Endothelial Dysfunction in Non-Alcoholic Fatty Liver Disease

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ABSTRACT | Non-alcoholic fatty liver disease (NAFLD) is a potent hepatopathy. The metabolic syndrome combined with vascular complications is the leading cause of morbidity among NAFLD patients. Endothelial dysfunction is defined as the impairment of endothelial function, which is associated with the decrease in nitric oxide (NO) production. NO is a well-known vasodilator and a modulator of vascular tone and insulin secretion. L-Arginine is converted to nitric oxide and citrulline by the action of nitric oxide synthases (NOS). Asymmetric dimethylarginine (ADMA) is an endogenous inhibitor of all three isoforms of nitric oxide synthases (NOS) and is degraded by dimethylarginine diamino hydrolase (DDAH) in the liver. The decrease in DDAH activity results in accumulation of ADMA and reduction in NO bioavailability, subsequently leading to endothelial dysfunction in NAFLD. This review provides an overview of endothelial dysfunction in NAFLD and possible therapies for this common disorder.

KEYWORDS | Asymmetric dimethylarginine; Dimethylarginine diamino hydrolase; Endothelial dysfunction; Nitric oxide; Non-alcoholic fatty liver disease

ABBREVIATIONS | ADMA, asymmetric dimethylarginine; ATP, adenosine triphosphate; CCL, chemokine ligand; cGMP, cyclic guanosine monophosphate; DDAH, dimethylarginine diamino hydrolase; eNOS, endothelial nitric oxide synthase; ET-1, endothelin; FMD, flow-mediated dilation; FXR, farnesoid X receptor; HCC, hepatocellular carcinoma; HFD, high fat diet; 1H-MRS, proton magnetic resonance spectroscopy; HSC, hepatic stellate cells; IL, interleukin; iNOS, inducible nitric oxide synthase; KLF2, Kruppel-like factor 2; LSECs, liver sinusoidal endothelial cells; MCP, monocyte chemoattractant protein; MMA, monomethyl L-arginine; mtDNA, mitochondrial DNA; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; NOS, nitric oxide synthase; PPAR, peroxisome proliferator activated receptor; PRMT, protein arginine methyltransferase; ROS, reactive oxygen species; SDMA, symmetric dimethylarginine; TAG, triacylglycerols; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor; VLDL, very low-density lipoprotein

REVIEW ARTICLES

CONTENTS

1. INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is one of the emerging chronic liver diseases. Even though the awareness of NAFLD has increased over the years, it remains poorly diagnosed due to lack of knowledge, as NAFLD is not considered as a clinically important diagnosis [1]. The prevalence of NAFLD is high among people with obesity and diabetes. It is characterized by deposition of fat in the hepatocytes and persistent abnormalities in hepatic marker enzymes in the absence of alcohol consumption [2, 3]. Continuum of NAFLD ranges from simple steatosis to steatohepatitis, fibrosis, and cirrhosis, and is often associated with type 2 diabetes, insulin resistance, obesity, and hyperlipidemia. The hallmark feature of NAFLD is primarily the accumulation of triacylglycerols (TAG) in the liver and adipose tissue. NAFLD is closely associated with metabolic syndrome and is considered as a risk factor for the development of cardiovascular diseases with the excess of intracellular fatty acids, oxidative stress, energy depletion, and mitochondrial dysfunction causing cellular injury [4]. Major complications of patients with metabolic syndrome are of vascular origin [5, 6].

2. EPIDEMIOLOGY

NAFLD affects 42% of the general adult population and 70–90% of diabetic and obese patients. It has rapidly become the most common cause of abnormal liver biochemistry in many developed and developing countries [7]. In addition, NAFLD is likely to be the major underlying etiology for liver transplantation by 2020 in Western countries [8, 9]. Histological studies in apparently healthy candidates for liver donation found that the prevalence of NAFLD was 12–18% in Europe [10] and 27–38% in the United States (US) [11, 12]. This high prevalence of NAFLD is consistent with studies in unselected populations that used proton magnetic resonance spectroscopy (1H-MRS), in which ~31% of 2,349 US adults (45% of Hispanic individuals, 33% of white, and 24% of black people) in the Dallas Heart Study were found to have NAFLD as defined by an intrahepatic triglyceride content >5.56% [13, 14]. There is an increase in the incidence of diabetes, obesity, and insulin resistance in India over the last two decades. The Indian Council of Medical Research (ICMR) has estimated that up to 32% of Indian population suffers from NAFLD, from over and under nutrition, and this figure is even more among alcoholics. NAFLD in Indian population ranges from 5 to 28%, which is comparable to the West [15]. Prevalence of NAFLD in urban south Indian populations was studied in relation to different grades of glucose intolerance and metabolic syndrome [16].

3. SPECTRUM OF NAFLD

3.1. Overview

NAFLD consists of various clinicopathological entities. It begins with a condition being simple steatosis,

to steatohepatitis which can progress to cirrhosis and hepatocellular carcinoma (HCC) [17]. **Figure 1** shows the progression of HCC from simple steatosis. Simple steatosis is a benign condition when the fat deposition in the liver exceeds 5% of the liver volume without any significant inflammation or injury [18]. Steatosis gradually progresses into a more severe condition called nonalcoholic steatohepatitis (NASH) that is outlined by fat deposition along with hepatic ballooning, inflammation, and injury [19]. Pathogenesis of NASH can be multifactorial. Insulin resistance is considered to be the central event for the pathogenesis of NASH. However, increased intracellular fatty acids, adenosine triphosphate (ATP) depletion, oxidant stress, and mitochondrial dysfunction also play a major role in hepatic injury and inflammation. NASH is a severe condition which may progress further to fibrosis marked by different grades of necro-inflammatory changes such as perisinusoidal fibrosis, perisinusoidal with periportal fibrosis, bridging fibrosis, and fully developed cirrhosis [20–22]. These changes are often accompanied by distortion of hepatic vasculature [23]. Cirrhosis is often characterized by damage to the hepatocyte function, increased intrahepatic resistance (portal hypertension), and ultimately develops to HCC. HCC can also arise from a non-cirrhotic liver with the presence of metabolic syndrome that plays a key role in the progression of the disease. A growing number of cases have also demonstrated that HCC arises from NAFLD patients without the occurrence of cirrhosis [24].

3.2. Non-Alcoholic Steatohepatitis

NASH is the inflammatory form of NAFLD and is viewed as a result of "two hits hypothesis" (**Figure 1**). The first hit is the accumulation of fat and insulin resistance. Insulin resistance increases the hepatic lipogenesis and impairs the lipolysis in the adipose tissue resulting in an increase in the efflux of free fatty acids from adipose tissue to the liver. The second hit promotes the hepatic injury through oxidative stress attributed to lipid retention within the hepatocytes. This generates reactive oxygen species (ROS) at different intracellular levels with the release of cytokines [25, 26]. Oxidative stress is associated with the accumulation of lipid peroxidation products, elevation of proinflammatory cytokines, and mitochondrial dysfunction, and is often considered as a mechanism of hepatocellular injury in steatohepatitis. Patients with NASH have a deregulated cytokine production and elevated amounts of tumor necrosis factor-alpha (TNF-α) and TNF-α-inducible cytokines in serum, and as thus, TNF-α plays an important pathogenic role in NASH [27, 28]. Similar to TNF-α, interleukin 6 (IL-6) favors the progression of NASH by interfering with insulin signaling [29]. Furthermore, it has been reported that TNF-α levels are elevated in the adipose tissue of rodent models of obesity [30]. An increase in IL-1β, which is a proinflammatory cytokine, amplifies the inflammation and sensitizes the hepatocytes to TNF-α-induced liver damage as a result of the activation of inflammasome complex [31]. The serum from NAFLD and NASH patients showed the increased levels of IL-6, chemokines, such as CC-chemokine ligand 2 (CCL2), monocyte chemoattractant protein-1 (MCP-1), and CCL19 [32]. MCP-1 plays a major role in recruiting inflammatory cells and acts as a mediator for NASH. Increase in CCL2 can be directly linked to lipid accumulation in the hepatocytes by activation of peroxisome proliferator-activated receptor-α (PPAR-α) gene expression. Overexpression of CCL2 in adipose tissue can also lead to insulin resistance and increase in triglyceride accumulation [33, 34].

4. ENDOTHELIAL DYSFUNCTION

Normal vascular endothelial cells are known to regulate the blood flow and macromolecules between the tissues, prevent leukocyte activation, and also aid in immune surveillance from pathogens, thus maintaining the normal homeostasis. Any injury or cell death can disrupt these activities, resulting in their dysfunction. Under shear stress, normal endothelium produces vasodilators to control the blood pressure in blood vessels. In diseased states, this property is lost and is termed as endothelial dysfunction [35]. It is one of the key pathological processes of NAFLD that occurs during the early stages of the disease, before liver fibrosis and inflammation [6, 35, 36].

The presence of endothelial dysfunction with decreased nitric oxide (NO) production is considered to be the cornerstone for the development of NAFLD and cardiovascular diseases [6]. Endothelial dysfunction is observed in patients with NAFLD, indicated by decreased brachial artery flow-mediated dilation (FMD). A recent study has suggested an association
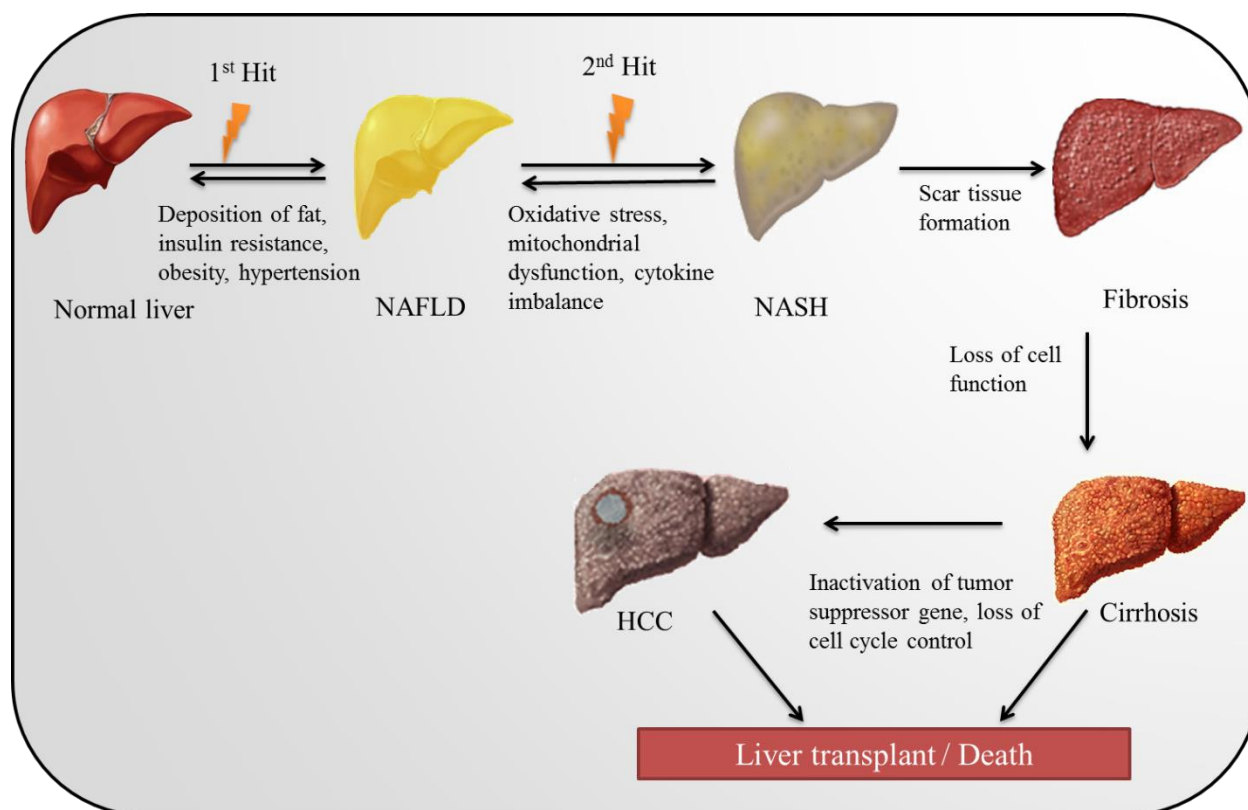
FIGURE 1. Spectrum of non-alcoholic fatty liver disease (NAFLD) and its progression to hepatocellular carcinoma (HCC). See text (Section 3) for detailed description.

between the impairment of endothelial nitric oxide synthase (eNOS) function and decrease of NO production (i.e., reduced NO bioavailability) in the progression of NAFLD [37]. Liver sinusoidal endothelial cells (LSECs) play a key role in maintaining the liver integrity and regeneration. Studies have shown that LSECs act as a "gatekeeper" and can prevent the progression of simple steatosis to NASH. LSEC injury can activate the Kupffer cells leading to various liver injuries [38, 39].

Furthermore, insulin resistance, which is the cornerstone for NAFLD, is known to exacerbate endothelial dysfunction by disrupting the balance between the NO production and secretion of endothelin-1 (ET-1). The decrease in NO production can be either due to an increase in ROS, which causes the breakdown of NO, or change in downstream signaling of cyclic guanosine monophosphate (cGMP), which is the second-messenger of NO, in vascular smooth muscle cells [40].

4.1. Role of Oxidative Stress in Endothelial Dysfunction

Oxidative stress is a pathophysiological condition that arises due to an imbalance in the reactive cellular oxygen and the body's mechanism to detoxify the reactive intermediates. Oxidative stress and redox imbalance have been shown to enhance the progression of NASH and atherosclerosis as they have a negative effect on endothelial cells [41, 42]. ROS are generated during an injury or inflammation, which at low concentrations act as signaling molecules, but in high concentrations, cause cellular injury. ROS play a critical role in the pathophysiology of several vascular diseases and disorders as they target the vascular endothelium that regulates the passage of macromolecules and circulating cells from blood to tissues and promote leukocyte adhesion leading to impairment in endothelial barrier function [43]. In addition, rats fed with oxidized edible oil have an in-

creased lipid peroxidation compared to non-oxidized edible oil [44]. Induction of lipid peroxidation also leads to inflammation and fibrogenesis, through the activation of hepatic stellate cells (HSC). ROS have also been shown to inhibit the secretion of very low-density lipoprotein (VLDL), which in turn increases the fat accumulation [42]. Lipid peroxidation alters the function of mitochondrial DNA (mtDNA) to inhibit the electron transfer along the respiratory chain which further increases the ROS production. This causes a propagating cycle of oxidative stress and accumulation of ROS [45, 46]. Zhu and Fung have shown the protective role of NO against liver injury by inhibiting the lipid peroxidation [47]. NO helps in maintaining vascular homeostasis, modulating vascular tone, regulating the local cell growth and protecting the vessel from injury, and maintaining the endothelial permeability [48].

4.2. Role of Nitric Oxide Synthases

NO maintains homeostasis in liver vasculature by increasing cGMP production via the activation of soluble guanylyl cyclase, which thereby catalyzes the conversion of GMP to cGMP. The NO-cGMP downstream signaling leads to endothelial processes like vasodilation and inhibition of platelet aggregation [49]. In LSEC, shear stress-induced NO release is mediated by the activation of Kruppel like factor 2 (KLF2) and eNOS expression [50]. In NAFLD, fat accumulation causes blockage of fenestrae in LSECs leading to decrease NO bioavailability [35, 51] and paving the way for portal hypertension. NO production is also affected by insulin resistance, that affects the eNOS-NO signaling pathway and promotes vasoconstriction and vascular damage [37, 52].

NOS catalyzes the conversion of L-arginine to nitric oxide and L-citrulline. Three isoforms of NOS are known to exist: eNOS, inducible NOS (iNOS), and neuronal NOS (nNOS). eNOS is constitutively expressed and functions to regulate blood flow and maintain vascular homeostasis [53]. On the other hand, iNOS expression is initiated by immunological stimulation; it causes a prolonged release of NO thereby contributing to the inflammatory state in liver diseases [54, 55]. iNOS is found to be upregulated in diseased conditions. However, in fibrosis and NASH-related fibrosis animal models, iNOS has been shown to have both positive and negative roles [55–59].

In NAFLD, it is widely speculated that reduced sinusoidal NO production is caused by eNOS dysfunction. Chronic eNOS inhibition in obese rat models led to hepatic steatosis, indicating the role of eNOS in NAFLD progression [60]. In an NAFLD mouse model, reduction in eNOS levels contributed to NASH progression [61]. Furthermore, high-fat diet (HFD) fed eNOS$^{-/-}$ mice showed signs of NASH, suggesting the involvement of eNOS in NAFLD pathogenesis [62, 63]. A recent study conducted in NAFLD patients also showed that the impaired vascular function occurs due to reduced eNOS activity [37].

4.3. Asymmetric Dimethylarginine and Dimethylarginine Diamino Hydrolase Pathway

Asymmetric dimethylarginine (ADMA) is one of the important molecules behind eNOS dysfunction and belongs to the family of methylarginines (**Figure 2**). Methylarginines are formed by the proteolytic action of protein-arginine methyl transferases (PRMTs) on methylated proteins [64]. Other known methylarginines are symmetric dimethylarginine (SDMA) and monomethyl-L-arginine (MMA). Both ADMA and MMA are guanidino-substituted analogues of arginine, which inhibit eNOS by competing with L-arginine for the active site of the enzyme. Since MMA is present in low levels in mammals, there is not much information available except for its function. SDMA, on the other hand, is an inactive stereoisomer of ADMA, which interferes with NO generation by competing with the other methylarginines for cationic amino acid transporters [65].

In normal physiological conditions, intracellular NOS is saturated with substrate L-arginine and the reaction favors towards NO production. However, in pathological conditions, ADMA levels increase and the reaction equilibrium favors towards NO inhibition. Therefore, the state of eNOS activation is dependent on the proportion of ADMA to L-arginine [65]. Many studies have reported an increase in ADMA levels in NAFLD patients. This increase in ADMA levels corresponded to insulin resistance and was independent of disease markers (stage of fibrosis, grade of steatosis) and inflammation [66–68]. Intracellular ADMA is metabolized in the liver by dimethylarginine diaminohydrolase (DDAH) 1 and 2 to citrulline and dimethylamine, or transported to the plasma by the cationic amino acid transporters [69].
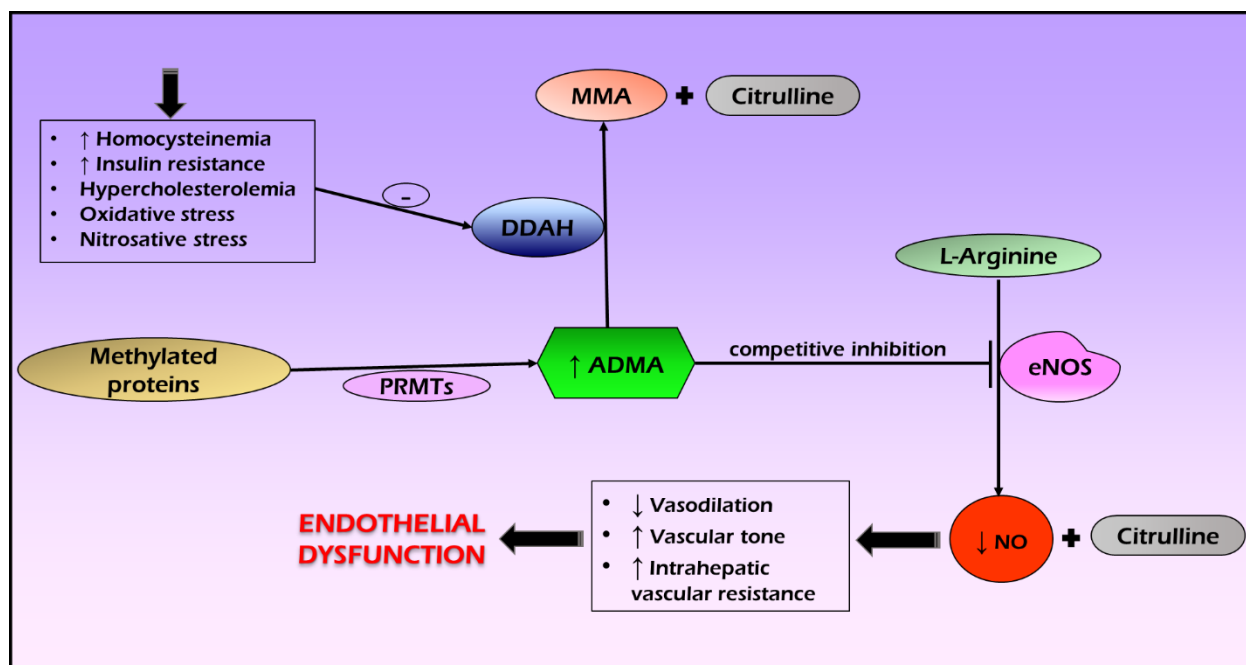
FIGURE 2. Mechanisms of endothelial dysfunction in non-alcoholic fatty liver disease (NAFLD). See text (Section 4) for detailed description.

DDAH1 and DDAH2 are normally found in tissues expressing nNOS and eNOS, respectively. Overexpression of DDAH1 increases NO production by reducing ADMA levels [70].

DDAH contains a sulfuryl group, which is vulnerable to oxidative stress. Therefore, in pathological conditions, reduced DDAH activity is observed [71]. This leads to accumulation of ADMA which interferes with the L-arginine/NO pathway, subsequently leading to decreased levels of NO and endothelial dysfunction [72, 73] (**Figure 2**). Overexpression of DDAH1 in human endothelial cells showed a 3-fold modest increase in NO concentration [74]. DDAH1 is one of the target genes of farnesoid X receptor (FXR) and treatment with an FXR agonist in cirrhotic rats restored NO levels [75]. This strongly suggests that DDAH1 could be a potential drug target for treating endothelial dysfunction in NAFLD. HFD-fed DDAH1-knockout mice displayed liver inflammation, hepatic steatosis, and higher NAFLD activity score compared to HFD fed wild type mice. Glucose tolerance and insulin sensitivity were also impaired in HFD-fed transgenic mice than their wild-type counterparts, indicating a relation between obe-sity-related genes and DDAH1 in the pathogenesis of NAFLD [76].

4.4. Vascular Endothelial Growth Factor (VEGF)

VEGF is a proangiogenic signaling molecule that promotes the proliferation of endothelial cells and growth of blood vessels. VEGF, secreted by hepatocytes and HSCs, stimulates the release of NO from LSECs, thereby mediating the expression of NO [49]. It is also thought to play a role in the pathogenesis of NAFLD [77]. In animal models of NASH, VEGF was increased during the transition of simple steatosis to NASH. Treatment with an inhibitor of vascular endothelial growth factor receptor (VEGFR) A prevented the progression of simple steatosis to NASH by attenuating inflammation and fibrosis [78]. However, results were conflicting in the clinical setting. Serum VEGF levels were reported to be significantly higher in simple steatosis and NASH patients when compared to healthy controls [79, 80]. In contrast, other studies have reported no change in VEGF levels [81] or even decreased VEGF levels in NASH patients [82].

4.5. Endothelin-1

Endothelin-1 is a potent vasoconstrictor that exerts its effects by binding to receptors endothelin A or B, present in endothelial cells and HSCs. In an NAFLD mouse model, increase in liver and serum ET-1 levels was observed. Increased ET-1 levels may be behind the intrahepatic resistance in severe steatosis [83]. Serum ET-1 levels were found to be increased in NASH patients compared to steatosis patients, and were shown to correspond to the severity of fibrosis in NASH patients [84]. The use of ET-1 antagonists in an NASH mouse model attenuated the progression of fibrosis, indicating their therapeutic potential [85].

5. THERAPEUTIC APPROACHES TO PREVENTING ENDOTHELIAL DYSFUNCTION IN NAFLD

Treatment of NAFLD falls into two main categories: treating steatosis or intervening the progression of disease. Treatment options for NAFLD are continuously being researched with many clinical trials underway. Currently, many types of drugs like antioxidants, insulin sensitizers, weight loss medications, lipid lowering drugs, and nuclear receptor agonists are under investigation [86]. Insulin sensitizers such as biguanides (e.g., metformin) and glitazones

(e.g., pioglitazone and rosiglitazone) have positive effects on biochemical parameters. Metformin activates adenosine monophosphate activated protein kinase, a master regulator of glucose and lipid metabolism while glitazones increase insulin sensitivity by decreasing insulin resistance via activation of peroxisome proliferator-activated receptor-gamma (PPAR-γ) [87, 88]. Antioxidants and cytoprotective agents treat NAFLD by disturbing the lipid peroxidation reaction and stabilizing the phospholipid membrane [89]. Foster et al. demonstrated the role of atorvastatin, vitamin E, and vitamin C in treating NAFLD. Their study demonstrated that 20 mg of atorvastatin combined with vitamins E and C reduced the progression of NAFLD to NASH by 71% [90]. Use of natural compounds, such as polyphenols, seems to lower the lipid content in the liver by regulating the expression of gene involved in de novo lipogenesis and fatty acid oxidation. Green tea is a rich source of polyphenolic catechins with potential antioxidant, anti-inflammatory, antifibrogenic, and anti-lipogenic properties that can prevent NAFLD progression [91, 92]. Physical activity and life style interventions induce weight loss and reduce the severity of NAFLD. Decrease in sucrose and fructose, which are rich in soft drinks, can also help in the reduction of insulin resistance and lipogenesis [93]. Some of the available drugs involved in improving endothelial dysfunction are summarized in **Table 1**.

TABLE 1. Therapeutic agents attenuating endothelial dysfunction in NAFLD		
Therapeutic agent	**Decrease in endothelial dysfunction**	**Ref.**
Resveratol	Promotes antioxidant activities and inhibits TNF-α	[94, 95]
Sildenafil-leucine-metformin	Suppresses oxidative stress; activates eNOS leading to NO/cGMP signaling	[96]
GW4064	Increases DDAH1 and L-arginine levels; reduces ADMA accumulation	[97, 98]
V-PYRRO/NO	Increases NO production	[99]
Nitro-aspirin	Reduces iNOS and cyclooxygenase-2 expression	[100]
Simvastatin	Increases eNOS expression; decreases iNOS; inhibits HSC activation	[101]
Arginine	Reduces oxidative stress; decreases TNF-α; restores eNOS and antioxidant enzymes	[102]
Vitamin B6	Prevents endothelial dysfunction and hepatic lipid accumulation	[103]
Vitamin E	Reduces oxidative stress and HSC activation	[104, 105]
Vitamin C	Reduce oxidative stress	[106, 107]
Omega 3 fatty acids	Promotes anti-inflammatory effects; reduces TNF-α	[108]
Exercise	Improves endothelial dysfunction by increasing NO generation	[109, 110]
Note: GW4064 is an agonist of farnesoid X receptor; V-PYRRO/NO is a liver-selective NO donor.		

6. CONCLUSION

NAFLD is a metabolic disease that largely affects obese and diabetic patients, resulting in multi-organ complications. In this review, we have described the involvement of NO and its modulators in the pathogenesis of NAFLD. Endothelial dysfunction is an early event, and therefore, NO regulators like DDAH can be considered as a potential target for biomarker and therapeutic research. Treating NAFLD with NO modulators might also prevent its progression to cirrhosis and HCC. Receptors, which target DDAH, such as FXR may be used to increase NO bioavailability and possibly reverse the disease condition. However, further research must be done to understand DDAH and exploit its therapeutic potential in NAFLD.

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