible loss of membrane potential, activation of mitochondria-mediated apoptosis, disruption of local cell renewal, microenvironmental imbalance and accelerated fibrotic scar expansion (129). Multiple *in vitro* and animal studies of NASH have shown that iNOS and ARG2 mRNA levels are significantly higher in activated HSCs than in quiescent states, reflecting the simultaneous amplification of urea detoxification and NO generation, driving HSCs into a state of high metabolic stress and promoting progression to irreversible fibrosis (120). Furthermore, high levels of NO produced by HSCs can diffuse into neighboring hepatocytes, where S-nitrosylation inhibits CPS1 and ARG1, further impairing the urea cycle, causing increased arginine and ammonia leakage and establishing a positive feedback loop between HSCs and hepatocytes that exacerbates NASH/NAFLD-related fibrosis (28).

6. Hepatic macrophages

Alterations of the urea cycle and NO pathways in macrophages. In recent years, hepatic macrophages, including resident Kupffer cells and infiltrating monocyte-derived macrophages (MoMFs), have garnered wide attention for their roles in NAFLD and its progressive form, NASH. In pathological contexts, hepatic macrophages contribute to inflammatory responses, matrix remodeling and fibrogenesis by secreting inflammatory mediators, phagocytosing apoptotic cells and regulating intercellular metabolic signaling, thus exerting multilayered control over the hepatic microenvironment (130-133). In NAFLD/NASH, metabolic stressors such as lipid accumulation and insulin resistance drive metabolic reprogramming of hepatic macrophages, inducing dynamic switching between pro-inflammatory (M1) and anti-inflammatory/repair (M2) polarization states, which mediate divergent functional outcomes at different disease stages (134).

In the early inflammatory phase, Kupffer cells and infiltrating macrophages preferentially polarize toward the M1 phenotype, characterized metabolically by a reliance on glycolysis for ATP production and regulatory metabolite generation and by robust upregulation of iNOS. Through iNOS, L-arginine is converted to large amounts of NO, amplifying pro-inflammatory signaling and inducing high expression of cytokines such as TNF- α , IL-1 β and IL-6, which further exacerbate oxidative stress and apoptosis in hepatocytes

and propagate inflammatory infiltration throughout hepatic tissue (30,135). Evidence shows that NO not only directly damages mitochondrial function and increases ROS, but also transiently suppresses TGF- β 1 signaling in HSCs, briefly delaying collagen matrix secretion. However, excessive NO production intensifies hepatocyte toxicity, causing more severe liver injury and accelerating inflammatory deterioration (31,136). Under M1 polarization, macrophage arginine flux is directed predominantly toward the iNOS pathway, with relatively suppressed Arg1 expression, maximizing NO production and amplifying the local pro-inflammatory milieu (137). IL-1 β further acts on hepatocytes via its receptor, on the one hand upregulating lipogenic genes and promoting triglyceride accumulation and on the other hand impairing insulin signal transduction, thereby increasing the susceptibility of hepatocytes to lipotoxic and inflammatory insults (138,139). Meanwhile, TNF- α secreted by Kupffer cells can activate the hepatocellular TNFR-JNK axis, inducing changes in mitochondrial permeability and cytochrome *c* release; in dietary steatohepatitis mouse models, pharmacological or genetic blockade of TNF- α markedly attenuates hepatocyte apoptosis and hepatic inflammation (140). A study has further shown that Kupffer cell-derived TNF- α can induce hepatocytes to secrete the chemokine C-X-C motif chemokine ligand 1, thereby driving massive neutrophil recruitment and amplifying necrotic liver injury, which establishes a TNF- α -CXCL1-mediated amplification loop between macrophages and hepatocytes (141).

Conversely, in M2-polarized hepatic macrophages, Arg1 drives the urea cycle, converting L-arginine into urea and L-ornithine. This reaction helps eliminate excess nitrogen through urea excretion while providing ornithine for proline synthesis, a step that is essential for matrix remodeling and tissue repair (142). M2 macrophages also downregulate iNOS, suppress NO generation and secrete anti-inflammatory mediators, attenuating excessive immune responses and stabilizing tissue during late-stage fibrosis (143,144). This Arg1 driven metabolic shift not only curbs NO overproduction, but also provides macrophages with energy and precursors for extracellular matrix and collagen synthesis, thus accelerating fibrogenesis and tissue remodeling (145). At the level of metabolic crosstalk between macrophages and hepatocytes, alternatively activated Kupffer cells secrete anti-inflammatory cytokines such as IL-10, which act via the hepatocellular IL-10 receptor to suppress excessive STAT3 phosphorylation and acute-phase protein expression, thereby limiting triglyceride accumulation and impairment of insulin signaling (146). In high-fat diet-fed mice, depletion of Kupffer cells using clodronate liposomes or global knockout of IL-10 both lead to a marked reduction in hepatic IL-10, enhanced STAT3 signaling and increased intrahepatic triglyceride levels, indicating that Kupffer cell-derived IL-10 plays a pivotal role in restraining hepatocyte lipotoxicity (146). In obesity-related models, chimeric mice reconstituted with PPAR δ -deficient bone marrow cells exhibit downregulation of hepatic fatty acid oxidation genes and pronounced steatosis; in follow-up conditioned culture experiments, supernatants from PPAR δ -deficient macrophages added to primary hepatocytes reduce the expression of oxidative genes such as Cpt1a and Acox1, lower mitochondrial oxygen consumption

and exacerbate triglyceride accumulation, demonstrating that M2-associated metabolic mediators can directly remodel hepatocyte lipid metabolism via paracrine mechanisms (147). In the early phase of high-fat feeding, free fatty acids released from hepatocytes, particularly palmitate, activate Kupffer cells, inducing robust TNF- α secretion that acts on hepatocyte TNFR1 to drive citrate accumulation, increased *de novo* lipogenesis and enhanced fatty acid uptake; blockade of TNF- α signaling markedly reduces hepatocellular lipid droplet burden, suggesting that metabolites and cytokines together establish a bidirectional crosstalk loop between macrophages and hepatocytes (148). Taken together, these bidirectional cytokine- and metabolite-driven interactions are not only central to lipotoxic and inflammatory amplification, but also provide a mechanistic context in which hepatocellular metabolic stress, mitochondrial dysfunction and altered arginine handling may converge to impair urea cycle function in NAFLD/NASH.

In NAFLD/NASH patients, global hepatic urea cycle activity is clearly impaired. Studies show that mRNA and protein expression of key enzymes such as CPS1 and OTC are markedly reduced in NASH tissues, severely compromising the capacity for urea synthesis in both hepatocytes and hepatic macrophages. This contributes to hyperammonemia and worsens liver injury (8,31). The degree of CPS1 down-regulation (as the rate-limiting urea cycle enzyme) correlates positively with the loss of urea synthetic capacity in NASH patients, while declining OTC expression is associated with greater fibrosis and worse clinical outcomes (8). Thus, functional impairment of the urea cycle spans the full spectrum of NASH, underpinning hyperammonemia and hepatocellular damage and requiring compensatory ammonia clearance via macrophage Arg1 dependent mechanisms.

Despite global urea cycle impairment, Arg1 expression in hepatic macrophages is often upregulated across various research models. This apparent paradox may reflect a compensatory hepatic strategy: Enhancing local urea cycling in macrophages to mitigate ammonia toxicity. However, when Arg1 activity becomes excessive, arginine is quickly depleted, limiting the substrate for iNOS, lowering NO production and weakening the early pro-inflammatory removal of cytokines and apoptotic cells, which ultimately slows lesion resolution and tissue repair (135,149). At the same time, abundant ornithine generated by Arg1 is utilized by HSCs for proline and collagen precursor synthesis, accelerating matrix deposition and fibrosis progression; this is supported by evidence from both NASH animal models and clinical liver tissue (144,149). Hence, precise regulation of Arg1 activity is critical for balancing hepatic inflammation clearance vs. fibrogenic progression in NAFLD/NASH.

Mechanistic studies further demonstrate that Arg1 knockout or inhibition in macrophages restores iNOS-mediated NO production, thereby enhancing the early pro-inflammatory clearance capacity and reducing intrahepatic inflammation, oxidative stress and lipid peroxidation in NASH mouse models. Nonetheless, this situation can perpetuate mitochondrial dysfunction, boost superoxide generation and amplify oxidative stress, which collectively heighten hepatocellular necrosis and apoptosis and accelerate fibrogenesis, underscoring the intricate role Arg1 plays in regulating inflammation (31,149). Conversely, in late-stage NASH, excessive Arg1 activity may

acutely clear ammonia via urea production and protect hepatocytes, but surplus ornithine synthesis further drives HSC activation and collagen deposition, thereby worsening fibrosis, increasing hyperammonemia and potentially predisposing to hepatic encephalopathy and other complications (144,149). Therefore, the delicate balance between Arg1 and iNOS activities in hepatic macrophages ultimately determines whether the liver manifests inflammatory or fibrotic phenotypes during NAFLD/NASH progression.

At the microenvironmental level, excess free fatty acids activate TLR4 signaling, upregulate iNOS and promote M1 polarization, while pro-inflammatory cytokines further reinforce the M1 phenotype. Conversely, IL-4, IL-13 and PPAR γ/δ agonists enhance Arg1, driving M2 polarization, so hepatic macrophages display remarkable phenotypic and metabolic plasticity in response to complex microenvironmental cues (31,143). Additionally, negative regulators such as galectin-12 inhibit Arg1 in macrophages; galectin-12 deficiency promotes M2 polarization in Kupffer cells with elevated Arg1 and TGF- β 1, enhancing HSC activation and collagen deposition, indicating a key regulatory role for galectin-12 in maintaining M1/M2 metabolic balance (150). Various transcription factors altered in NASH also directly regulate urea cycle gene transcription (CPS1, OTC and ASS1), while mTOR signaling influences downstream effectors (S6K and 4EBP1) to control macrophage metabolic fate, indirectly affecting Arg1 and iNOS levels. For example, mTORC1 hyperactivation stabilizes HIF-1 α , promoting iNOS and M1 polarization, whereas mTOR inhibition favors M2 polarization and Arg1 transcription, substantially shaping hepatic macrophage phenotype and function (114). At the epigenetic level, histone acetylation and DNA methylation changes in early NASH also affect expression of key arginine metabolic enzymes, further complexifying the regulatory landscape of macrophage metabolic reprogramming.

Single cell transcriptomic analyses demonstrate pronounced heterogeneity among hepatic macrophages in NASH. Inflammation related subsets often show contrasting levels of Arg1 and iNOS, cells with abundant Arg1 cluster within fibrotic regions, whereas those with higher iNOS drive the early inflammatory response (150-152). For example, in NASH mouse models, 15-20% of MoMFs differentiate into Arg1^{hi}/Retnla^{hi} M2-like cells under certain inflammatory stimuli, whereas about 30% of Kupffer cells display high iNOS, TNF and IL-1 β , indicative of strong M1 features. Such spatial and temporal dynamics underscore the distinct contributions of macrophage subpopulations to fibrosis progression and inflammatory spread (153,154).

Inflammatory state changes mediated by urea and NO cycle alterations. At the level of intercellular interactions, M2-polarized macrophages secrete large amounts of L-ornithine and its proline derivatives, providing precursors for collagen synthesis by HSCs. Additionally, various metabolites (such as urea) can modulate TGF- β 1/Smad signaling in HSCs in a concentration-dependent manner during fibrosis: low urea concentrations promote HSC survival and collagen synthesis, whereas high urea may induce HSC apoptosis, potentially facilitating matrix remodeling and fibrosis regression at late stages (144,149). On the cytokine level, NO secreted by M1

macrophages can directly inhibit TGF- β 1 signaling in HSCs, briefly suppressing collagen deposition; however, excess NO becomes cytotoxic to hepatocytes, disrupting mitochondrial membrane potential, inducing apoptosis and releasing DAMPs (damage-associated molecular patterns) that recruit and activate further macrophages, fueling a vicious cycle and ultimately accelerating fibrosis (31,136).

As well as metabolic interplay with HSCs, hepatic macrophages also secrete chemokines and cytokines that tightly interact with hepatocytes. M1-derived TNF- α and IL-1 β can induce hepatocyte MCP-1 expression, enhancing monocyte recruitment and establishing a pro-inflammatory circuit; conversely, M2 macrophage IL-10 and unsaturated fatty acid-binding proteins, via PI3K/Akt signaling, suppress hepatocyte apoptosis and promote proliferation and repair, conferring both protective and pro-fibrotic actions during pathology and recovery (114,144). HSCs, in turn, not only respond to M2-derived ornithine and collagen precursors, but can secrete IL-33, CXCL12 and other factors that induce macrophage CD163 and MERTK, further promoting M2 polarization and creating complex positive-feedback networks that stabilize and amplify the fibrotic microenvironment (155).

7. Clinical biomarkers and therapeutic targets

Biomarker value of urea cycle enzymes and arginine metabolites in serum and liver tissue

Liver tissue assessment. At the tissue level, consistent with the central role of CPS1 and other urea-cycle enzymes in hepatic ammonia detoxification aforementioned, multiple investigations using gene-expression profiling, proteomics and enzyme-activity assays have documented sharp reductions in these rate-limiting enzymes in NAFLD and NASH. One hepatic mRNA-sequencing study comparing 20 non-diabetic patients with NAFLD (eight with simple steatosis and 12 with NASH) to healthy controls reported a roughly 3.5-fold decrease in CPS1 transcripts ($P<0.0001$); this decline closely paralleled diminished urea-synthetic capacity ($P=0.03$) (75). Given that CPS1 catalyzes the entry step of the urea cycle, its downregulation provides a direct mechanistic explanation for impaired ammonia detoxification and the tight correlation between CPS1 expression and urea production supports CPS1 as a mechanistically grounded tissue biomarker of urea-cycle failure in NAFLD/NASH. The same analysis and other studies observed significant downregulation of OTC, ASS1 and ASL, whereas GS expression rose by more than 1.5-fold and showed a negative correlation with CPS1 ($P=0.004$) (8). This enzyme-expression pattern mirrors the shift from urea-based detoxification toward glutamine trapping of excess ammonia described in experimental models, indicating that quantitative measurement of CPS1, OTC, ASS1, ASL and GS in liver tissue reflects not only the severity of metabolic remodeling but also the specific route by which ammonia is handled. Indeed, human liver specimen analyses demonstrate that lower CPS1, OTC, ASS1 and ASL expression is strongly associated with higher serum ALT and AST concentrations and more advanced fibrosis, indicating that these enzymes can serve as pathophysiologically informed tissue biomarkers for staging NAFLD/NASH and assessing hepatic injury (8,31).

Complementing transcriptomic data, studies utilizing western blotting, immunohistochemistry and *in vitro* enzyme assays in a rat NASH model and liver biopsies from 35 human fatty liver patients further confirmed that CPS1 and OTC protein levels and activities were markedly reduced (30). Importantly, promoter hypermethylation of CPS1 and OTC, triggered by a high-fat, high-cholesterol diet, was tightly associated with the loss of enzyme expression, whereas switching back to a normal diet partly restored both methylation status and enzyme levels (30). These findings link a specific epigenetic mechanism (promoter hypermethylation) to measurable loss of CPS1/OTC function and underscore that tissue CPS1/OTC expression simultaneously captures an actionable regulatory lesion and a potential therapeutic target.

Mechanistically, suppression of urea-cycle flux leads to intrahepatic and systemic ammonia accumulation, which in turn drives mitochondrial dysfunction and HSC activation as outlined in sections *Fundamentals of the urea cycle and arginine metabolism* and *Metabolic reprogramming in hepatocytes*. Clinically, this is reflected by a study showing that peripheral blood ammonia levels in NASH patients are significantly higher than in healthy controls ($P<0.01$) and intrahepatic ammonia content is positively correlated with fibrosis stage ($r=0.62$; $P<0.001$) (30). Thus, hyperammonemia is not only an effector of fibrogenesis but also serves as a circulating biomarker that integrates upstream defects in CPS1/OTC/ASS1/ASL into a clinically accessible readout of NAFLD severity.

Taken together, these data indicate that downregulation of CPS1, OTC, ASS1 and related enzymes and the resulting hyperammonemia, are not only central drivers of mitochondrial dysfunction and fibrogenesis but also provide mechanistic justification for using urea-cycle enzyme expression and systemic ammonia levels as biomarkers to stage NAFLD/NASH.

Blood-based detection. In serum and plasma, measurement of ammonia and arginine metabolites not only reflects hepatic urea cycle impairment but also reveals systemic metabolic disturbances. Research showed that blood-ammonia concentrations are significantly higher in NASH patients ($P<0.01$). Circulating ammonia also closely mirrors intrahepatic ammonia content and fibrosis stage ($r=0.62$; $P<0.001$), supporting blood ammonia as a non-invasive biochemical marker of urea-cycle impairment and fibrosis progression (30).

For arginine derivatives, one of the clinical study measured plasma ADMA in 70 patients with NAFLD (53 NASH; 17 NAFLD) and 12 healthy controls, finding that ADMA was significantly higher in the NAFLD group (0.81 ± 0.25 vs. 0.48 ± 0.24 $\mu\text{mol/l}$; $P=0.005$) and that this elevation was independent of insulin resistance or body composition, suggesting ADMA as a sensitive early warning marker for endothelial dysfunction and cardiovascular risk in NAFLD (156).

A Chinese cohort study reported that serum homocitrulline was significantly elevated in patients with NAFLD, serving as a diagnostic metabolic biomarker (157). A plasma metabolomics study of non-diabetic patients with NAFLD and NASH, found significantly decreased homogluthathione and glutamyl-dipeptide, with distinctive fluctuations in citrulline, ornithine, arginine and other urea cycle substrates

Table I. Approved and investigational drugs targeting the urea cycle.

Authors, year	Drug	Mechanism/target	Drug type	Development stage	Indications	(Refs.)
Sugiyama <i>et al</i> , 2020	Carglumic acid (Carbaglu)	CPS1 activator (N-carbamoyl- L-glutamate)	Small molecule	Approved	NAGS deficiency; organic acidemia- related hyperammonemia	(166)
Butterworth <i>et al</i> , 2019 and Conte <i>et al</i> , 2022	L-ornithine-L- aspartate (LOLA)	Supplement ornithine/aspartate to enhance urea cycle	Small molecule/ amino-acid complex	Clinical (Phase II/III; approved in multiple countries)	Hepatic encephalopathy; NAFLD/NASH (improvement in liver enzymes, triglycerides and liver/spleen CT ratio)	(163,167)
Naing <i>et al</i> , 2024	CB-1158 (INCB001158)	Arginase 1 inhibitor	Small molecule	Phase I/II clinical	Metastatic solid tumors (tumor- microenvironment immunomodulation)	(168)
Fenwick <i>et al</i> , 2024	BCT-100	PEGylated recombinant human Arginase 1 (arginase)	Biologic (enzyme- replacement therapy)	Phase I/II clinical	Arginine-dependent adult and pediatric tumors	(169)
Russo <i>et al</i> , 2024	Pegzilarginase	PEGylated human Arginase 1	Biologic (enzyme- replacement therapy)	Phase III clinical	Arginase 1 deficiency (ARG1-D)	(170)

CPS1, carbamoyl phosphate synthetase 1; NAGS, N-acetyl-L-glutamate synthase; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; CT, computed tomography.

and derivatives, along with widespread dysregulation of branched-chain amino acids, glutamate and pyruvate. These metabolic fingerprints reflect global amino acid metabolic imbalance and tight linkage to disease progression, providing data to support multi-marker diagnostic models (158).

Genetic testing. At the genetic level, studies of polymorphisms provide biomarkers for NAFLD/NASH susceptibility and risk stratification. Large-scale association and clinical phenotype analyses have identified that the A allele of the CPS1 gene SNP rs1047891 confers partial protection against diet-induced urea cycle impairment and is associated with a significantly reduced risk of NAFLD progression, indicating this SNP as a genetic marker for early risk screening (31).

Potential therapeutic strategies targeting the urea cycle. In hepatocytes, the urea cycle is the principal pathway for detoxifying ammonia (8,31); efficient arginine-citrulline cycling not only maintains nitrogen homeostasis but also promotes mitochondrial biogenesis and fatty-acid oxidation through nanomolar nitric-oxide production (159,160). In late-stage disease or under high-fat/high-glucose conditions,

downregulation of key urea-cycle enzymes and substrate depletion trigger hyper-ammonemia and energy-metabolic stress (8,31). By contrast, activated HSCs and M2 macrophages preferentially channel arginine into ornithine and polyamine synthesis to fuel proliferation, extracellular-matrix production and fibrogenesis (60,75), whereas pro-inflammatory M1 macrophages, via iNOS, generate excessive NO and peroxynitrite, amplifying oxidative and inflammatory injury (161,162).

Given this cell- and stage-specific metabolic reprogramming, the urea and NO cycles emerge as regulatable therapeutic nodes. Maintaining adequate substrate levels and balanced enzyme activity in hepatocytes, while continuously tracking blood ammonia, urea and NO derivatives, can restore or enhance physiological urea synthesis and beneficial NO signaling, yet prevent excessive substrate diversion into pro-fibrotic or pro-inflammatory pathways (163,164).

Accordingly, successful NASH therapies must precisely modulate arginine availability and urea-cycle homeostasis, demanding high target specificity plus ongoing monitoring of hepatic function, nitrogen metabolism and fibrosis markers, with dose adjustments based on efficacy and tolerance. As metabolic wiring varies across cell types and disease stages,

Table II. Approved and investigational drugs targeting the NO-sGC-cGMP signaling pathway.

Authors, year	Drug	Mechanism/target	Drug type	Development stage	Indications	(Refs.)
Lian <i>et al</i> , 2017	Riociguat (Adempas)	sGC stimulator	Small molecule	Approved	PAH; CTEPH	(171)
Trujillo <i>et al</i> , 2023	Vericiguat (Verquvo)	sGC stimulator	Small molecule	Approved	HFrEF; reduces risk of heart-failure-related death and hospitalization	(172)
Hanrahan <i>et al</i> , 2020 and Schwartzkopf <i>et al</i> , 2022	Praliciguat (IW-1973)	sGC stimulator	Small molecule	Phase II clinical	Type 2 diabetes with hypertension; diabetic kidney disease and other metabolic disorders	(173,174)
Hingorany <i>et al</i> , 2011	Cinaciguat (BAY 58-2667)	sGC activator	Small molecule	Phase II clinical	Acute decompensated heart failure; symptomatic heart failure	(175)
Gawrys <i>et al</i> , 2025	Mosliciguat (BAY 1237592)	sGC activator	Small molecule	Clinical (inhaled)	Pulmonary arterial hypertension (inhalation therapy)	(176)
Sawabe <i>et al</i> , 2019	TY-55002	Short-acting sGC activator	Small molecule	Preclinical	Acute decompensated heart failure	(177)
Bhogal <i>et al</i> , 2019	Sildenafil (Revatio)	PDE5 inhibitor (enhances NO-cGMP signaling)	Small molecule	Approved	PAH	(178)
Rosenzweig <i>et al</i> , 2010	Tadalafil (Adcirca/Cialis)	PDE5 inhibitor (enhances NO-cGMP signaling)	Small molecule	Approved	PAH; ED; BPH	(179)

NO, nitric oxide; sGC, guanylate cyclase; cGMP, cyclic GMP; PAH, pulmonary arterial hypertension; CTEPH, chronic thromboembolic pulmonary hypertension; HFrEF, heart failure with reduced ejection fraction; ED, erectile dysfunction; BPH, benign prostatic hyperplasia.

these pathways remain versatile drug targets: Dynamically optimizing arginine or citrulline supplementation, with real-time read-outs of blood ammonia, urea and NO derivatives, can enhance ammonia detoxification and hepatoprotective NO signaling while limiting flux into polyamine or excess NO production (163,164). Finally, integrating single-cell transcriptomics and epigenomics allows patient stratification through deep molecular profiling (165), whereas multimodal serum and metabolomics monitoring throughout treatment helps gauge hepatic architecture, fibrosis progression and metabolic performance, achieving a balanced profile of efficacy and safety.

Although multiple drugs targeting the urea (Table I) (163,166-170) and NO cycles (Table II) (168-176) have been approved or are in clinical trials for other diseases, their application in NASH/NAFLD remains to be further validated and refined for targeted therapy.

In children with CPS1 deficiency, carnitine acid administered in combination with other ammonia-lowering measures can maintain relatively low blood ammonia and glutamine levels and improve tolerance to dietary protein intake,

suggesting that supplementation with a cofactor mimetic related to CPS1 activation may enhance urea synthesis in inherited urea cycle disorders with residual enzymatic activity (166). By contrast, L-ornithine-L-aspartate does not directly activate a specific rate-limiting enzyme, but primarily supports ammonia detoxification and related metabolic conversion by providing ornithine and aspartate. Available clinical summaries indicate that oral L-ornithine-L-aspartate in patients with NAFLD/NASH is associated with improvements in liver enzymes, triglycerides and imaging parameters. As well as ammonia reduction, its potential benefits may also involve increased synthesis of glutamine, arginine and glutathione, as well as antioxidant, anti-inflammatory and microcirculatory effects (163,177). INCB001158 has shown measurable pharmacodynamic activity and an overall acceptable tolerability profile in advanced or metastatic solid tumors; however, its clinical responses have not clearly exceeded the background response rates of the respective tumor types, suggesting that increasing arginine availability alone may be insufficient to produce durable clinical benefit (168). Unlike ARG1 inhibition, BCT-100 and pegzilarginase represent strategies based on

systemic arginine depletion and enzyme replacement therapy, respectively. BCT-100 has achieved sustained depletion of circulating arginine in children and adolescents with relapsed or refractory tumors, supporting the clinical feasibility of systemic metabolic remodeling mediated by exogenous arginase (169). Pegzilarginase, in turn, has demonstrated efficacy in reducing plasma arginine levels and improving functional outcomes in patients with ARG1 deficiency, indicating that enzyme replacement therapy can confer benefit through correction of circulating metabolic abnormalities (170).

In NAFLD and NASH, dysregulated arginine metabolism is reflected not only by altered utilization of urea cycle substrates and impaired ammonia clearance, but also by redistribution toward the NO-producing branch. Accordingly, the NO-sGC-cGMP pathway may be regarded as a druggable downstream node of the arginine metabolic network. Riociguat and vericiguat are both sGC stimulators that directly stimulate soluble guanylate cyclase and enhance its responsiveness to endogenous NO, thereby increasing cGMP production; they have been approved for diseases such as pulmonary arterial hypertension, chronic thromboembolic pulmonary hypertension and heart failure with reduced ejection fraction (171,172). Praliciguat is likewise an sGC stimulator and has entered clinical development for metabolic disorders. It has been evaluated in a randomized placebo-controlled trial in patients with type 2 diabetes and hypertension and has also shown metabolic effects in diet-induced obese mice, supporting its translational relevance at the interface between arginine-NO signaling and metabolic homeostasis (173,174). By contrast, cinaciguat, mosliciguat and TY-55002 are sGC activators. Rather than enhancing the coupling between endogenous NO and native sGC, these agents activate oxidized or heme-free forms of sGC. At present, cinaciguat has mainly been investigated in acute decompensated heart failure, mosliciguat is under clinical development as an inhaled agent and TY-55002 remains at the preclinical stage. Collectively, these agents suggest that even when arginine flux is diverted toward the NO branch, downstream signaling can still be pharmacologically activated without directly supplementing NO itself (175-177). Sildenafil and tadalafil represent another level of intervention. As PDE5 inhibitors, they prolong the duration of existing NO-cGMP signaling by inhibiting cGMP degradation. Their pharmacological significance therefore lies in preserving the second-messenger effects already generated by the arginine-NO branch, rather than directly promoting the upstream urea cycle or arginine resynthesis (178,179).

8. Conclusion and future perspectives

NAFLD, particularly its advanced form, NASH, has emerged as a leading cause of chronic liver disease, significantly increasing the global burden of metabolic disorders (80). This comprehensive review highlighted the pivotal role of the urea cycle and arginine metabolism dysregulation in the pathogenesis and progression of NAFLD/NASH. Liver hepatocytes exhibit a profound impairment in key urea cycle enzymes, including CPS1, OTC, ASS1 and ASL (30). This enzymatic downregulation is closely associated with epigenetic modifications such as DNA hypermethylation and histone modifications, which result in decreased urea synthesis and

elevated intrahepatic ammonia levels (22). The accumulation of ammonia not only exacerbates hepatic injury by disrupting mitochondrial oxidative phosphorylation and inducing oxidative stress but also promotes HSC activation and fibrosis progression (81).

Importantly, the arginine-NO axis emerges as a critical metabolic switch that governs hepatic metabolic homeostasis (7). In early stages, physiological levels of NO generated by eNOS enhance mitochondrial biogenesis, oxidative phosphorylation and fatty acid oxidation, providing a protective metabolic phenotype (82). However, sustained inflammation in NASH results in increased iNOS expression in Kupffer cells and hepatic infiltrating monocyte-derived macrophages, elevating NO and reactive nitrogen species production to pathological levels (134). This pathologic NO elevation leads to extensive mitochondrial damage, metabolic dysfunction and intensified hepatic inflammation and apoptosis (85). Moreover, activated HSCs reprogram their arginine metabolism, favoring polyamine synthesis pathways via arginase, which drives fibrogenesis through enhanced collagen deposition and extracellular matrix remodeling (60). Concurrently, the interplay between macrophages and HSCs modulates local metabolic fluxes, exacerbating hepatic fibrosis through intricate feedback loops involving NO and polyamine metabolism (102).

A clinical study highlights the potential of urea cycle metabolites and arginine derivatives as biomarkers for diagnosing and monitoring NAFLD/NASH, particularly blood ammonia levels, plasma ADMA and CPS1 genetic variants, which correlate with hepatic dysfunction and cardiovascular risk (156). Advances in single-cell transcriptomics and metabolomics have further clarified hepatic cellular heterogeneity, emphasizing the therapeutic value of precisely targeting distinct metabolic pathways (153). Thus, strategies aiming to restore urea cycle functionality and rebalance arginine-NO metabolism (such as supplementation with citrulline or arginine, modulating enzyme activities through epigenetic and post-translational regulation and selective inhibition of harmful pathways like iNOS-driven NO overproduction or excessive polyamine synthesis) represent promising therapeutic approaches (164). Successful implementation of these therapies necessitates rigorous clinical trials, detailed biochemical monitoring and molecular profiling to achieve optimal efficacy and personalized intervention, potentially revolutionizing NAFLD/NASH management by mitigating liver injury, fibrosis progression and systemic metabolic dysfunction.

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Competing interests

The authors declare that they have no competing interests.

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