The Riddle of Nonalcoholic Fatty Liver Disease: Progression From Nonalcoholic Fatty Liver to Nonalcoholic Steatohepatitis

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Nonalcoholic fatty liver (NAFL) is an emerging global epidemic which progresses to nonalcoholic steatohepatitis (NASH) and cirrhosis in a subset of subjects. Various reviews have focused on the etiology, epidemiology, pathogenesis and treatment of NAFLD. This review highlights specifically the triggers implicated in disease progression from NAFL to NASH. The integrating role of genes, dietary factors, innate immunity, cytokines and gut microbiome have been discussed. (J CLIN EXP HEPATOL 2015;5:147–158)

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Abbreviation: AGE: Advanced glycation end products; ALT: Alanine aminotransferase; AMPK: AMP-activated protein Kinase; APPL1 and 2: Adaptor protein 1 and 2; ATP: Adenosine tri-phosphatase; BMI: Basal Metabolic Index; CD: Cluster of differentiation; COL13A1: Collagen, type XIII, alpha 1; DAMP: Damage associated molecular pattern molecules; EFCAB4B: EF-hand calcium binding domain 4B; FA: Fatty acid; FDFT1: Farnesyl-diphosphate farnesyltransferase 1; FFA: Free fatty acid; GSKR: Glucokinase regulatory protein; GLUT 5: Glucose transporter type 5; GWAS: Genome wide association studies; HDL: High density lipoprotein; Hh: Hedgehog; HMGB1: High-mobility group protein B1; HOMA-IR: Homeostatic model assessment-insulin resistance; HSC: Hepatic Stellate Cells; IL6: Interleukin 6; IR: Insulin Resistance; KC: Kupffer Cells; LPS: Lipopolysaccharides; LYPLAL1: Lypophospholipase like 1; MCP: Monocyte chemotactic protein; NAD: Nicotinamide adenine dinucleotide; NAFL: Nonalcoholic fatty liver; NAFLD: Nonalcoholic fatty liver disease; NASH: Nonalcoholic steatohepatitis; NCAN: Neurocan gene; NF-KB: Nuclear Factor Kappa B; NK: Natural Killer; NKL: Natural Killer T cells; NLR: NOD like receptor; NNMT: Nicotinamide N-methyltransferase gene; OX-LAM: Oxidized linolenic acid metabolite; PAMP: Pathogen-associated Molecular pattern; PARVB: Beta Parvin Gene; PDGF: Platelet-derived growth factor; PNPLA3: Patatin-like phospholipase domain-containing protein 3; PPAR- α : Peroxisome proliferator activated receptor alpha; PPP1R3B: Protein phosphatase 1 R3B; PUFA: Poly unsaturated fatty acid; PZP: Pregnancy-zone protein; ROS: Reactive oxygen species; SAMM: Sorting and assembly machinery component; SCAP: SREBP cleavage-activating protein; SFA: Saturated fatty acid; SNP: Single nucleotide polymorphism; SOCS3: Suppressor of cytokine signaling 3; SOD2: Super-oxide dismutase 2 gene; SREBP-1C: Sterol regulatory Element–Binding Protein 1-C gene; TLR: Toll like receptor; TNF α : Tumor necrosis factor Alpha; UCP3: Uncoupling protein 3 gene

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Non alcoholic fatty liver disease (NAFLD) is an emerging medical problem worldwide which affects a significant proportion of the western population and there is gradual spread of this epidemic to south-east Asian countries. NAFLD encompasses two entities: Non-alcoholic fatty liver (NAFL) and Non-alcoholic steatohepatitis (NASH). NAFL is defined as the evidence of hepatic steatosis without inflammation either by imaging or by histology in individuals without significant alcohol consumption in whom secondary causes of steatosis are absent.¹ NASH on the other hand, is characterized by the presence of both steatosis and inflammation with evidence of hepatocyte injury in the form of ballooning with or without fibrosis.¹

The prevalence of NAFLD has been gradually increasing and one third adult Americans have NAFLD.² The prevalence in obese population may be as high 57.55–74%.^{3,4} The global spread of this epidemic is evidenced by the presence NAFL in 36.8% of Mediterranean, 21.5% of Iranians and 27% of urban Chinese adults.^{5–7} However, the prevalence varies between countries and continents. The prevalence in Europe is 20–30% while that in Japan varies between 9 and 30%^{8,9} and in China between 5 and 24%.¹⁰ In India, the prevalence of NAFLD in urban population is 16%–32% while that in rural areas is approximately 9%.^{11–13} Among the Asian countries the lowest prevalence is observed in Singapore at 5%.¹⁰ Globally NAFLD has been related to obesity and sedentary lifestyle. Interestingly both NAFL and NASH have been observed in non-obese subjects in Asians, which is referred to as the Asian paradox.^{11,14,15} Mere presence of fat in the hepatocytes is not considered as a disease. As most of the subjects with NAFL do not progress to NASH, differentiation between these two conditions is paramount. Though liver histology is the

gold standard in diagnosis of NAFL, the commonest method used is transabdominal ultrasonography which has a sensitivity of 100% and specificity of 90% when fat on liver biopsy exceeds 20%.¹⁶ Other non-invasive modalities used to diagnose NAFL includes transient elastography (Fibroscan) and Acoustic Resonance Magnetic Imaging (ARFI) which measures liver stiffness and corresponds to presence of fibrosis. Magnetic resonance spectroscopy is a quantitative method of measuring liver fat but is limited in clinical use due to lack of widespread availability and cost.¹⁷ However, none of these available noninvasive tests can distinguish simple steatosis from NASH. Although raised aspartate aminotransferase and alanine aminotransferase levels are considered by some as a marker of NASH, yet these enzymes may be normal in many subjects with biopsy proven NASH.¹⁸ A recent study using non-invasive tools have found 81% probability of differentiating NAFL from NASH using Bayesian approach combining clinical, laboratory, and imaging data.¹⁹ Based on the modality used to diagnose NAFL, the detection of disease varies. Ultrasound based study from India has shown the prevalence of NAFLD to be 16.6%¹² while in a study based on liver biopsy the presence of NASH was 53%.²⁰ In another study from coastal eastern India, one fourth of patients had evidence of NASH on liver biopsy on presentation.²¹ In another Asian study involving 52 patients, biopsy proven NASH at presentation was found in 32.6% patients while 23% patients with baseline NAFL progressed to NASH.²² In contrast, a recent study from the West with mean 6.6 years follow up, baseline NASH was found in 75% patients with disease progression from NAFL to NASH in 44% patients. Among those patients with simple steatosis fibrosis progression was observed in follow up liver biopsy which was statistically more in diabetic steatosis thereby suggesting that simple steatosis can progress to clinically significant fibrosis.²³ Similar data from west suggests that among those with NASH only 21–26% progress to cirrhosis over 8.2 years.²⁴ Meta-analysis have demonstrated that NAFLD increases the risk of all-cause mortality.²⁵ Therefore, aggressive management in the form of dietary and lifestyle modification is required in patients who has NASH when compared to those who have simple steatosis. The riddle of progression from NAFL to NASH and subsequently to cirrhosis is poorly understood. In this review, we present new insights into progression of NAFL to NASH, integrating the role of genes, diet, immunological profile, cytokines, liver cell types, and gut microbiota.

JOURNEY FROM NONALCOHOLIC FATTY LIVER DISEASE TO NONALCOHOLIC STEATOHEPATITIS

The spectrum of NAFLD ranges from simple steatosis to NASH. The accumulation of fat above the physiological level (<5%) in the hepatocytes (steatosis) is the pre-

requisite for development of NASH. Although NAFL is almost universally present in obese individuals yet not all of them progress to NASH. Similarly there are many individuals who may be non-obese physically but may be metabolically obese and have NASH. This indicates that multiple factors other than obesity and insulin resistance are involved in the disease progression. The original “two-hit hypothesis” considered steatosis to be the “first hit”, which increased the sensitivity of the liver to the “second hits” that leads to hepatocyte injury and ultimately inflammation (NASH) and fibrosis.²⁶ However, emerging evidence suggests that the pathogenesis involves “multiple hits”²⁷ which progress parallel to each other and progression to NASH depends on the close interaction and cross talk between host genes, environmental influences, gut microbiota and host immune system.²⁸ The progression from NAFL to NASH is poorly understood. The following sections will elaborate the various factors implicated in disease progression (Figure 1).

GENES IN NONALCOHOLIC FATTY LIVER DISEASE

Considerable variation in severity of the disease and rate of progression suggests a genetic predisposition for the disease. It is a polygenic disease with involvement of multiple loci, environmental and nutrient interactions. Furthermore, in a substantial percentage (25–35%) of individuals, the disease is thought to be contributed by a genetic component.²⁹ Common genetic variants at various loci have consistently been associated with fatty infiltration. Studies have shown that Asian Indians have a genetic predisposition to have up to twofold increased hepatic fat accumulation despite having a low BMI due to their tendency to have more visceral adiposity.¹⁵ The major locus among these is 22q13.31 which harbors the PNPLA3 gene. A particular single nucleotide polymorphism (SNP) namely rs738409 showed strongest association with NAFLD across various Genome wide association studies (GWAS) in various populations^{30–32} and this is probably one of the very few SNPs that were identified by GWAS which was significant across various ethnicities. The SNP is located in the 3rd exon of the gene and is mainly expressed by liver and adipose tissue. The gene is involved in triglyceride hydrolysis activity and the SNP introduces an amino acid substitution from isoleucine to methionine (I148M) that abolishes the function.³³ PNPLA-I148M polymorphism has been shown to be associated with increased necroinflammation, severe steatohepatitis and advanced fibrosis.³⁴ In India, higher frequency of C/G and G/G genotypes of the rs738409 polymorphism was noted in NAFLD subjects and this was associated with significantly higher fasting insulin, HOMA-IR, alanine transaminase and aspartate transaminase levels.³⁵ In addition to predisposing patients to severe disease,

Figure 1 Graphical illustration showing the unknown factors in the riddle of progression of NAFL to NASH.

patients with PNPLA3 rs738409 GG homozygous genotype has also fivefold increased risk of developing hepatocellular carcinoma though the findings needs to be validated in larger studies.³⁶ On the other side of the spectrum PNPLA3 S453 polymorphism is associated with lower hepatic fat content in African Americans, making them the group at lowest risk of NAFLD. This protective effect is independent of the presence of PNPLA3–I148M polymorphism.³⁷

Other GWA studies identified common genetic variants in or near LYPLAL1, PPP1R3B, NCAN and GCKR that are associated with steatosis apart from NASH, fibrosis and metabolic traits in patients with European ancestry.³⁰ Importantly, the rs780094 SNP was associated with a higher risk of type2 diabetes, higher levels of triglycerides, reduced fasting plasma glucose levels and lower Homeostatic model assessment–insulin resistance (HOMA-IR) in Japanese population. Furthermore, it was hypothesized that the effect of the SNP on diabetes is probably mediated through impaired beta cell function rather than through obesity.³⁸ Likewise another study in Japanese identified SAMM50, PARVB and PNPLA3 as risk loci associated with NAFLD. A GWA study in Caucasians identified FDFT1, COL13A1, EFCAB4B, PZP as causative loci for steatosis, NAFLD activity score, degree of fibrosis, lobular inflammation and serum levels of alanine amino transferase (ALT). A pooled genetic study that identified SNPs from the above GWA studies and genotyped the same in Indian NAFLD patients identified PNPLA3, SAMM50, PARVB and PZP as risk loci for NAFLD apart from association of variants in NCAN and PZP being associated with

higher levels of ALT.³⁹ Individual hypothesis or candidate gene approach based studies have identified various loci that are either protective, or confer susceptibility to NAFLD, steatosis or faster progression. It was shown that the SCAP rs2101247 “A” allele might decrease the risk of NAFLD especially in females with metabolic syndrome, however the reasons and mechanisms are unclear.⁴⁰ SNPs namely rs2276736, rs3772630 and rs3772627 appear to be protective especially in the Indians against NAFLD.⁴¹ A Val277Ala substitution in the PPAR- α (peroxisome proliferator activated receptor alpha) was associated with NAFLD. However it was associated with decreased waist circumference and waist-to-hip ratio suggesting a protective role in obesity.⁴² In contrast, variants in uncoupling protein 3 gene (UCP3),⁴³ Sterol Regulatory Element-Binding Protein 1C gene (SREBP-1C),⁴⁴ Adaptor protein (APPL1 and 2),⁴⁵ Nicotinamide N-methyltransferase gene (NNMT)⁴⁶ and mitochondrial superoxide dismutase 2 gene (SOD2)⁴⁷ conferred susceptibility.

Another gene which has been linked to NAFLD is apolipoprotein 3 (APOC3) gene. Carriers of APOC3 variant alleles (c-482T, T-455C or both) in non-Asian population had increased NAFLD and insulin resistance.⁴⁸ Further studies in Asian Indians has shown varied results. Polymorphism T-455C in APOC3 gene has been associated with NAFLD and higher serum triglyceride levels in Southern Indian population.⁴⁹ Another study in the same region of India, on the other hand, found that PNPLA3 rs738409 polymorphism to be associated with NAFLD but failed to show any independent association of APOC C3 gene rs2854116 and rs2854117 polymorphism with NAFLD.⁵⁰

Environmental influences on the genotype and the effect they have on the phenotype have been studied to a certain extent in the context of NAFLD. Since identifying disease susceptibility alone would not necessarily aid in disease outcomes, understanding the environmental interactions with the genotype will help in managing the disease in a better way. Recent studies have identified the effect of n3 and n6 poly unsaturated fatty acids (PUFAs) on the genotype. It was seen that there was significantly higher fatty infiltration in individuals who consumed more of n6 versus n3 PUFAs and with GG genotype in the PNPLA3 gene.⁵¹ Similarly another study which included subjects with both GG and CC genotype, showed that. Individuals in both groups lost 3.1 kg weight on an average. However, individuals in the GG genotype group lost 45% as against only 18% of liver fat in the CC genotype group, suggesting that individuals with the GG genotype may have a better advantage with weight loss and fatty infiltration.⁵² In another significant study it was seen that the G allele of the rs738409 in the PNPLA3 gene was associated with both alcoholic and non-alcoholic fatty liver disease, so it is prudent for an individual with the GG genotype to avoid alcohol for an overall better health of the liver.⁵³

The role of hepatic iron in progression of NAFL to NASH is unclear. Dysregulation of iron regulatory molecules or genetic factors may be responsible for iron overload in NAFLD patients. The C282Y mutation (HFE gene) which is common in Europeans are not found in Indian population. However, H63D heterozygosity was found in 16.9% of Asian Indians with NASH when compared to only 12% in controls, suggesting that primary iron overload in Indians is non HFE type.⁵⁴ Although genetic susceptibility has been identified for NAFLD, further research should focus on gene–gene and gene–nutrient interactions employing advanced genomic techniques.

OBESITY, INSULIN RESISTANCE AND DIETARY FACTORS IN NONALCOHOLIC FATTY LIVER DISEASE

Lipids and Lipotoxicity

The composition of food intake plays an important role in NAFL. Not all lipids are equally harmful. Polyunsaturated fatty acids (PUFAs) are of two type (i) n-6 PUFAs which includes linoleic acid and arachidonic acid and (ii) n-3 PUFAs which includes alpha-linolenic acid, eicosapentanoic acid and docosahexanoic acid. The n-6 PUFAs have the capability of producing proinflammatory eicosanoids⁵⁵ and are harmful while the n-3 PUFAs have anti-inflammatory properties leading to decreased lipogenesis and lower hepatic steatosis.^{56,57} Studies have shown that a high ratio of n6:n3 PUFA in hepatocytes and circulation is associated with severity of NAFLD.⁵⁸ Based

on this, a meta-analysis showed n-3 supplementation may decrease hepatic fat, though heterogeneity of the study population warrants the need of well-designed randomized control trials.⁵⁹ Reducing n-6 intake is an interesting alternative as linoleic acid is associated with formation of oxidized linoleic acid metabolite (OXLAMs) which are found increased in NASH and correlate with severity of NASH.⁶⁰

The “lipotoxicity” represents the major mechanism underlying hepatocyte dysfunction leading to disease progression in NASH. Lipotoxic injury appears to occur in the setting of excess free fatty acid (FFA) traffic, especially saturated fatty acids (SFAs), rather than due to simple triglyceride accumulation. This view is supported by studies that demonstrate that increased hepatocyte triglyceride formation⁶¹ or reduced export of lipid⁶² does not, of itself, increase inflammation. Furthermore, evidence suggests that triglyceride accumulation may actually be a protective mechanism to counter lipotoxicity.⁶³ A number of other studies have shown lipotoxic injury is reduced if alternative pathways of fatty acid (FA) disposal are available.⁶⁴ This suggests that triglycerides themselves are unlikely to be the cause of hepatocyte injury in NASH and probably occurs in parallel with the generation of toxic metabolites, with these lipotoxic metabolites being primarily responsible for disease progression.⁶⁵ The current theory of lipotoxicity centres on an increase in the flux of FFAs within hepatocytes. This is a direct consequence of increased influx (through increased dietary intake of SFAs as well as *de novo* lipogenesis and adipose lipolysis in the setting of insulin resistance and impairment of compensatory oxidative processes.⁶⁶ The net result is the generation of toxic lipid metabolites, such as ceramides, diacylglycerols, lysophosphatidyl choline, and oxidized cholesterol metabolites, which act as reactive oxygen species (ROS),⁶⁷ although the absolute and relative levels of each of these substances in NAFLD and its progression remain unconfirmed.

Insulin Resistance

Obesity and metabolic syndrome are risk factors for NAFL and NASH in the western world. However, in Asians, it has been observed that subjects with no components of the metabolic syndrome can still have insulin resistance and also significant NASH.⁶⁸ Abdominal obesity has been found in only 7.1% subjects among lean Asian Indians with NAFL.⁶⁹ Visceral adipose tissue content was initially thought to be the most important factor in pathogenesis of NASH among Asians. However, in an Indian study involving biopsy proven NAFL patients, both subcutaneous adipose tissue and total adipose tissue contents has been found to be positively correlated with the disease severity.⁷⁰ Indian studies have shown that presence of metabolic syndrome among Indian NAFL to be between

21% and 41%.^{71,72} However, in contrast, a recent study from India has shown that 50% of Indian subjects with NAFLD did not have insulin resistance and one third of these patients had significant fibrosis.⁷³ The lack of insulin resistance may be associated with less severe disease in lean Indian fatty liver subjects.⁶⁹ In contrast, other studies from India found the prevalence of insulin resistance in around 97.5% subjects with NAFLD.⁷⁴ In support of the previous study, another study from the subcontinent has found that 98% of Indian NAFL subjects have reduced insulin sensitivity and 50% of them were associated with features of metabolic syndrome.²⁰ The Indian cohort of NAFLD appears to be a heterogeneous subset of patient with variable insulin resistance with more resistance in subjects with features of metabolic syndrome and lesser in lean NAFLD.

Fructose

Fructose is an isomer of glucose with a hydroxyl group on carbon-4 reversed in position.⁷⁵ Over the years, the consumption of fructose has enormously increased as it has been increasingly used as artificial sweetener in preference over sucrose due to its lower cost and ability to induce less satiety.⁷⁶ With the help of fructose specific Glucose transporter type 5 (GLUT 5), fructose is absorbed from the small intestine, and reaches the liver via the portal vein. In the liver, it undergoes phosphorylation to produce 3 carbon intermediates.⁷⁷ Fructose may cause hepatotoxicity indirectly by worsening metabolic syndrome and has been linked in humans to increase triglyceride levels and decrease HDL cholesterol. Peroxisome proliferator activated receptor gamma coactivator 1 beta also plays a crucial role in fructose induced insulin resistance.^{78,79}

Fructose metabolism involving hexokinase causes depletion of ATP which induces metabolic stress.⁷⁰ High fructose intake has also been linked to intestinal dysbiosis, which activates the gut-liver axis and induces a low grade endotoxemia which in turn predisposes to NASH.⁸⁰ Copper depletion and iron overload which are indirectly associated with NASH has been found with increased fructose consumption.^{81,82} Whether visceral fat accumulation is directly related to progression of NAFL remains an area of research. However, some studies have linked increased fructose to significant increase in waist circumference and the proportion of visceral adipose tissue with no change in total body fat.^{83,84} The direct effects of fructose toxicity may be due to the accumulation of fructose 1-phosphate in the hepatocytes. Fructose produces advanced glycation end products (AGE) 17 times faster than glucose. High AGE levels, common in western diet, exacerbate liver injury, inflammation and fibrosis via oxidative stress, cytokine synthesis (TNF- α and IL-6), and through hepatic stellate cell activation.⁸⁵ Fructose over consumption, thereby contributes to development of obesity, dyslipidemia, and impaired glucose

tolerance, producing AGEs responsible for producing dysfunctional proteins.⁸⁶ Therefore, AGE and its receptor pathway could be considered a new target for nutritional or pharmacological strategy to slow NAFLD progression.

INNATE IMMUNITY AND CYTOKINES

Immune Cells

Lymphoid cells of liver encompass resident lymphocytes, which are functionally and phenotypically distinct from their counterparts in the peripheral circulation and in other organs as well as hordes conventional (i.e. B cells, CD4+ and CD8C+ T cells, natural killer cells) and non-conventional lymphoid cells(i.e., $\gamma\delta$ TCR+ T cells, natural killer T cells). A key role in specific immune function is also exerted by mucosal associated invariant T cells that are a highly specialized T cell population in the vascular network of the liver.⁸⁷ Regulatory T cell populations seem to have an important role in maintaining a beneficial balance in the liver between immuno-tolerance and activation.⁸⁸ In addition to classic parenchymal hepatocytes and cholangiocytes, the liver contains other cell types responsible for the homeostasis of the innate and adaptive immune system. Among the non-lymphoid cells, Kupffer cells (KC) and dendritic cells (DC) from the myeloid lineage have a major role in the immune response. While dendritic cells are the primary antigen-presenting cells of the liver, the cholangiocytes can also act as antigen-presenting cells⁸⁹ thus playing an additional role in the hepatic immune function. Dendritic cells in patients with NAFLD exhibits immature yet functionally activated phenotype in response to lipo-polysaccharide stimulation which secrete inflammatory cytokines and contribute to worsening of disease.⁹⁰ The innate immune response is an important effector of parenchymal inflammation in liver diseases, such as NASH, and is mediated by innate immune cells, including neutrophils, macrophages (Kupffer cells), natural killer (NK) cells, and natural killer T (NKT) cells. The following section elaborates the role of each cell types in NAFL.

Resident or monocyte-derived Kupffer cells are the largest population of mononuclear phagocytes in the body. They are present throughout the liver, but there is variation in the population density, cytological characteristics, and physiologic functions of KC in different zones of the hepatic acinus/lobule. During liver injury and diseases, monocytes rapidly differentiate into mature cells that are indistinguishable from genuine KC, independently from the circulating monocytes.⁹¹ They are strategically located in liver sinusoids, therefore they are the first cells to come in contact with exogenous immunoreactive material or endogenous signals phagocytosing, processing and presenting antigen, and secreting various pro-inflammatory mediators such as cytokines, prostanooids, nitric oxide,

and reactive oxygen intermediates. Expression of the Fc receptor results in non-specific phagocytosis of immune complexes as well as antibody-coated particles such as microorganisms and allows KC to have a significant role in control of inflammatory and immunologic processes. It has been shown that high-fat diet increase Kupffer cells number and induce their pro-inflammatory status i.e. attainment of M1 phenotype from M2 status. Pro-inflammatory activated Kupffer cells by lipid promote hepatic NKT cell over-activation and cell death, which lead to further hepatic NKT cell deficiency in the development of NASH.⁹² Generally, KCs are exposed to low levels of gut-derived lipopolysaccharides (LPS). This stimulus allows KC to trigger an escape mechanism that involves IL-10, which in turn contributes to the down-regulation of pro-inflammatory cytokines.⁹³ On the other hand, following massive Toll like receptor 4 (TLR4) stimulation, KC produce several chemokines and cytokines, such as TNF- α , IL-1 β , IL-6, IL-12, and IL-18.⁹⁴ Conversely, adiponectin was recently shown to shift KC polarization to the M2/anti-inflammatory phenotype, preventing progression of NASH in mice. Adiponectin decrease, as well as adiponectin gene deletion, induces hepatic steatosis progression, fibrosis, and tumor development.⁹⁵ Moreover, KCs have also metabolic function, regulating fatty acids oxidation, increasing hepatic lipid storage and insulin resistance (IR), as mechanisms of adaptation to increased caloric intake. This event which is triggered by secretion of inflammatory cytokines, suggests a beneficial role for alternatively M2-activated KCs in metabolic derangements.^{96,97}

Natural killer cells are abundant in the liver and may have a role in the progression of NAFLD as they are capable of inducing apoptosis of both HSCs and hepatocytes, via IFN γ .⁹⁸ Although circulating NK cell numbers and cytotoxic activity appear to be reduced in obesity,⁹⁹ it is likely that the hepatic NK cell population is significantly increased in NASH.¹⁰⁰ NKT cells regulate host responses to tissue damage by inducing an adaptive immune response (both Type 1 and Type 2) and are seen in association with necroinflammation.¹⁰¹ Although animal models and studies in other chronic liver diseases have shown conflicting roles in hepatic fibrogenesis,¹⁰² NKT cells are activated by lipid antigens and therefore may contribute to the progression of fibrosis in NAFLD. Studies in humans and animals suggest that NKT cells may play a protective role in limiting steatosis but a deleterious role in the progression of inflammation and fibrosis.¹⁰³

Hepatocytes apart from their metabolic and detoxifying functions, express TLR4, although they require high doses of LPS to induce significant effects.¹⁰⁴ Under inflammatory conditions, the expression of TLR2, rather than TLR4, is up-regulated, indicating a major responsiveness to TLR2 activation and involvement of downstream MyD88 signaling pathway following an insult.¹⁰⁵ In com-

parison to KCs, hepatocytes are more able to clear LPS from systemic circulation, through its uptake and release into the bile, where TLR4, CD14, and myeloid differentiation (MD)-2 have an obligatory role for LPS uptake by hepatocytes.¹⁰⁶ Furthermore, TLR4/MyD88 signaling in hepatocytes has been shown to play a pivotal role during the early progression of high fructose diet induced NAFLD, in which free High-mobility group protein B1 (HMGB1) served as a Damage-associated molecular pattern molecules (DAMP) mediating TLR4 activation.¹⁰⁷

Hepatic stellate cells (HSCs) trans-differentiate from quiescent to active state under the influence of activators like platelet-derived growth factor (PDGF) and transforming growth factor β 1 (TGF- β 1) secreted by activated KC, infiltrating monocytes, platelets, and damaged hepatocytes.¹⁰⁸ The resting HSCs may acquire adipogenic or myogenic phenotype during the trans-differentiation,¹⁰⁹ determined by adipogenic and myogenic gene expression. Indubitably, adipogenic genes are down-regulated under ischemia and inflammation and up-regulated by peroxisome proliferator-activated receptor- γ (PPAR- γ). Other factors implicated in HSCs activation include the Hedgehog (Hh) pathway,¹¹⁰ cytokine stimulation (particularly TNF- α , IL-1 β , and IL-6),¹¹¹ and leptin.¹¹² Conversely, adiponectin released from adipose tissue reduces HSC migration and proliferation.¹¹³ Finally, TLR4 contributes to the activation of HSCs through the MyD88-NF- κ B-dependent pathway.¹¹⁴

Murine biliary cells express CD14, MD-2, and TLR2, 3, 4, and 5 and after LPS stimulation, murine biliary cells activates the NF- κ B pathway and synthesized TNF α .¹¹⁵ Human biliary epithelial cells express TLR 1 to TLR10. Subsequent to TLR2 and TLR4 activation, Homeobox protein CDX-2 and mucus core protein-2 expression increases.^{115,116} The progression of NAFLD in humans has been related to the increase in bile ductules, and their senescence markers. Moreover, such senescent bile ductules express chemotactic protein, such as Monocyte chemotactic protein 1 (MCP-1), which are likely responsible for HSC activation.¹¹⁷

Hepatic dendritic cells are the antigen-presenting cells in the liver. In inflammatory conditions, they are recruited in to liver sinusoids, and migrate to periportal and pericentral areas. They express TLR4/MD-2 complex, produce inflammatory cytokines (i.e. IL-12 and TNF- α), and express co-stimulatory molecules (CD40, CD80, and CD86) following several stimuli, such as LPS, peptidoglycan, poly-I: C, and cytidine-phosphate guanosine (CpG)-DNA.¹¹⁴

Cytokines

The synthesis of cytokines, such as TNF- α and IL-6, both involved in inflammatory and metabolic alterations, characterizes the earliest phases of liver injury, leading to the

synthesis of other cytokines that, jointly, induce cell migration and initiate healing processes, including fibrosis.¹¹⁸ A correlation has been found between TNF- α levels and fibrosis degree in NASH patients,¹¹⁹ indeed gene expression of either TNF- α or its receptor is significantly elevated in their hepatic and adipose tissues.¹²⁰ Similar correlation has been found in NAFLD patients, whose circulating TNF- α are significantly elevated concomitantly with the increase in the Nonalcoholic steatosis (NAS) score which is a histologic scoring system recognized as standard reference in the evaluation and gradation of hepatic inflammation and damage.¹²¹ In addition, progression of NAFLD correlates with polymorphisms in the TNF- α promoter region and serum level of soluble Tumor Necrosis Factor (TNF) receptor-2.¹²²

It has been also shown that adipose tissue-derived IL-6 regulates hepatic IR via up-regulation of Suppressor of cytokine signaling 3 (SOCS3).¹²³ Indeed, over expression of SOCS-3 in the mouse liver causes IR and an increase in sterol regulatory element-binding protein (SREBP-1c) that regulates fatty acid synthesis. Conversely, inhibition of SOCS3 in obese diabetic mice improves insulin sensitivity, normalizing the increased SREBP- 1c expression.¹²⁴ In humans, a significant association of high IL 6, IR and increased oxidative stress have been found in NAFLD subjects and correlated with advanced disease on histology.¹²⁵ When oxidative stress and cytokine levels of NAFLD subjects were compared with those of chronic viral hepatitis and healthy volunteers, NAFLD patients showed significantly higher levels of oxidative stress as well as cytokine levels.¹²⁶ Similarly, higher oxidative stress has also been documented in both diabetic and non-diabetic Indian NAFLD patients.¹²⁷

Recent studies have established a link between NASH and increased IL-1 β production,^{128,129} potentially via an increase in inflammasome activation.¹²⁹ Activation of the inflammasome and release of IL-1 β requires 2 signals: an endogenous danger signal and a TLR ligand.¹³⁰ A recent study has demonstrated that saturated fatty acids (SFAs) can elicit inflammasome activation.¹³¹ SFAs are known to increase sensitivity of primary hepatocytes to LPS *in vitro*, and administration of exogenous LPS *in vivo* can enhance IL-1 β levels and inflammasome activation in the setting of steatohepatitis. Evidence that SFAs induce hepatocyte apoptosis^{132,133} and DAMPs from dying hepatocytes can induce inflammasome activation and IL-1 β further suggests that SFAs “prime” the fatty liver for LPS-induced inflammasome activation.¹³³

Adiponectin/Visfatin/Leptin

Adiponectin, a hormone synthesized and secreted by adipocytes is considered as an anti-inflammatory adipokine¹³⁴ which trim down inflammation by stimulating secretion of anti-inflammatory cytokines (e.g., IL-10), slab-

ing NF-KB activation, and stalling release of TNF- α , IL-6 and chemokines. It has been shown that adiponectin levels were reduced in NASH in comparison to control and simple steatosis patients.¹³⁵ Nevertheless, adiponectin levels was inversely associated only with intrahepatic fat but not with inflammation and fibrosis.¹³⁶ It has been suggested that adiponectin may represent a link between hepatic fat and insulin resistance.¹³⁷

Visfatin, an adipocytokine predominantly expressed and secreted by visceral adipose tissue is of central importance in Nicotinamide adenine dinucleotide (NAD) biosynthesis, innate immunity and inflammation.¹³⁸ Our study showed that there was a significant decline in visceral adipose tissue visfatin level which was associated with degree of steatosis in NAFLD patients.¹³⁹ Moreover, our own unpublished data documented reduction of body weight as well as liver weight in high fat diet induced obese rats upon exogenous administration of visfatin in comparison to the untreated group of obese rats. It is to be noted that visfatin being important in NAD biosynthesis may be playing a role in energy balance which needs further investigations.

Leptin an adipocyte-secreted, negative feedback hormone whose levels are elevated in obesity is considered to have a lipostatic function, and is released into the bloodstream upon increase in the amount of fat stored in the adipocytes. Acting indirectly through the central neural pathway, and directly through the activation of AMPK (AMP-activated protein kinase), it prevents the occurrence of NAFLD where leptin levels are directly correlated to the severity of the disease.¹⁴⁰ However, the correlation of circulating levels of leptin in NAFLD progression is still obscure. Increased level of leptin in nonalcoholic steatohepatitis has been reported with higher in patients with advanced disease which was independently of BMI.¹⁴¹ An increase in serum leptin levels associated with a decrease in soluble leptin receptor, has been observed in subjects with moderate to severe steatosis. This strong negative correlation between serum leptin and soluble leptin receptor found in NAFLD implicates the role of peripheral leptin resistance in these patients.¹⁴²

ROLE OF GUT MICROBIOTA

Gut microbiota is now considered as a major metabolic internal organ, composed of $>10^4$ microorganisms and containing a second genome which is up to 100–400 times that of humans.¹⁴³ The intestinal microbiome is composed of bacteria, viruses, yeast and archae.^{144,145} Recently, there has been a growing body of evidence that implicate gut microbiota in NAFLD and its progression to NASH.¹⁴⁶ The advances of “culture independent techniques” focused on 16s ribosomal RNA gene sequencing, having led to linking of gut microbiota to obesity related NAFLD in humans as well as in animal models through the concept of gut-

liver axis gut derived products like lipopolysaccharides, bacterial DNA and peptidoglycans reach the liver through the portal vein which activates cell surface receptors and triggers a cascade of signal transductions leading to hepatic inflammation and fibrosis.¹⁴⁷ The microbiome composition of obese humans are distinct from normal weight individuals with a lower proportion of bacteroidetes and a higher proportion of firmicutes-Bacteroidetes ratio in NASH¹⁴⁸ though a recent study only found a relative lower abundance of bacteroidetes in NASH which was independent of BMI and energy intake from diet. The same was not found in simple steatosis and healthy controls.¹²⁷ An intact intestinal mucosal barrier acts as a filter to release of products of gut microbiome into circulation. A study by Miele et al has shown that both intestinal permeability and the prevalence of small intestinal bacterial overgrowth are increased in patients with NAFLD and correlate with the severity of steatosis. They have also suggested that the disruption of tight junction integrity in NAFLD leads to cross talk between the gut and the liver. The microbiota causes increased release of endogenous ethanol,¹⁴⁶ metabolizes dietary choline causing deficiency and releases LPS.¹⁴⁹ TLRs on hepatocytes, recognize these pathogen associated molecular patterns (PAMPs) associated with LPS, unmethylated DNA and lipopeptides and trigger pro inflammatory gene expression and nod-like receptors (NLR) family which upon activation forms molecular machines (“inflammasomes”) that regulate immune responses by processing cytokine precursors into active forms. In a study by Zhu et al, the composition of gut bacterial communities of NASH, obese and healthy children were determined by 16s ribosomal RNA pyrosequencing revealed that each group of subjects had a unique gut microbial composition. Proteobacteria, Enterobacteriaceae and Escherichia were the only phylum, family and genus types, exhibiting significant difference between obese and NASH microbiomes. Alcohol producing bacteria were also significantly higher in NASH microbiome possibly leading to increased oxidative stress and hepatic inflammation.¹⁵⁰

Whether the gut microbiome in non-obese NASH is similar to obese NASH is still an area of active and interesting research. The key is to identify a metagenomic sequence which will co relate to NAFL or NASH and translate it into a non invasive diagnostic tool for risk stratification of progression to NASH. Microbiota modulation to prevent or improve NAFL/NASH is an area on developing new therapeutic targets.

CONCLUSION

In the setting of NAFL, increased influx of FFAs within hepatocytes along with enhanced de novo lipogenesis with associated derangement of compensatory oxidative and endoplasmic reticulum (ER) stress processes can

generate Damp molecules to induce TLRs in various liver cells. These in turn induce downstream production of proinflammatory cytokines to augment inflammatory processes towards progression to NASH. Alternation in gut microbiota or a leaky gut can further aggravate the TLR sensitivity to LPS resulting in a shift from tolerance to immune-active condition in the liver contributing inflammatory processes towards NASH. In addition, emerging evidence suggest that there is a genetic susceptibility for hepatic steatosis in subjects who may or may not have the conventional risks. SNPs in PNPLA3 have been reported to be strongly associated with NAFL and progression to NASH. Whether all the above cross talks between genes, diet, metabolic abnormalities, immunological changes and gut microbiome translate phenotypically to disease progression from NAFL to NASH is still an area of future research.

CONFLICTS OF INTEREST

All authors have none to declare.

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