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Review Article

Non-alcoholic fatty liver disease: Genetic susceptibility

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Abstract

The prevalence of Non-alcoholic Fatty Liver Disease (NAFLD) is increasing, particularly in individuals who consume minimal to no alcohol. Currently, NAFLD affects at least 32% of the global population. Although not all but approximately 5-10% cases of NAFLD progress to non-alcoholic steatohepatitis (NASH), a condition arising from lipotoxicity. Patients with NASH may further develop liver fibrosis, cirrhosis, and hepatocellular carcinoma (HCC). However, it is important to note that progression from NAFLD to NASH and HCC is not inevitable for all patients. The pathogenesis of HCC in the context of NAFLD and NASH remains poorly understood, however, fat accumulation, often due to obesity or metabolic syndrome, leads to lipotoxicity. In individuals with type 2 diabetes (T2D), elevated insulin levels promote lipolysis in adipose tissue and activate lipid-synthesizing enzymes such as fatty acid synthase (FAS) and stearoyl-CoA desaturase 1 (SCD1), which contribute to lipid accumulation in the liver. Additionally, high glucose levels in T2D can alter gene regulation by activating carbohydrate response-element binding protein (ChREBP) and insulin can increase the activity of sterol regulatory element binding protein (SREBP-1c). These lipogenic transcription factors are known to directly or indirectly activate patatin-like phospholipase domain-containing protein 3 (PNPLA3). Recent research has found elevated levels of angiotensinogen (AGT) and des-angiotensin in both experimental animals and patients with NAFLD. Emerging evidence highlights the significance of genetic factors in the risk of developing NAFLD. Here we explore the association between specific genes and hepatosteatosis, detailing the roles of T2D and obesity in the progression from NAFLD to NASH, and involvement of hepatic stellate cells in liver fibrosis. Additionally, we examine the impact of genes such as PNPLA3, MBOAT7, TM6SF2, GKRP, CCN3, and AGT as well as role of single nucleotide polymorphisms in gene regulation and their contribution to the risk of NAFLD.

Keywords: Hepatosteatosis; Non-alcoholic fatty liver disease; Non-alcoholic steatohepatitis; Obesity; Type 2 diabetes mellitus

Introduction

Non-Alcoholic Fatty Liver Disease (NAFLD) is a widespread condition affecting an estimated 32.4% of the global population and playing a significant role in the onset of liver cirrhosis and hepatocellular carcinoma (HCC). Liver steatosis, or the accumulation of fat in the liver, may not invariably lead to inflammation, known as Non-Alcoholic Steatohepatitis (NASH). However, the coexistence of steatosis and inflammation can hasten the progression to liver fibrosis, more so than in NAFLD cases without inflammation. NAFLD is often linked with metabolic syndrome and Type 2 Diabetes (T2D), with one manifestation of metabolic syndrome presenting as NAFLD itself [1]. Consequently, NAFLD has been reclassified as metabolic-dysfunction associated fatty liver disease (MAFLD) and more recently as steatotic liver disease (SLD). Metabolic syndrome encompasses a range of interconnected clinical features, including insulin resistance, elevated fasting blood sugar, abnormal cholesterol levels, central obesity, and high blood pressure [2,3]. NASH is characterized by the infiltration of fat in the liver, particularly in hepatocytes, where inflammation levels increase due to macrophage activation, in the absence of significant alcohol consumption [2].

Multiple cellular interactions among liver cells, such as hepatocytes, hepatic stellate cells (HSCs), and hepatic macrophage, the Kupffer cells (KCs) occur during the development of NAFLD [4,5]. HSCs become activated by exosomes released from hepatocytes under stress from lipotoxicity, which carry various signaling molecules crucial for intercellular communication. This activation is pivotal in liver pathology leading to fibrosis by producing excessive extracellular matrix components and accelerating liver disease progression [6]. Furthermore, dysbiosis, or the imbalance of the gut microbiome, leads to increased intestinal barrier permeability allowing more bacterial endotoxins into the bloodstream. These toxins, transported to the liver via the portal vein, contribute to various health issues including liver inflammation and damage. High-fat diets, obesity, and NAFLD are associated with intestinal dysbiosis [7]. In the liver, endotoxins activate KCs, promoting the release of a myriad of pro-inflammatory cytokines, e.g., tumor necrosis factor alfa (TNFα), interleukins (IL-1, IL-6), contributing to the fibrotic transformation of the liver following hepatocyte death [8]. Further, transforming growth factor-β, which is a profibrotic mediator, activates HSCs, which in turn might contribute to the fibrotic transformation of the liver after hepatocyte death [8]. Notably, both alcoholic and non-alcoholic forms of steatohepatitis can lead to significant liver damage, highlighting the liver's remarkable regenerative ability to heal itself through a HSC-mediated repair process [9,10]. Besides, HSCs play a critical role in the development of liver fibrosis by secreting fibrin, by which a life-threatening disease, known as liver cirrhosis, might develop [11].

NAFLD is particularly common in industrialized countries, affecting 20-40% of the population. Within this group, 10-20% may develop NASH, a more severe condition often associated with metabolic syndrome [12]. The prevalence of NAFLD is alarmingly high among individuals with morbid obesity or diabetes, reaching up to 90% and 70% respectively [13,14].

Complex interactions among liver cells (hepatocytes, HSCs, and KCs) driven by overnutrition, can trigger pro-inflammatory reactions, initiating a cascade of events including inflammasome activation, hepatocyte death, and tissue regeneration, ultimately contributing to fibrogenesis [15,16]. The progression from NAFLD to NASH involves a complex interplay of various factors, including insulin resistance, and initiation of complex inflammatory reactions. Often increased levels of insulin are found among T2D individuals while the values of insulin could be lower in individuals with increased insulin clearance [17]. The transition from NAFLD to NASH involves multiple factors including insulin resistance and hypoadiponectinemia, which together with inflammation and visceral adiposity, create a pro-inflammatory state that facilitates liver damage and fibrosis. This multifactorial environment highlights the need for addressing these underlying factors for effective management and treatment of the disease. The progression of NAFLD and NASH is further illustrated by changes in gut microbiota, leading to increased TNFα and toll-like receptors (TLR4 and TLR9) agonists in the portal circulation [18,19]. **Figure 1** shows the key mechanisms involved in the initiation and progression of NAFLD.

Insulin and Activation of Transcription Factors

Insulin interacts with the insulin receptor, a specific type of receptor known as a receptor tyrosine kinase, initiating a complex signaling cascade within cells. This interaction triggers a series of downstream effects primarily through the activation of G-protein coupled receptors. These receptors then facilitate the activation of critical signaling pathways, including the Raf serine/threonine kinases (Raf), which activates the MAPK/ERK kinase (MEK) followed by the extracellular signal-regulated kinase (ERK), (Raf/MEK/ERK), and the PI3K (phosphoinositide 3-kinase)/protein kinase B (also known as Akt), as well as phospholipase C gamma (PLCγ) pathways. These pathways are instrumental in mediating various cellular responses,

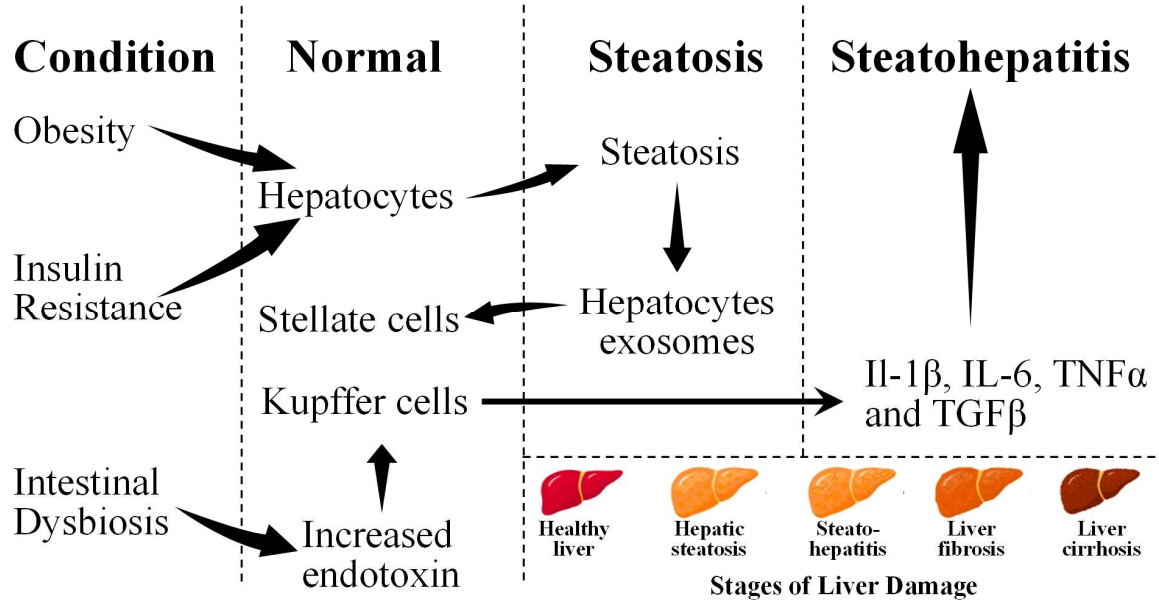

Figure 1. Key mechanisms implicated in the initiation and progression of non-alcoholic fatty liver disease (NAFLD). Adopted from one of our earlier publications on this series [197].

including metabolism, growth, and survival of the target cells [20]. Insulin receptor induces biosynthesis of early growth response protein 1 (Egr-1) transcription factor [21] which is activated by ETS Like-1 protein (Elk-1) [22]. In addition to pancreatic β-Islets of Langerhans, Egr-1 activation also occurs in hepatocytes and lack of Egr-1 delays the hepatocyte mitotic progression [23]. Additionally, Elk-1 regulates activator protein-1 (AP-1) transcription factor [24]. Activation of Elk-1, Egr-1, and AP-1 are important in glucose homeostasis [25-27]. Egr-2 also called as Krox20, an Elk-1 related transcription factor is also activated in adipocytes, thus insulin plays an important role in the regulation of adipogenesis [28].

Insulin-mediated increased transcription of genes from upstream stimulatory factors (USF-1 and USF-2) have been observed [29]. USF binds to E-box (5'-CANNTG-3') and can synergize with sterol regulatory element binding protein (SREBP-1c) resulting in the activation of lipogenic genes [30]. USF regulates gene transcription of fatty acid synthase (FAS). FAS converts acetyl-CoA and malonyl-CoA into palmitic acid. The FAS gene contains two E-boxes, one sterol-response element (SRE), and the liver X receptor response element (LXRE) in the proximal promoter [31]. Additional genes integral to lipogenesis, including acetyl-CoA carboxylase, ATP-citrate lyase, and mitochondrial glycerol-3-phosphate acyltransferase, also possess E-box and SRE. These genes are modulated by insulin and various nutrients, indicating a complex regulatory network that controls fat synthesis in the body. This regulation underscores the intricate interplay between genetic factors and metabolic processes, highlighting the role of specific genetic elements in the body's response to nutritional and hormonal signals [32]. Posttranslational modification by insulin which is phosphorylation and nutrients involving acetylation play a significant role in the activation of genes of fatty acid synthesis by USF [33,34].

The SREBP produces two active forms, SREBP-1a and SREBP-1c, and is involved in the regulation of lipogenic genes. It binds to SRE (5'-ATCACCCCAC-3') or its variations. Insulin treatment causing activation of the FAS gene is mediated by SREBP-1c [35,36]. SREBP expression is regulated by LXR and glycogen synthase kinase-3 (GSK-3). c-AMP mediated phosphorylation of LXR activates SREBP-1 gene while GSK-3 phosphorylation of SREBP results in its degradation by ubiquitin ligase [37-39]. Insulin inhibits GSK-3, thus protecting SREBP-1c from its degradation, thus activating the lipogenic genes. SREBP-1c functions as a negative regulator of phosphoenolpyruvate carboxykinase (PEPCK) in the liver, the enzyme responsible for gluconeogenesis. Insulin treatment shuts down PEPCK expression, thus blocking gluconeogenesis. It occurs due to the presence of two SRE sites in the proximal promoter of PECPK [40].

SREBP-1c, which is the principal transcription factor activator, reduces fatty acid synthesis by 50%, in SREBP-1c knock-out mice indicating involvement of other transcription factor(s) [41]. The glucose influx by glucose facilitator, GLUT4 after insulin administration results into activation of carbohydrate response-element binding protein (ChREBP) which is another lipogenic transcription factor and is expressed in both hepatocytes and adipocytes [42-44]. ChREBP is a single gene and expressed by alternate promoters to produce two isoforms, ChREBPα and ChREBPβ. ChREBP heterodimerizes with Mlx and binds to 5'-CAYGNGNNNNNCNCRTG-3' [45,46]. ChREBP is retained in cytoplasm by protein 14-3-3 and breaking of the 14-3-3- from ChREBP causes ChREBP to translocate to nucleus and mediate the transcription from carbohydrate response element (ChORE) [42]. A ChORE has been identified in acetyl-CoA carboxylase, FAS, and SCD [47]. Together with SREBP-1c, ChREBP regulates the lipogenic genes, ChREBP also regulates enzymes of glycolysis and pentose phosphate pathway, thus providing essential intermediates for lipogenesis [47,48].

Insulin mediated enhanced activity of LXR has been observed in primary hepatocytes [49] and two of LXR, LXRα and LXRβ are abundantly present in adipose tissues. LXR is a member of the nuclear receptor superfamily and heterodimerizes with the 9-cis retinoic acid receptor (RAR) and binds to 5-AGGTCANNNNAGGTCA-3' to control insulin-mediated lipogenesis by activating FAS, ACA, and SCD genes [50]. Knock-down of LXRα and LXRα/β abolishes the expression of genes of the lipogenic processes [49,51]. LXR also activates SREBP and ChREBP, thus activating the process of lipogenesis [52,53].

Presently, there are no treatments for NAFLD or NASH except lifestyle changes which include weight reduction and less fat intake [54]. Raising the insulin sensitivity is another option for treatment which is done by peroxisome proliferator-activated receptor-γ (PPARγ) agonist; glitazones and SREBP agonist; pioglitazone which is an insulin sensitizer [55]. The ability of these drugs is manifested in reducing the levels of blood aminotransferases; alanine aminotransferase and aspartate aminotransferase (ALT,AST) which are markers of liver injuries and whose levels are increased in the event of liver injuries [56]. These drugs have been shown to decrease insulin resistance and resolve liver inflammation, but they are ineffective in resolving liver fibrosis and cirrhosis [57,58].

Role of Angiotensins Peptides in Progression of NAFLD/NASH to Fibrotic Liver

NAFLD and NASH patients which progress to fibrosis were thought to progress by increased levels of angiotensin-II (Ang-II). Ang-II is produced from precursor angiotensinogen (AGT) which is involved in wound healing and repair [59]. Ang-II binds to its receptor, type-I, and type-II, to mediate its effects. Ang-II type-I receptor blockers are common treatment currently in use for the management of hypertension. In animal models, type-I receptor blockers, such as losartan and telmisartan were observed to provide protective effects against the development of NAFLD and NASH [60,61]. In a recent study, these blockers were found to offer no benefits in resolving NAFLD and NASH [62]. The study revealed that no differences in NAFLD progression to fibrosis were observed among NAFLD patients but suggested that these blockers might prevent NAFLD development and its progression among certain individuals [62]. Correcting insulin resistance is necessary but not good enough to ameliorate NASH in most of the patients [63]. There is a need for a broader hepatoprotective drug that can resolve NAFLD/NASH and stop the process of liver fibrosis [64].

In a study, the Ang-II precursor, AGT has been implicated in liver steatosis and the development of NAFLD [65]. Using the selective expression of AGT in the liver, which is the source of circulating AGT, it was observed that liver AGT causes liver steatosis when the Western diet (WD: containing 22% w/w fat equivalent to 40 kcal% fat) is fed to mice [65]. Remarkably, overeating and lipolysis in fatty tissues release free fatty acids in the blood which infiltrate to liver causing inflammation [66]. Circulating AGT produces Ang-II

which activates nuclear factor-kappaB (NF-κB) transcription factor which increases the levels of TNFα which is the one of the markers of inflammation. Increasing the levels of circulating AGT will serve as the source of increased Ang-II and source of inflammation [67].

In addition to the production of Ang II from AGT, another peptide Ang1-7 is also produced via the action of angiotensin-converting enzyme 2 (ACE2) [68]. Ang1-7 offers protective effects on liver fibrosis [69]. Ang 1-7 binds to mas-receptor and protects the liver from fibrosis by promoting collagen degradation and resolution of inflammation. The mas receptor antagonist [7-D-Ala]-Ang-(1–7) (A779) increases experimental liver injury as evidenced by TGF-β1 and hydroxyproline levels [70]. Infusion of Ang 1-7 peptide attenuated liver fibrosis as evidenced by hydroxyproline and type 1 collagen content in bile-duct ligated rat model of liver fibrosis [71]. Inhibition of HSCs was also observed as the expression of alpha smooth muscle actin (α-SMA) was reduced [71]. Inhibition of ACE with Ang 1-7 is also observed while downregulation of mas receptor is also observed with Ang 1-7. Ang 1-7 treatment decreases the expression of connective tissue growth factor (CTGF, also known as CCN2) and vascular endothelial growth factor (VEGF), these are the factors involved in fibrosis. These observations indicate that Ang 1-7 can protect the liver from fibrosis [71]. Further studies with the knock-out of ACE2 in mice establish that ACE2 is involved in the regulation of liver fibrosis [72]. Despite being a vasodilator, Ang 1-7 has failed to exhibit any vasodilatory effect in either normal or cirrhotic liver [72,73]. The vasodilatory effects of Ang 1-7 occur due to the release of nitric oxide (NO) in cirrhotic liver, the NO-mediated vasodilation is compromised due to endothelial cell dysfunction [74,75]. It is evident that Ang II and Ang1-7 are two arms of the renin-angiotensin-aldosterone system (RAAS) which possess opposite actions. For too long, ACE inhibitors as classical RAAS inhibitors have been attempted in liver diseases such as fibrosis while it is known that these inhibitors also affect the alternate pathway, i.e. Ang1-7 mediated effects. Following chronic treatments with ACE inhibitors and AT1R (Ang II type 1 receptor) blockers, plasma renin and Ang I level rise [76]. Chronic ACE inhibitor treatment results into increased Ang II and/or aldosterone production that is described as Ang II reactivation or aldosterone escape [77-79]. Hypothetically, activating alternate axis (Ang 1-7) in comparison to "classical axis" (Ang II) by ACE inhibitors and angiotensin II receptor blockers (ARBs) might offers protective effects for liver fibrosis [80,81].

The HSC play a pivotal role in liver fibrogenesis [82]. Ang II activates these quiescent cells and dedifferentiates into myofibroblast. Ang II promotes the release of inflammatory cytokines and the deposition of extracellular matrix (ECM). Ang II acts through the AT1R in the liver which is predominant while AT2R is also present which is comparatively less than AT1R. Gene deletion studies in mice point out that AT1R deletion protects hepatic fibrosis while AT2R deletion exacerbates fibrosis [83,84]. Animal studies defining the role of inhibitors of ACE which converts Ang I (a decapeptide produced from precursor AGT by the action of renin) into Ang II (an N-terminal octapeptide of precursor AGT, the Ang II) which is a vasopressor molecule and participates into fibrotic process after loss of hepatocytes are controversial. Treatment with losartan, an AT1R blocker failed to offer any protection against liver injury and fibrosis in NAFLD [85]. In a different study, the administration of olmesartan in a NASH model demonstrated a remarkable 70% efficacy in mitigating fibrosis [86]. Despite its widespread adoption for hypertension management, the effectiveness of AT1R (Ang II Type 1 Receptor) blockers in curbing fibrosis remains underreported, with most evidence coming from animal studies. This discrepancy in observed benefits may stem from the prolonged nature of fibrosis development in humans, coupled with the presence of only two angiotensin receptors (AT1R and AT2R) in humans, in contrast to the four receptors (AT1Ra, AT1Rb, AT2Ra, AT2Rb) identified in mice. Furthermore, the use of losartan has shown promise in diminishing liver fibrosis mediated by chronic hepatitis C virus infection with significant improvements noted after a six-month treatment regimen in patients relative to a control group [87]. In another study involving cirrhotic compensated children, 48 weeks of candesartan treatment resulted in a significant decrease of hyaluronic acid, which is a marker of fibrogenesis [88]. With no improvement in other serum markers of fibrosis, no histological data or architectural changes were reported [88]. Patients with mild fibrosis after hepatitis C infection were treated with losartan and ursodeoxycholic acid, were found to have reduced serum markers of liver fibrosis, such as transforming growth factor β1 (TGFβ1), and type IV collagen compared to only ursodeoxycholic acid. However, fibrosis scores between losartan and ursodeoxycholic vs ursodeoxycholic were not significantly different [89]. In another research investigation, the combined application of perindopril and low-dose interferon in individuals afflicted with the hepatitis C virus was shown to be effective in diminishing the levels of serum markers associated with fibrosis. These markers include hyaluronic acid, type IV collagen 7S, and procollagen III-N-peptide. However, it is important to note that histological examinations, which could provide further insight into the tissue changes, were not conducted in this study. This limitation suggests the need for future research to comprehensively assess the therapeutic impact of this treatment combination on liver fibrosis [90]. Studies using AT1R blockers on NASH patients are lacking. In a study where hypertensive patients with defined NASH were treated with losartan (50 mg/day for 48 weeks), reduction in fibrotic markers, TGF-β1, ferritin, and serum transaminases and necroinflammation were observed among five patients out of seven treated patients [91]. In another study, improvement in necroinflammation in NASH compared to NAFLD patients was observed with losartan treatment (50 mg/day for 48 weeks). The patients with NASH have less activated HSC after 48 weeks of losartan treatment [92]. In patients with advanced cirrhosis, the plasma levels of renin, Ang II, and aldosterone are increased compared with healthy individuals [93]. Chronic use of ACE inhibitors and their inhibition may not lead to sustained decreased levels of Ang II because of hyper-reninemia and possible generation of Ang II by liver chymase during ACE inhibition [94,95].

Genetic Basis of NAFLD

PNPLA3 and hepatosteatosis

NAFLD is linked to heritable components where genetic differences between individuals developing NAFLD are estimated to be 20-70% [2]. It has been found that a single nucleotide polymorphism (SNP) in the patatin-like phospholipase domain-containing protein 3 (PNPLA3) gene plays a significant role in the development of NAFLD and this SNP of PNPLA3 is a susceptibility gene of NALFD [96-98]. PNPLA3 was cloned in 2001 from mouse 3T3 preadipocytes which were feeding-inducible genes, and hence named adiponutrin [99]. It is also known as calcium-independent phospholipase A2- epsilon (IPLA2-epsilon), and chromosome 22 open reading frame20 (C22orf20) [99,100]. PNPLA family contains

9 members (PNPLA 1-9) [101]. All family members of the PNPLA family possess the patatin-like phospholipase domain (PNPLA1-9) [102]. Human PNPLA3 contains 9 exons and codes for 481 amino acids. It is localized on chromosome 22; (22q13.31) [102]. The human PNPLA3 gene is bigger (481 AA) compared to mice which is smaller (384 AAs) [101-103]. High expression of the human PNPLA3 gene has been observed in the liver while moderate expression is found in the brain, kidney, skin and adipose tissues [104]. Human PNPLA3 possesses a single nucleotide polymorphism (rs738409) which changes the amino acid, isoleucine into methionine (I148M). In a genome-wide association study (GWAS) among Hispanic, African American, and European American individuals, a strong association was observed between the 148M variant of PNPLA3 and hepatic fat levels [98]. In addition, multiple studies have linked a strong association between liver cirrhosis and the PNPLA3 148M variant [105-107]. Additionally, this variant is also associated with alcohol liver disease which proceeds first with hepatosteatosis followed by steatohepatitis [108,109]. The variant 148M is implicated in the development of fatty liver in a transgenic mouse model of NAFLD [110]. The role of PNPLA3 is controversial. The knock-out of the PNPLA3 gene in mice results in neither fatty liver nor abnormal plasma and hepatic triglyceride [111,112]. However, the knock-in of human PNPLA3/148M in mice causes hepatosteatosis after sucrose feeding, and similarly, inflammation is observed after feeding with the NASH diet [113-115].

The purified PNPLA3 protein when expressed by baculovirus in Sf9 cells has been shown to possess triacylglycerol lipase and acylglycerol transacylase activities [100]. Similarly, when Huang *et al.* used Sf9 purified PNPLA3 protein, observed only lipase activity against triacylglyceride, diacylglyceride, and monoacyl glyceride while transacylase activity was absent [116]. The presence of triglycerides hydrolase and retinyl esterase activities using retinyl-palmitate as substrate were observed when PNPLA3 was expressed in lower eukaryotes, such as *Sacchromyces cerevisiae* [117,118]. When expressed in mammalian cells, such as human embryonic kidney cells (HEK293), the purified human PNPLA3 possessed lipase activity [119]. Human PNPLA3 mutant (148M) expressed in Sf9 cells using baculovirus was shown to lose triglyceride hydrolase activity in the presence of triolein as a substrate [120].

The mechanism of action of PNPLA3 in lipid metabolism is not well understood. Protein PNPLA3 remains associated with lipid droplets in mammalian cells [120-122]. Similar to wild-type PNPLA3, the mutant 148M exhibits an abnormally increased association with lipid droplets, leading to impaired lipid metabolism and elevated lipid levels within mammalian cells. Moreover, the turnover of the mutant 148M, either through ubiquitination or autophagy-related mechanisms, is decreased compared to wild-type turnover, which remains normal across feeding and fasting cycles [123,124]. It is presumed that the activity of another homolog, PNPLA2 which is also referred to as adipose triglyceride lipase (ATGL) is inhibited because the activator protein, comparative gene identification 58 (CGI-58) also referred as abhydrolase domain containing 5 (ABHD5) can no longer competitively associate with PNPLA2 but associates at higher levels with mutant PNPLA3 [122,125,126]. PNPLA3 fails to localize to lipid droplets in CGI-58 liver knock-out cells, thus CGI-58 is needed to direct PNPLA3 association with lipid droplets [122]. There is a rare variant of PNPLA3 (rs2294918), in this variant amino acid glutamic acid (E) is changed to lysine (K) at 434 (E434K). The variant E434K PNPLA3 attenuates the I148M mediated impact on steatosis and blood enzyme levels of liver injury markers, like AST and ALT [127,128].

Regulation of the PNPLA3 Gene in T2D and Metabolic Syndrome

Regulation of the PNPLA3 gene by ChREBP and SREBP-1c are shown in **Figure 2**. Both ChREBP and SREBP-1c regulate PNPLA3 in human hepatocytes and in mouse liver [129-131]. SREBP-1c levels are increased in T2D because of increased levels of circulating insulin levels [132]. The binding of SREBP-1c to the promoter of PNPLA3 is increased by insulin treatment. Treatment with the inhibitor of PI3K (LY 294002) reduces the insulin-mediated promoter activity of PNPLA3 and SREBP-1c expression in HepG2 cells. The response element of SREBP-1c (SRE) is located at -97/-88 from the start site [130]. SREBP-1c cooperates with the NFY transcription factor to increase the expression of PNPLA3 [130]. Overexpression of SREBP-1c in HepG2 cells increases the expression of PNPLA3 [130]. PNPLA3 gene is regulated by ChREBP and SREBP-1c in

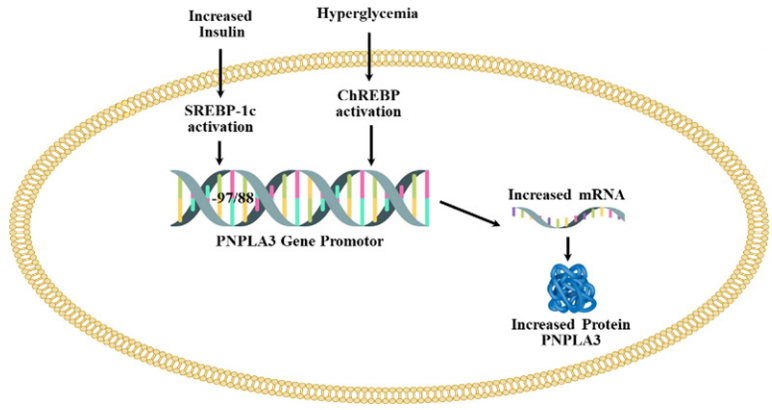

Figure 2. The upregulation of PNPLA3 by hyperglycemia and increased level of insulin. Adopted from one of our earlier publications on this series [197].

response to hyperglycemia and increased levels of insulin which is shown in **Figure 2**.

ChREBP is activated by higher blood glucose levels [133]. ChREBP transcriptional activity is regulated by intermediate products of glycolysis (glucose-6-phosphate (G-6-P), and fructose 2,6- bisphosphate), pentose phosphate pathway (xylulose-5-phosphate) [134-138], and acetylation which is a posttranscriptional modification mediated by cyclic-AMP-response element binding (CREB) protein/p300 [139]. ChREBP exists in two isoforms (ChREBPα and ChREBPβ) and in normal physiological conditions, ChREBPα resides in the cytosol and upon glucose stimulation, translocates to the nucleus and induces the transcription of ChREBPβ, thus linking both isoforms in the regulation of activity. ChREBPβ remains localized in the nucleus which lacks the glucose inhibitory domain (LID) that is present in ChREBPα and thus ChREBPβ possesses more potent transcriptional activity [140,141]. Under low glucose levels, LID-mediated inhibition of ChREBPα occurs while ChREBPβ remains constitutively active [140] ChREBP is involved in *de novo* lipogenesis, deletion of ChREBP genes decreases liver triglycerides levels via inhibition of *de novo* lipogenesis [142]. Knock-down of ChREBP in ob/ob mice is shown to reduce the hepatic triglyceride levels [143]. Liver-specific silencing of ChREBP using an adenoviral-based silencing expression system reduced hepatosteatosis in ob/ob mice [144]. PNPLA3 remains in direct control of ChREBP and also SREBP-1c in both mouse and human hepatocytes. Infection of adenoviruses expressing ChREBP and SREBP-1c to mouse and human hepatocytes increases the expression of PNPLA3 [129]. Therefore, both of these transcription factors upregulate the expression of PNPLA3. Additionally, ChREBP is activated by the LXR, which forms a heterodimer with retinoid X receptors and binds to LXR response elements. Two LXR binding sites are located 2.4 kb upstream (+1) of the ChREBPα promoter, thereby inducing the expression of the ChREBP gene. It's noteworthy that ChREBPα can activate ChREBPβ, as previously mentioned [53]. The site is a response element of the nuclear hormone receptor superfamily which can also bind to thyroid receptors (TR), however, only TRβ but not TRα can activate ChREBP in liver and white adipose tissues [145]. There is an indirect link of PNPLA3 mutant with the fibrogenesis of the liver. Retinoic acids (all-trans) activate RAR and retinoid X receptor (RXR) transcription factors which downregulate fibrotic genes in HSCs [146-148]. The mutant PNPLA3 (148M) decreases retinol levels causing downregulation of RAR/RXR target genes in the hepatic stellate cell line [104].

Membrane-bound O-acyltransferase domain-containing protein 7 (*MBOAT7*)

MBOAT7 encodes a member of membrane-bound O-acyltransferase which is integral to the membrane. The enzymatic function of the protein is not well defined, however, its transfers arachidonic acid to lysophosphatidylinositol from arachidonic-CoA, therefore known as lysophosphatidylinositol acyl-transferase 1 (LPLAT1 or LPIAT11) [149] (https://www.ncbi.nlm.nih.gov/gene/79143). MBOAT7 is involved in Land cycling in which reacylation of membrane phospholipids occurs (https://www.ncbi.nlm.nih.gov/gene/79143). Multiple variants of MBOAT7 are produced due to alternate spicing. Loss of MBOAT7 causes increased triglyceride synthesis and accumulation in hepatocytes. The substrate for triglyceride synthesis, diacylglycerol is produced due to increased turnover of phosphatidylinositol [150]. A similar decrease of MBOAT7 in stellate cells of the liver leads to an increased fibrogenic phenotype [150]. An exact opposite observation was made when hepatocyte-specific deletion of MBOAT7 was made. The cholesteryl esters were increased while no effects were observed with triglycerides [151]. Hepatic fibrosis was observed with hepatocyte-specific deficient MBOAT7 mice when treated with diet-inducing NAFLD. Initially, the polymorphism rs641738 C>T of MBOAT7 was found associated as a genetic modifier with alcohol-mediated cirrhosis and later it was linked to NAFLD and NASH [152,153]. European liver biopsy samples and Dallas heart study samples were linked to lower MBOAT protein expression and changes in the plasma phosphatidylinositol level [153]. MBOAT7 variation, rs641738 C>T was predominantly associated with Italian NAFLD and HCC patients when T was present [154]. The MBOAT7 rs641738 T allele increases an approximately 80% risk of HCC [154]. However, others did not find evidence of its association with NAFLD [155,156].

Transmembrane 6 superfamily member 2 (TM6SF2)

The function of TM6SF2 is unknown, however, studies in mice revealed that loss of TM6SF2 function causes reduced lipid export by very low-density lipoprotein (VLDL). TM6SF2 knock-out mice develop NAFLD, and hypocholesterolemia exhibiting the characteristics of human NAFLD [157]. The variant rs58542926 of TM6SF2 converts amino acid "glutamine" into lysine at amino acid position 167 when T is present and allele rs58542926 T has been associated with the increased risk of NAFLD across children and adults [158]. The loss of expression of TM6SF2 was also associated with reduced expression of PNPLA3 [157]. The variant is associated with lower levels of blood cholesterol, triglycerides and LDL-cholesterol and thus protective against cardiovascular diseases [159,160].

Glucokinase regulatory protein (GKRP)

Glucokinase is a crucial enzyme responsible for initiating glucose metabolism through glycolysis. Its activity is tightly regulated by the glucokinase regulatory protein (GKRP). Typically found in the cytoplasm, glucokinase undergoes modulation under conditions of low glucose concentration. During such times, GKRP binds to glucokinase, facilitating its translocation into the nucleus of hepatocytes. This regulatory mechanism ensures precise control over glucose utilization in hepatic cells [161]. When glucose levels rise after the meal, GKRP is released from glucokinase, thus glucokinase is transported to the cytoplasm [161]. Overexpression of GKRP, decreases fasting glucose levels and increases insulin sensitivity in diabetic mice [161]. It is proposed that GKRP increases the stability of glucokinase [161]. An SNP, rs1260326 results in the conversion of amino acid 446 from proline to leucine (P446L) in GKRP when cytosine (C) converts to thymine (T) [162]. The P446L conversion attenuates GKRP's ability to inhibit glucokinase [163]. The L446 (P446L) of GKRP has been associated with NAFLD [155,163]. Another SNP, rs780094 which is in linkage disequilibrium with rs1260326 has also been found with NAFLD and liver fibrosis severity [164-166].

Cellular communication network factor 3 (CCN3/NOV)

CCN3 is a novel adipokine that is a member of the CCN matrix protein family [167]. It is being synthesized and secreted by adipose tissue and is involved in regulating various processes, such

as inflammation, organogenesis, and fibrosis [168,169]. CCN3 is encoded by the nephroblastoma overexpressing (NOV) gene [170]. Studies demonstrate association of NOV with glucose and lipid metabolism disorders, diabetes and obesity [171]. Animal studies demonstrate impairment of adipose tissues in obesity and thus increase in inflammatory molecules, including CCN3 while a decrease in adiponectin [171]. Higher serum levels of CCN3 among NAFLD patients were observed compared to controls [172]. Research indicates that CCN3 offers a protective role against apoptosis in the liver and CCN3 peptides are being attempted for treatment of NASH [173].

Adipose Tissue and Crosstalk with Liver

Adiponectin and leptin are hormones secreted by adipocytes, playing pivotal roles in metabolic regulation. Adiponectin functions to reduce liver glucose production while enhancing the utilization of glucose and fatty acids in muscle tissue. Moreover, it suppresses the secretion of inflammatory cytokines, thereby exhibiting potent anti-inflammatory properties. Reduced circulating levels of adiponectin have been associated with conditions such as NAFLD, characterized by elevated lipid accumulation in the liver [174]. Administration of adiponectin to ob/ob mice (leptin knock-out mice) results in decreased steatosis and liver inflammation [175]. The other adipocyte-derived cytokine, leptin is involved in food intake. Obese individuals have higher levels of leptin. Leptin regulates T-cell and inflammatory response [176,177]. Leptin activates stellate cells and may be involved in liver fibrosis [178]. A putative active form of adiponectin is not well defined while leptin treatment is ineffective in obese individuals. Another potent proinflammatory cytokine is resistin, its higher levels are observed among T2D patients. Resistin levels correlate with the levels of TNFα whose levels are higher among T2D patients and also NAFLDs [179,180]. TNFα plays a pivotal role in the intricate network of insulin signaling, exerting a significant influence that leads to insulin resistance. This interference underscores the complexity of metabolic regulatory mechanisms, where TNFα's interaction with insulin signaling pathways highlights its detrimental impact on glucose homeostasis. Additionally, other cytokines originating from visceral adipose tissues, including visfatin and vaspin, contribute to this regulatory landscape. Although the precise mechanisms of their actions remain partially understood, evidence suggests they play a role in reducing glucose levels. This points to a nuanced interplay between various cytokines and insulin, indicating a multifaceted regulatory environment that impacts glucose metabolism [181,182]. The cytokines from visceral fat play a significant role in NAFLD [183]. Other cytokines, such as TNFα, IL-6, and CC chemokine ligand 2 (CCL2), also known as monocyte chemoattractant protein 1 (MCP1), are also secreted by adipose tissue [181,182]. Hepatosteatosis leads to an inflammatory response whose mechanism has never been defined. In addition to adipose-derived cytokines in inflammation, liver resident Kupfer's cell activation which is liver resident macrophage occurs which further pushes the inflammatory condition in NAFLD [184]. Chemokines also increase inflammatory cell recruitment [185].

Angiotensinogen

As mentioned above for the role of RAAS in NAFLD, the levels of AGT and des-AGT are increased in hepatosteatosis [186]. After cleavage of 10 amino acids of the N-terminal region (Ang I) of secreted AGT, the remaining portion, desAngI-AGT has been observed to possess Ang II independent function [186]. In mice lacking AGT, (Agt -/-), if desAngI-AGT is expressed using adeno-associated virus (AAV-desAngI-AGT) or AGT, caused hepatosteatosis [186]. The mechanism of AGT-mediated hepatosteaosis is unclear. It is understood that increased plasma AGT after renin action leads to increased desAngI-AGT which can mediate hepatosteatosis and so will AGT. Variation in the expression of AGT among individuals can be anticipated due to its genetic variation among humans. The human AGT gene (hAgt) possesses a number of SNP in the proximal promoter region [187]. The increased circulating levels of AGT have been associated with hypertension, although experimentally the link of these SNP with hepatosteatosis is lacking because studies were solely evaluating hypertension but not hepatosteatosis. Studies that focus on the association between AGT locus and blood pressure include linkage and genetic studies. Studies were initially demonstrated in 1992 in pairs of hypertensive siblings from American and French populations, and this study is considered the first report that SNP plays a significant role. AGT coding variants (M235T) and (T174M) [188] have been linked with the increased plasma level of AGT in hypertensive subjects when 235T and 174M were found among two independent population cohorts derived from France and Utah [189].

In addition, hAGT possesses several SNPs in the proximal promoter associated with increased gene expression and hypertension when -6A, -217A, -776T, -793A and -1074T were present, which are in linkage disequilibrium (-6A/G, -217A/G, -776T/C, -793A/G, and -1074T/G). In vitro, gene reporters have shown that haplotypes possessing -6A, -217A, -793A, and -1074T possess increased transcriptional activity [190]. In a transgenic study, Kumar *et al.* showed that -217A and -6A promoter variants cause increased hAGT expression in the liver, kidney and plasma [191-193]. In genetic association studies, meta-analyses of variants were conducted to investigate the correlation between the homozygous -6A and 235T alleles and hypertension [192]. Nevertheless, to date, no studies have elucidated the mechanism underlying the increased synthesis and secretion of AGT, particularly through transcription factor-mediated activation at the -6A locus [193]. One study focusing on the African-American population shows that an A/G polymorphism at -217 in the promoter of the AGT gene plays a significant role in increasing the expression of AGT and is responsible for the risk of preeclampsia, a form of hypertension occurring during pregnancy [191,194]. Other SNPs in the promoter region are still unclear and not characterized for their mechanism affecting hAGT regulation, such as -776T/C and -793 A/G. The studies from our lab demonstrate that -776T/C can bind to AP-1 transcription factors when -776C is present. Similarly, AP-1 can also bind to -793 A/G when –793A is present (unpublished data). These two sites also become the target of alcohol-mediated AGT regulation since alcohol metabolism activates the AP-1 transcription factor. The human AGT gene is an acute phase response type II secreted by the liver and regulated by inflammatory cytokines, especially IL-6, in addition to estrogen receptors (ERs), glucocorticoid receptors (GRs), and signal transducer and activator of transcription (STAT3). IL-6 activates STAT3. Activated STAT3 binds to acute phase response-II (APR II) element in the proximal promoter of AGT (-273 locus) and regulates the activation of hAGT [195]. There is one SNP present at the -1074 locus, which is variant T/G, that can bind with hepatocyte nuclear factor-3beta (HNF3β) and opens the possibility of regulation of hAGT by liver-specific orphan transcription factor(s). Increased

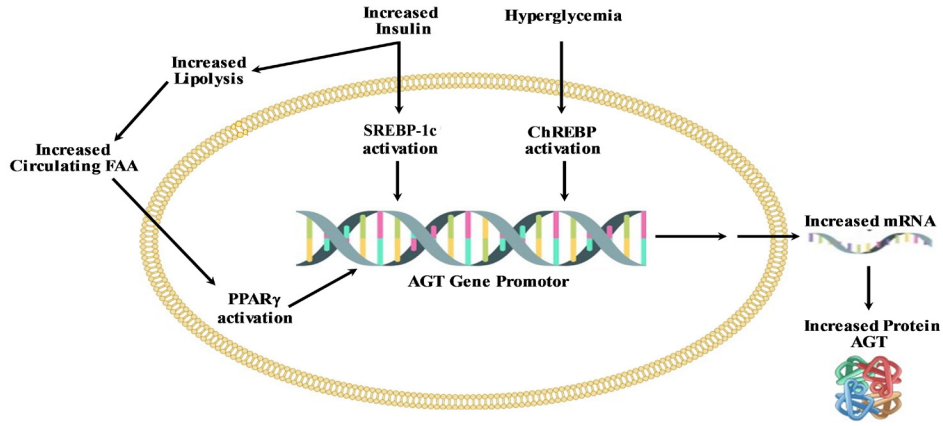

Figure 3. Key mediators of upregulation of AGT in obesity and T2D.

levels of AGT have been implicated in NAFLD which is common among T2D and metabolic syndrome patients [196]. Hence, individuals harboring the -1074T variant may become susceptible to NAFLD/ALD (alcohol liver disease) following ethanol consumption due to heightened levels of IL-6-mediated AGT secretion from the liver. The genetic sequence surrounding the 1074T/G variant aligns with the consensus HNF3β sequence when T is present, whereas it exhibits two mismatches when G is present. Consequently, the genotype characterized by -6A, -217A, -776T, -793A, and -1074T may trigger enhanced AGT synthesis by the liver and elevate plasma AGT levels, thereby elucidating its implication in NAFLD. This is significant as ARBs either obstruct or mitigate the effects of Ang II in hepatosteatosis. **Figure 3** represents key factors involved in the increased expression of AGT during T2D and obesity.

Conclusions

NAFLD poses a significant and escalating health challenge, not just within the United States but globally. Alarmingly, treatment expenses alone in the USA have soared to a staggering $103 billion. The severity of this issue is further accentuated by the fact that liver diseases, including cirrhosis, currently rank as the 12th leading cause of mortality in the USA, according to data from 2014. The surge in NAFLD prevalence can be attributed to the escalating incidence of obesity and metabolic syndrome over the past two decades. These interlinked conditions have substantially contributed to the rise in cases of fatty liver disease, with NAFLD prevalence ranging from 30% to 90% among individuals with obesity and approximately 70% in those with T2D. Presently, diagnosing NAFLD or its more severe manifestation, NASH, relies on a multifaceted assessment of clinical parameters rather than a singular definitive marker. However, despite the mounting prevalence and associated economic burdens, currently there are no pharmaceutical interventions specifically endorsed for the treatment of either NAFLD or NASH. Effectively tackling NAFLD and NASH demands a comprehensive understanding of the intricate molecular mechanisms governing the initiation and progression of hepatic steatosis. Pinpointing these pivotal events holds the potential to pave the way for targeted therapeutic interventions aimed at arresting or even reversing the disease trajectory. Such advancements offer a glimmer of hope for the millions affected by these debilitating conditions, promising improved outcomes and quality of life.

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