Deciphering the role of aberrant DNA methylation in NAFLD and NASH∗

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ABSTRACT

The global incidence of nonalcoholic fatty liver disease (NAFLD) is mounting incessantly, and it is emerging as the most frequent cause of chronic and end stage liver disorders. It is the starting point for a range of conditions from simple steatosis to more progressive nonalcoholic steatohepatitis (NASH) and associated hepatocellular carcinoma (HCC). Dysregulation of insulin secretion and dyslipidemia due to obesity and other lifestyle variables are the primary contributors to establishment of NAFLD. Onset and progression of NAFLD is orchestrated by an interplay of metabolic environment with genetic and epigenetic factors. An incompletely understood mechanism of NAFLD progression has greatly hampered the progress in identification of novel prognostic and therapeutic strategies. Emerging evidence suggests altered DNA methylation pattern as a key determinant of NAFLD pathogenesis. Environmental and lifestyle factors can manipulate DNA methylation patterns in a reversible manner, which manifests as changes in gene expression. In this review we attempt to highlight the importance of DNA methylation in establishment and progression of NAFLD. Development of novel diagnostic, prognostic and therapeutic strategies centered around DNA methylation signatures and modifiers has also been explored.

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is increasing at an alarming rate and has become a major health concern worldwide. NAFLD is the leading cause of liver fibrosis, cirrhosis and hepatocellular carcinoma (HCC) [1]. NAFLD is defined as a clinical condition with the evidence of hepatic steatosis in more than 5% of hepatocytes in individuals with a history of very little or no alcohol consumption [2]. Histologically NAFLD is broadly categorized into nonalcoholic fatty liver (NAFL) and nonalcoholic steatohepatitis (NASH) based on its pathophysiology. NAFL exhibits a more benign course, having hepatic steatosis with no evidence of hepatocellular injury, whereas NASH is the more aggressive form displaying steatosis, hepatocyte inflammation and ballooning with or without fibrosis which might progress to cirrhosis, liver failure and mortality [3]. Metabolic derangements in NAFLD along with its heterogeneous pathogenesis have led to modification in its nomenclature by several experts to metabolic fatty liver disease (MAFLD) [4]. The diagnosis of MAFLD in patient population can be sub-grouped into obese-MAFLD, lean-MAFLD and diabetic-MAFLD having variable severity, prognosis, and diagnostic parameters. This sub-grouping of patients may help in defining the heterogeneity of NAFLD. Dietary habits, physical activity, genetic, epigenetic, and immunological factors contribute to NAFLD heterogeneity [5]. Identification of these additional factors that could modulate the clinical outcomes of NAFLD may help in understanding the disease heterogeneity and approaches to personalized medicine.

Globally, the occurrence of NAFLD is estimated to be 15–30% of the total population, however in the obese population the prevalence of NAFLD is 50–90% [1, 6]. The prevalence of NAFLD is increasing even in pediatric population with White and Asian children exhibiting a higher prevalence than African American children [7]. A recent mathematical model has projected the prevalence of NASH to rise by 56% by the year 2030 in various countries [8]. According to epidemiological data, 3–15% of obese patients having NASH develop cirrhosis and 4–27% of obese-NASH-cirrhosis patients progress to HCC. HCC has also been observed in patients suffering from NASH without cirrhosis [9]. It has been projected that NAFLD is poised to become the prime trigger for development of end stage liver diseases and HCC over the next decade [10].

Early diagnosis of NAFLD is imperative for this silently progressive disease which is mostly asymptomatic till its transition to NASH or other degenerative liver disorders. NAFLD diagnosis has traditionally been
confirmed by liver biopsy which is sparingly used being invasive, expensive and not well accepted by the patients [11]. Various diagnostic tests and biomarkers based on combination of several indicators of steatosis exist that can be employed in clinical practice for early diagnosis and have been extensively reviewed [12]. Noninvasive or minimally invasive diagnostic methods include Visceral adiposity index (VAI), Fatty liver index (FLI), NAFLD liver fat score (NLFS), Triglyceride and Glucose Index (TyG), and Hepatic steatosis index (HSI). Fatty liver index which comprises body mass index (BMI), waist circumference, serum triglyceride, and gamma-glutamyltransferase levels is considered as the most practical, economical and precise in diagnosing NAFLD [12]. Imaging techniques like magnetic resonance imaging-Proton density fat fraction (MRI-PDFF), elastography, and controlled attenuation parameter (CAP) are emerging as definitive diagnostic criterion for assessing liver fat content [13].

Genetic predisposition as well as epigenetic modifications are of paramount significance in the development and pathogenesis of various diseases including NAFLD [14]. DNA methylation is a key epigenetic event that plays a pivotal role in regulation of transcription, embryonic development, genomic imprinting, genomic stability as well as chromatin structure [15, 16]. Abnormalities in the DNA methylation system has been implicated in the pathogenesis of various human malignancies [16, 17]. Aberrant DNA methylation patterns affect cellular homeostasis with hypermethylation and hypomethylation of global genomic DNA resulting in altered gene expression [18, 19]. DNA methylation patterns appear to be reversible and have been demonstrated to be modulated by various environmental and lifestyle stimuli in a number of studies [20]. Interplay of genetic and epigenetic factors, with obeseogic exposures, sedental lifestyle and high calorie/carbohydrate diet seem to be operational in fatty liver diseases [21]. In this review we highlight recent studies on aberrant DNA methylation profiles found in NAFLD and NASH patients and their alteration by lifestyle determinants. Vast clinical spectrum and diverse outcomes associated with NAFLD warrant an in-depth study of its pathogenesis and epigenetic aspects for better understanding and management approaches.

For this review, DNA methylation studies dealing with the establishment and progression of NAFLD to end stage liver disorders were searched in the PUBMED and NLM database during July 2021–May 2022. The keywords used were DNA methylation and NAFLD; DNA methylation and NASH; DNA methylation and HCC; Epigenetics and NAFLD; Epigenetics and NASH; NAFLD and Sex; NAFLD diagnosis and management; NAFLD Progression; NAFLD/NASH and Epidemiology; Epigenetics and Liver disease; DNA methylation and obesity; DNA methylation patterns and epigenetic memory; Exercise and DNA methylation; NAFLD/NASH and DNMT; TET enzymes and NAFLD/NASH; Homocysteine and NAFLD/NASH; Dietary methyl deficiency and liver carcinogenesis; Nutrition epigenetic and liver diseases. A wide range of reports were found in the database from which initially abstracts were screened after which relevant studies, review articles were selected and consulted for this review. Both animal and human studies were included in the review. Major findings of these studies have been discussed in the following sections.

2. Pathogenesis and risk factors of NAFLD

NAFLD is a multifactorial disease exhibiting complex and incompletely understood pathophysiology. The progression of NAFLD was initially explained using a “two hit hypothesis”, where the “first hit” is lipid accumulation in hepatocytes enhancing the vulnerability of the liver to “second hit” including a variety of damaging factors such as oxidative stress, genetic polymorphisms, epigenetic modifications, apoptosis, inflammatory pathways, dysregulated adipokine secretion, and stimulation of hepatic stellate cells [22]. With time, it has evolved into “parallel multiple hit theory” of NAFLD progression [23]. Insulin resistance acts as the “first hit” resulting in augmented uptake and synthesis of free fatty acids leading to ectopic fat deposition and steatosis in hepatocytes. Varied parallel processes contribute to the development of steatosis and liver inflammation including adipokine deregulation, endoplasmic reticulum stress, oxidative stress, mitochondrial dysfunction, inflammatory cascades, unfolded protein response, lipotoxicity, altered gut microbiota, chemokines, cytokines and innate immunity [24].

Dyslipidemia, insulin resistance (IR), metabolic syndrome, obesity and type-2 diabetes mellitus (T2DM) are noteworthy risk factors for NAFLD [25]. IR is the most familiar irregularity associated with NAFLD and a useful predictor of its severity and progression [26, 27]. IR leads to NAFLD either through increased de novo lipogenesis (DNL) or by increased free fatty acid (FFA) transport to the liver from adipose tissue [26]. Also, hyperinsulinemia secondary to IR leads to increased expression and activity of the sterol response element binding protein 1c (SREBP-1c), a transcription factor, which stimulates the expression of all enzymes essential for DNL [26, 28]. Therefore, the gravity of IR is strongly linked to liver steatosis and the prevalence of NAFLD among individuals having hyperglycemia and T2DM [29, 30]). Obesity is known to cause excessive levels of circulating FFA, augmenting the risk of fatty liver and development of IR. Notable association of NAFLD with overall fat mass, waist circumference and abdominal adiposity has been well documented [31]. The endocrine function of adipose tissue specifically visceral fat has been implicated in producing multiple pro-inflammatory cytokines mediating the clinical manifestations of NAFLD and its progression [32]. Most observational studies suggest a bi-directional correlation of NAFLD with metabolic syndrome [33]. NAFLD seems to display sexual dimorphism with higher prevalence in males, although in females this protection seems to wane after menopause [34, 35, 36]. NAFLD has been found to be widespread in non-obese asymptomatic population as well, further confounding its diagnosis and management [37].

Hypernutrition and high fat diet (HFD) can greatly increase the circulating levels of FFAs which get deposited in adipose tissue and liver leading to low grade chronic inflammation and IR [38]. The contributors of fats that lead to hepatic steatosis include lipolysis from peripheral adipose tissue adding to the unesterified pool, DNL and dietary fatty acids. According to a study by Donnelly et al, most of the fat (nearly 60%) arises from the non-esterified fatty acids pool in the blood (the source of this being adipose tissue); a quarter from de novo lipogenesis; and only a small fraction (approximately 15%) from the diet [39].

NAFLD represents a heterogeneous patient population where some individuals exhibit steatosis alone, while others develop hepatoocyte damage and advanced fibrosis. This heterogeneous pathophysiology of NAFLD could be explained by genetic and epigenetic variants, sex, and comorbidities such as obesity, T2DM and many others that critically influence disease progression, prognosis, and differential response to treatments. Genome wide association studies reveal a connection between specific genetic variants and NAFLD onset and severity. Some of the key genetic variants associated with the NAFLD/NASH risk are TM6SF2, PNPLA3, MBOAT7, GCKR, APOB, lysophospholipase-like protein 1 (LPL), neurocan (NCAN), HSD17B1 and protein phosphatase-1 regulatory subunit-3B (PPP1R3B) [40, 41, 42]. These genetic variants do not account for all NAFLD individuals. Polygenic risk scores (PRS) is being explored as a helpful tool to recognize patients with high risk of developing NAFLD and its associated complications [43, 44]. The association of genetic variants with NAFLD development can be used to predict the disease trajectories which might aid diagnosis and better prognostication [45]. A recent study by Bianco et al., using a PRS system involving MBOAT7, PNPLA3, TM6SF2 and SREBP-2 might enhance HCC risk gradation in NAFLD [44]. Genetic risk scores using genetic variants PNPLA3, TM6SF2, and HSD17B13 was also shown to increase the risk of cirrhosis by 12 folds in patients with NAFLD [46]. Variants of PNPLA3 have been shown to correspond with NAFLD prevalence across ethnicities [47]. Mutations in PNPLA3, which encodes for triacylglycerol lipase correlated with the presence of NAFLD and NASH [48].

Different genetic variants of NAFLD were found to be associated with specific comorbidities. SREBP-2, TM6SF2, and transcription factor-7-like
2 (TCFL2) are predominantly present in individuals having increased risk of T2DM whereas adiponectin gene (ADIPOQ) and Src homology (SH2B1) are associated with obesity [49, 50, 51]. These genes are also linked with the dysregulated glucose and lipid homeostasis, IR, NAFLD, obesity and T2DM indicating greatly intertwined, shared mechanisms of disease pathogenesis [52, 53]. Therefore, these genetic variants are in agreement with the view that metabolic dysfunction is the prime driver of NAFLD. Interestingly some of the genetic variants associated with increased risk of NAFLD were associated with decreased risk of certain other metabolic diseases, for instance, GCKR P446L variant was reported to reduce the risk of T2DM [54]. The proposed mechanism for this observation was induction of DNL leading to decreased triglyceride levels, concomitant with improved hepatic glucose metabolism [55]. Similarly, PNPLA3I148M, and TM6SF2 variants were found to have inverse relationship with metabolic disorders such as T2DM and cardiovascular diseases [54, 56]. Thus, the genetics of fatty liver disease with metabolic variables is complex and warrants further studies. Pathogenesis of NAFLD might be cumulative of both metabolic as well as a genetic components which are fundamentally different [57].

Apart from the variants of a gene, its epigenetic modifications under different environmental conditions results in altered cell physiology. Epigenetics deals with reversible alterations in expression of gene and associated phenotype that are not due to the alteration of DNA sequence. Association of epigenetic dysregulation with the onset and progression of NAFLD is being extensively investigated [58]. Available literature has shown the association of circulating PPARγ methylation level with fibrosis in NAFLD patients and deacetylation of SIRT1was observed to improve hepatic steatosis [58, 59]. Therefore, targeting the epigenetic factors may be one of the mechanisms for drug action to attenuate the onset of NAFLD, for example resveratrol and glucagon like peptide-1 receptor agonists (GLP-1RAs), as activators for SIRT1 alleviate the diet induced fatty liver in animal models [60, 61, 62]. Besides, vorinostat, an inhibitor of Histone deacetylase (HDAC) that inhibits the lipid synthesis in liver cells in vitro, can be a candidate drug in the treatment of NAFLD [63]. Epigenetic modifiers are being investigated as potential drug targets for NAFLD. Over the past two decades epigenetic alterations leading to NAFLD have been investigated and are being recognized as major determinants in its pathogenesis [15, 64]. Recently Lonardo et al., have deliberated on the approaches targeting the genetic background, epigenetic modifiers and clinical features to achieve the goal of precision medicine in NAFLD patients [65]. Therefore, genetic markers and their epigenetic modification with clinical information would help in precise NAFLD classification and its management.

3. DNA methylation in NAFLD

Epigenetics is an indispensable biological process dealing with alteration in expression of gene or modification of cellular pathways by adaptive mechanism that are not associated with alteration in DNA sequence [66]. It encompasses many factors such as DNA methylation, acetylation, phosphorylation, copy number alterations, miRNA and chromatin modifications. Changes in gene expression due to aberrant epigenetic alterations leads to the development of various diseases [67]. DNA methylation is one of the prime epigenetic mechanisms that can regulate gene expression. During this modification methyl group is added to the fifth carbon of cytosine resulting in 5-methylcytosine (5mC) at CpG islands of DNA [68, 69]. CpG islands found in the regulatory sites of gene are mostly unmethylated whereas, those present in non-regulatory regions are typically methylated [70, 71, 72, 73]. It has been found that approximately 1% of DNA bases are methylated cytosines representing 70–80% of CpG dinucleotides [69].

DNA methylation is critically involved in gene expression with 5mC as an established and inherited epigenetic feature which typically suppresses transcription. Mechanism operating in the inhibition of gene expression by cytosine methylation is either the prevention of the interaction of transcription factors or through recruitment of DNA binding domains having affinity for 5mC for example, 5-methyl-CpG binding domain 1-4, methyl CpG binding protein 2 and Kaiso initiate the formation of compact structure by interacting with histone-modifying enzymes, thereby affecting the cell/tissue specific gene expression [74, 75, 76]. Also, cytosine methylation can affect the chromatin structure and participate in X chromosome inactivation, genome imprinting as well as cellular differentiation [77].

The process of DNA methylation is mainly carried out by a set of enzymes collectively called DNA methyl transferases (DNMTs). During their catalytic reactions they transfer the methyl group to 5mC from S-adenosyl l-methionine (SAM) to cytosine [78]. Among the different DNMTs present in humans, DNMT1, DNMT3A and DNMT3B are implicated in methyltransferase activities [73]. DNMT1 copies CpG methylation patterns and is vital for carrying forward such methylated patterns to the newly synthesized daughter strand during DNA replication. DNMT3A and DNMT3B are de novo methyltransferases responsible for installing DNA methylation patterns during gametogenesis and early embryogenesis [79]. For the stable maintenance of methylation status DNMT3A and DNMT3B conjoin with DNMT1.

Another base modification in DNA namely, 5-hydroxymethylcytosine of cytosine (5hmC) has added new layers to the understanding of epigenetic mechanisms in development of diseases. It influences both activation as well as repression of gene transcription by influencing DNA demethylation [80]. 5hmC is enriched in expressed genes and enhancer sequences in DNA. 5hmC exhibits tissue specific expression and depending on the cellular conditions it undergoes epigenetic modification in accordance with the environmental and metabolic state. 5hmC is abundant in human hepatic genomic especially in genes actively involved in energy metabolism [81]. Formation of 5hmC in DNA occurs through the oxidation of 5mC by a group of enzymes known as Ten-Eleven translocation (TET) enzymes. These iron-dependent enzymes have a dioxygenase activity. They act as 5mC hydroxylases using alpha-ketoglutarate as their co-substrate. TETs have the ability to reverse locus-specific DNA methylation. These 5mC oxidation products then mediate the transformation of 5mC to unmodified Cs [82]. TETs act as regulators of epigenetic processes as generation of 5hmC is critical for DNA demethylation [83]. Three TET enzymes have been identified in mammals so far, namely, TET1, TET2 and TET3 [82].

The mechanisms operating in methylation and demethylation of DNA, influenced by myriads of known and unknown factors clearly point toward a very complex and dynamic landscape of DNA methylation. DNA methylation is implicated in numerous cellular processes such as somatic, germline and embryonic development, repression and expression of genes, transposition and pathogenesis of diverse disorders. With the rapid pace of technical advancement and newer genetic manipulation tools, the future of mammalian DNA methylation and demethylation research holds great promise in deciphering novel mechanisms and therapeutic tools for various human diseases [84].

Altered methylation patterns of hepatic DNA for various genes has been depicted in Figure 1 [85]. Understanding the aberrations in DNA methylation system which might account for some principal features of NAFLD pathobiology will greatly augment our knowledge of the DNA methylation landscape in disease process. This has been dealt in the following sections.

3.1. Establishment and progression of NAFLD to NASH

Liver is the central organ participating in various metabolic pathways of carbohydrates, lipids, and proteins metabolism and is also involved in detoxification reactions [32]. Liver fat content is regulated by a dedicated set of enzymes and proteins involved in transport of lipids, lipogenesis and their oxidation inside the liver as well as their secretion from liver to extra hepatic tissues. Dysregulation in any of these processes can manifest in liver steatosis and NAFLD development [86]. As mentioned earlier, NAFLD can be broadly categorized into NAFL exhibiting simple benign form of steatosis and NASH representing the more advanced form
resulting in progressive fibrosis, cirrhosis and mortality [3]. Epigenetic mechanisms have been implicated in these changes in the liver physiology. The well-studied epigenetic modulation being DNA methylation and demethylation. Methyl donor for these methylation reactions comes from different pathways and methionine metabolism is prime among them, where SAM is sequentially demethylated to S-adenosylhomocysteine and homocysteine [87]. Elevated homocysteine levels have been observed in NAFLD patients compared to healthy controls suggesting an impaired methionine metabolism leading to decreased concentration of methyl donors such as methionine, choline and betaine in liver [88]. This could be a manifestation of hepatic injury, inflammation, oxidative stress and unfolded protein response owing to hepatic fat accumulation [88]. Elevated hepatic homocysteine levels with methionine deficiency, resulting from impaired homocysteine remethylation and aberrant methyltransferase reactions has been observed in diet induced NAFLD in mice. Further, decreased expression of DNMT3A gene was noticed in these mice although global pattern of methylation of DNA was unaltered [89]. Lai et al., observed significantly lower levels of global DNA

Figure 1. Aberrant DNA methylation in NAFLD: An illustration of the genes modified by epigenetic regulation due to their hypo-and hypermethylation in context of development and progression of NAFLD/ NASH. Transforming growth factor beta1 (TGFβ1), platelet derived growth factor-α (PDGFα), peroxisome proliferator activated receptor-α (PPARα), peroxisome proliferator activated receptor-δ (PPARδ), mitochondrial encoded gene NADH dehydrogenase-6 (MT-ND6), PPARγ coactivator 1a (PPARGC1A), hepatic dipeptidyl peptidase 4 (DPP4), pyruvate carboxylase (PC), ATP citrate lyase (ACLY), phospholipase C gamma 1 (PLCG1), insulin like growth factor 1 (IGF1), Insulin like growth factor binding protein-2 (IGFBP2), protein kinase C epsilon (PKCε), polypeptide N acetylgalactosaminytransferase like 4 (GALNTL4), glutamate ionotropic receptor delta type subunit 1 (GRIKD1) and inositol hexakisphosphate kinase 3 (IP6K3), Fibroblast growth factor 2 (FGFR2), caspase 1 (CASP1), methionine adenosyl methyltransferase (MAT1A), Acyl-CoA synthetase long chain family member 4 (ACSL4), carnitine palmityltransferase 1 C (CPT1C), ARH/RhoGEF, pleckstrin domain protein1 (FARP1) Apolipoprotein (APOs), Niemann-Pick C1-Like 1 (NPC1L1), STAR related lipid transfer domain 10 (STAR10), STAR related lipid transfer domain 4 (STAR4), Grainy head like transcription factor-3 (GRHL3), Cyclin dependent kinase inhibitor 2A (CDKN2A), Cytochrome P450 family 27 subfamily A member 1 (CYP27A1), Solute Carrier family 51 sub-unit alpha (SLC51A), Solute Carrier family 27 member 5 (SLC27A5), Solute carrier organic anion transporter family member 2B1 (SLCO2B1), Suppressor of cytokine signaling 1 (SOCS1), Ras association domain family member 1 (RASSF1), Hypermethylated in cancer (HIC1), Glutathione S-transferase pi1 (GSTP1), Solute Carrier family 47 member 1 (SLC47A1), Solute Carrier family 7 member 11 (SLC7A11), Branched chain amino acid transaminase-1 (BCAT1), Long intergenic nonprotein coding RNA 649 (LINC000649), Adenomatous polyposis coli (APC).
methylation in liver biopsies along with increased serum homocysteine levels in NAFLD patients compared to overweight controls. Furthermore, lower hepatic global DNA methylation levels and hyper-homocysteinemia was associated with increased hepatic inflammation grade, disease progression and histological severity of NAFLD [90]. This clearly points towards elevated levels of homocysteine to be an effect of aberrant DNA methylation rather than being a cause of metabolic abnormalities.

In a study of liver mitochondrial DNA methylation by Pirola et al., significantly higher methylation of mitochondrial gene NADH dehydrogenase-6 (MT-ND6) and lower mRNA levels was reported in NASH patients in comparison to simple steatosis patients. This points towards an association of hepatic methylation and transcriptional downregulation of MT-ND6 with the severity of NAFLD [91]. It was also observed that epigenetic alterations of mtDNA were potentially reversible using intervention programs like physical activity as status of hepatic MT-ND6 methylation in NASH group inversely correlated with regular physical activity [91]. Another observational study by Pirola et al., suggested that 5-hmc level was associated with development of NAFLD via dysregulation of the expression of PPARGC1A coactivator 1a (PGC-1a) [92]. Also, while exploring the levels of PPARGCA, an association was observed with a reduction in nonnuclear 5-hmc in NAFLD patients whereas liver PPARGCA mRNA levels were relatively associated with global 5-hmc levels. This suggested that its methylation status modulates its transcriptional activity [92]. PPARGCA encoded PGC-1a acts as a transcriptional coactivator regulating genes involved in cellular respiration, energy homeostasis, mitochondrial biogenesis, adaptive thermogenesis, adipocyte development, and lipid and glucose metabolism. Environmental signals have a great impact on its activity and synchronize the genes involved in metabolism by interacting with certain transcription factors and chromatin remodelers [93]. TET variants namely, p. Ile1123Met missense (TET1-rs9998860) and p. Ile1762Val substitution (TET2-rs2454206) were appreciably correlated with serum content of CK-18 fragment (cell death biomarker) and liver PPARGCA methylation as well as transcript level of the same. These genetic variants of TET demonstrate the involvement of epigenetic modification in regulation of hepatocyte apoptosis and TET induced regulation of transcription of PPARGCA in response to cellular metabolic milieu in NAFLD patients [92]. In another study, insulin resistance and liver transcriptional activity of PPARGCA displayed a close interaction in NAFLD where liver PPARGCA promoter methylation directly correlated with plasma fasting insulin levels and inversely correlated with liver mitochondrial DNA methylation level [94].

The advent of Genome wide DNA methylation profile analysis has facilitated screening large sample sizes from various human tissues [95]. Ahrens et al., using genome wide DNA methylation analysis from liver samples of 45 morbid obese patients in different stages of NAFLD, extracted 9 NAFLD associated genes exhibiting variation in methylation. These genes are primarily involved in intermediary metabolism and insulin signaling including pyruvate carboxylase (PC), insulin like growth factor-1 (IGF1), ATP citrate lyase (ACLY), glutamatecotropic receptor delta type subunit-1 (GRID1), phospholipase C gamma-1 (PLCG1), Inulin like growth factor binding protein-2 (IGFBP2), inositol hexakisphosphate kinase-3 (IP6K3), polyphenol O-acetylgalactosami- nytransferase like-4 (GALNT4), and protein kinase C epsilon (PRKCE) [96]. In a study of gene cohorts, methylation drifts in forty one genes involved in lipid metabolism and fourteen genes in vitamin D and energy balance were reported in NAFLD including novel genes namely, members of AMPK pathway (UDP-glucose transporter, NPC1L1, RAB7, STARD4 (cholesterol transport) and GRHL3 (energy balance)) [97]. In a related study using systematic analysis of drug methylation and bile acid homeostasis genes, it was shown that 64 genes were differentially methylated in NAFLD. Among those, altered methylation was significantly correlated with transcription of 26 genes including CYP27A1, OSTA, SLC27A5, SLC02B1, SLC47A1 and several UGT and CYP genes [98]. In another study, levels of hepatic dipeptidyl peptide 4 (DPP4), a hepatokine secreted by hepatocytes, was also found to be elevated in NAFLD and NASH patients [99].
APC/β-catenin destruction complex thereby displaying its anti-proliferative effect. This effect was not observed in male mice, suggesting that NAFLD development and its epigenetic regulation varies with sex [114]. Since most studies have used only male animals, it’s difficult to assess the extent of the sex-based differences in epigenetic alterations, warranting further investigation.

3.3. Progression from NASH to fibrosis and cirrhosis

Heightened inflammation in NASH can give rise to progressive fibrosis which can result in cirrhosis. 152ifferentially methylated CpG islands for transcription factors and developmental pathways were observed between NASH subjects and controls. Subjects with NASH also exhibited epigenetic age progression correlating with hepatic collagen content and extent of fibrosis [115]. Hence, DNA methyl signature is being suggested as a measure of chronological age as well as biological age [116].

Regulation of specialized enzymes including DNMTs and TETs, that catalyze DNA methylation system in a series of chemical reactions have the potential to reprogram genome-wide methylation patterns [117]. Any changes in their activity or expression can greatly influence global as well as gene specific methylation of DNA. In a study by Page et al., global alterations in Smc, 55 hmC and reduced expression of TETs with overall increased levels of DNMT3A, DNMT3B and DNMT1 were observed in animal and human fibrotic livers as well as in activated hepatic stellate cells. It was observed that a directed knock down of DNMT3A expression led to decreased pro-fibrotic phenotype in activated hepatic stellate cells [118]. In a targeted DNA methylation study of CpGs of five genes implicated in modulating fibrogenesis, hypomethylation of pro-fibrogenic genes including transforming growth factor beta1 (TGFβ1) and platelet derived growth factor-α (PDGFα); while hypermethylation of anti-fibrogenic genes including peroxisome proliferator activated receptor-α (PPARα) and peroxisome proliferator activated receptor-δ (PPARδ) was reported in advanced NAFLD patients compared to mild NAFLD patients. Thus at specific CpGs, methylation status of DNA might serve to predict severity and progression of NAFLD to liver fibrosis [119]. Decreased methylation of Cpg26 in Parvin beta variant1 (PARVB variant1) and increased methylation of Cpg99 in patatin like phospho lipase domain containing protein 3 (PNPLA3) was observed in livers of patients with severe NAFLD (stages 3 and 4) as compared with those having mild NAFLD (stages 1 and 2) and were shown to be correlated with severity of NAFLD [120]. In a study by Murphy et al., 69,247 differentially methylated CpG sites (76% hypomethylated, 24% hypermethylated) were reported in advanced NAFLD patients as compared to mild patients. It was found that 7% of these differentially methylated CpG sites correlated with transcript levels establishing a relationship between methylome and transcriptome of NAFLD patients. Various genes were found to be hypomethylated and over expressed including Fibroblast growth factor 2 (FGFR2), involved in epithelial mesenchymal transition and caspase 1 (CASP1) known to be implicated in inflammation and liver fibrosis. Also, genes involved in 1-carbon metabolism including methionine adenosyltransferase (MATIA) which is involved in synthesis of SAM, showed reduced expression due to increased methylation in advanced NAFLD compared to mild disease (based on histological severity of fibrosis) [121]. In a study comparing NAFLD patients with or without severe fibrosis using EWAS aberrant methylation of seven fibrosis related CpGs was reported. Cell type proportions of liver cells was well correlated with the development of fibrosis where natural killer cells were augmented whereas epithelial cell number dropped with increasing severity of fibrosis [122]. Hardy et al., reported promoter hypermethylation of PPPAR by plasma circulating cell free DNA (cfDNA) from NAFLD patients exhibiting severe form of fibrosis in comparison to NAFLD subjects having mild fibrosis [58].

Overexpression of branched chain amino-acid transaminase 1 (BCAT1) which converts alpha-ketoglutarate to glutamate plays vital role in citric acid cycleand oxidative phosphorylation has been linked to the occurrence and severity of NAFLD and NASH [123]. BCAT1 was found to be over expressed and hypomethylated in NAFLD patients experiencing adverse clinical outcomes [124]. Thus, it is evident that DNA methylation patterns could be used for detecting and predicting fibrosis in NAFLD patients.

3.4. The hepatocellular carcinoma (HCC) risk

The liver exhibits a unique characteristic of regeneration which concurrently makes it vulnerable to tumorogenesis. It has been observed that enduring hepatic disorders, damage or fatty infiltration frequently leads to liver cancer [125]. NAFLD along with NASH are well recognized hepatic pathologies which significantly increase the risk for development of HCC [1, 126, 127]. Distinctive modulations in gene expression due to anomalous DNA methylation patterns of some genes might promote the progression of NAFLD/NASH associated HCC. In a study by Dreval et al., investigating liver gene expression along with DNA methylation changes in mice using Stelic Animal Model (STAM) of NASH derived hepatocarcinogenesis, it was found that aberrantly expressed genes had greater accrual of DNA methylation abnormalities with majority of atypically methylated genes found in fully developed HCC. One of these genes, tubulin alpha 2B class II (Tubα2B), was found hypomethylated and overexpressed as liver carcinogenesis progressed [128]. This gene is a member of a family that encodes several microtubule cytoskeletal α- and β-tubulin proteins playing essential roles in cell differentiation, motility and shape which are abnormally expressed in human cancers including HCC [129, 130]. Another study by Margai et al., using relevant mice models (diet induced NAFLD mice, STAM of NASH derived HCC as well as choline and folate deficient diet mice models exhibiting NAFLD to HCC progression that resemble carcinogenesis in humans) characterized progressive increase in glycine N-methyltransferase (Gnmt) promoter methylation and corresponding reduction of Gnmt expression [131]. One consequence of GNMT decrease was found to be an increase in methylat ion along with an increase in SAM levels. GNMT is an abundant SAM dependent hepatic methyltransferase having folate binding and tumor suppressing features that transfers methyl group to glycine from SAM forming sarcosine and SAH [132, 133]. Decreased GNMT level was also observed in human HCC tissues as well as liver cancer cell lines [131]. In GNMT knockout mice, it was previously demonstrated that aberrant methylations result in steatosis and hepatocarcinogenic pathways in mice [134]. The authors suggested that assessing Gmnt methylation could be used for NAFLD stratification.

In a meta-analysis, decreased mRNA levels of PNPLA3 while increased expression of PARVB was correlated with NAFLD succession to HCC [135]. PNPLA3 protein is a lipid droplet associated protein having triglyceride hydrolyase activity. Altered expression and hypermethylation of PNPLA3 is associated with liver fat accumulation, inflammation, fibrosis, cirrhosis and HCC development [120, 136]. In another study it was shown that the liver biopsies of NAFLD patients with increased hepatocyte ballooning exhibited accrued oxidative DNA damage that could lead to promoter hypermethylation of tumor suppressor genes including HIC1, GSTP1, SOCS1, RASS1, Cdkn2a and APC, which could increase the risk of hepatocarcinogenesis in NAFLD subjects [137]. In a recent study, by performing differentially methylated region (DMR) network analysis, Kurokawa et al., identified two NAFLD specific DMR networks in NAFLD, viral hepatitis and HCC. The authors demonstrated that the methylation status of DMR-2 altered in a cooperative and/or synergistic manner during HCC establishment. Aberrant methylation levels of fatty acid binding protein-1 (FABP1), serum/glucocorticoid regulated kinase-2 (Sgk2) and hepatocyte nuclear factor 4 alpha (Hnf4α) were found from fibrosis to cancer making these as attractive therapeutic targets in NAFLD and associated HCC [138]. Using genome-wide DNA methylation analysis as well as mRNA expression levels in tissue samples of HCCs derived from precancerous background of NASH, Tian et al. found that deviant DNA methylation patterns resulted in altered gene expression in NASH associated HCC. It was observed that DNA
hypothesis as well as mRNA overexpression of tumor related genes including DCAF4L2, CKLF, UBEC2, TUBA1B, TRIM4 and PRCI occurred in NASH related SCC which were frequently linked to the necroinflammatory status of NASH and with poor tumor differentiation [139].

4. Lifestyle variables as DNA methylations modifiers

Over the past few decades, changes in lifestyle patterns have led to global rise in cluster of metabolic diseases including obesity, IR, T2DM and NAFLD. Dietary pattern and macronutrient composition impact theattenuation/development of NAFLD. Hypercaloric and HFD have been linked to the development of NASH by increasing the intrahepatic fat content [140, 141]. Several studies have shown that saturated fat consumption promotes hepatic fat accumulation [142]. Saturated fats are mostly found in processed food, high-fat dairy products red meat, cakes, cookies etc. [143]. Decreased consumption of carbohydrates having a high glycemic index, fructose content, sweeteners and sugar sweetened beverages have been reported to ameliorate NASH symptoms [144, 145]. Studies exploring the role of dietary proteins in relation to NAFLD [146, 147]. High intake of meat, particularly red meat and processed beverages have been reported to ameliorate NAFLD symptoms [144, 145]. Studies exploring the role of dietary proteins in relation to NAFLD have given conflicting results. Some reports have demonstrated positive association with NAFLD, whereas others have shown an inverse relation. These inconsistencies may be due to type of protein consumed [146, 147]. High intake of meat, particularly red meat and processed meats is well recognized to be associated with cardiovascular diseases, insulin resistance and T2DM [148]. Proteins from animal sources seem to promote NASH, whereas those from plant sources have the opposite effect [149]. Excessive intake of calories, sugars, saturated fats, fructose, animal protein, salt as well as cholesterol increase the incidence and progression of NAFLD and NASH [150]. Hence, dietary habits characterize the overall amalgamation of foods that might lead to synergistic health effects. One of the most well studied dietary pattern is the conventional Mediterranean diet, characterized by low intake of red meat, processed food, sugar and refined carbohydrate. Mediterranean diet has been consistently shown to protect against not only cardiovascular diseases and T2DM but also NAFLD [151, 152].

“Nutriepigenomics” a fast-evolving field that involves the study of nutrient-genome interactions via epigenetic alterations is rapidly gaining prominence in various complex human disorders [153, 154]. Food intake influences epigenome remodelling considerably all throughout life. Maternal dietary habits appear to be significant during pregnancy as well as postpartum, that leads to epigenetic alterations affecting the susceptibility to metabolic diseases by reprogramming the metabolic milieu [154]. In a study by Wankhade et al., it was concluded that maternal HFD negatively influences littersmate DNA methylation and gut microbiota to augment the development of NAFLD and its advanced sequelae [155]. Wang et al., showed that high glucose (HG) induced lipid accumulation in hepatocytes in cell culture and increased levels of nuclear 25-hydroxycholesterol (25SHC), an oxysterol, which specially activated the DNMT1. This significantly increased methylation of over 2000 genes belonging to 57 pathways of fuel metabolism such as PI3K, AMP mediated insulin secretion, diabetes and NAFLD signaling [156]. HG diet induced cholesterol as well as triglyceride biosynthesis and increased 25SHC levels. The HG diet also leads to excess accumulation of acetyl-CoA which is utilized for cholesterol as well as oxysterols (25SHC) biosynthesis. 25SHC has been shown to be a ligand of liver-X-receptors which are important in lipid metabolism. This increases the expression of vital enzymes of lipid biosynthesis like, ACC and FAS and increases the concentration of intracellular lipids through SREBP-1C cascade. OXysterols have adverse effects on liver and are associated with the pathogenesis of various disorders such as metabolic syndrome and NAFLD, thus exacerbating the role of HG in liver damage [157].

Dietary deficiencies of methyl donors like choline, Vitamin B12, folic acid and betaine are linked with altered methylation of DNA that favors the progression of NAFLD and liver carcinogenesis. These methyl donors are required for SAM synthesis thus diet can greatly influence DNA methylation [158]. A methyl deficient diet induces liver injury in mice analogous to human NASH. In an investigation using mouse gene expression model and CpG microarrays, it was found that CpG islands of 164 genes were demethylated in mouse livers, displaying an inverse relationship with expression of specific genes following a diet devoid of methyl donors. These 164 genes are involved in glucose and lipid metabolism, DNA repair, apoptosis, fibrosis and liver tissue remodeling [159]. In a study by Cordenò et al., newborn rats whose mothers were on an obesogenic diet had greater intrahepatic fat content. Hyperhomocysteinemia induced by maternal high fat-sucrose diet was prevented by maternal methyl donor supplementation. DNA methylation metabolism in liver was also affected by maternal dietary pattern due to perturbation in one-carbon metabolism [160]. Cordenò et al., also demonstrated in mice that methyl donor supplementation could revert HFD induced hepatic triglyceride buildup and altered DNA methylation pattern of fatty acid synthase (FASN) promoter [161]. Mice on choline deficient diet leading to disturbed one-carbon metabolism exhibited reduced PPARα expression and hypermethylation of PPARα promoter [162]. It was shown that betaine supplementation in HFD fed mice resulted in global methylation and decreased promoter methylation of microsomal triglyceride transfer protein (MTP) gene, promoting liver triglyceride export and reducing hepatic steatosis [163]. In another report it was shown that SAM supplementation attenuated triglyceride accumulation in alcoholic microgips with fatty liver disease [164]. McCurdy et al., showed that HFD feeding in non-primate mothers makes the developing fetus highly vulnerable to excess lipids triggered lipotoxicity in fetal livers increasing the risk of pediatric NAFLD [165]. It was shown by Wesołowski et al., that western style diet of mothers induces oxidative stress and altered intracellular transport while, shifting obese mothers to a healthy diet improved liver metabolites and lipotoxicity in primate models [166].

NAFLD is speculated to be reversed by modulating dietary patterns. In study by Kim et al., HFD fed mice were subsequently fed with a normal chow diet resulting in reversal of diet-induced NAFLD with altered DNA methylation in important hepatic genes through Genome wide DNA methylation analysis, although lipid regulating APOA4 gene remained hypomethylated [167]. In another study it was shown that maternal obesity during pregnancy and lactation in dams animal model, programs the development of NAFLD phenotype in offspring [168]. Offspring of HFD fed mothers in rats exhibit CpG hypomethylation and increased expression of hepatic cell cycle inhibitor CDKN1A suggestive of hepatic dysfunction [169]. In a related study by Chen et al., maternal high fat/cholesterol diet exacerbated the establishment of NAFLD in male offspring by low hepatic ApoB gene expression and hypermethylation of CpG dinucleotides in the promoter region of ApoB gene compared to male offspring born to chow fed mice. Increased methylation of ApoB gene is essential for VLDL assembly and low expression of Apo B in livers contributing to altered lipid profile and lipid transport from liver [170]. Deregulated Nuclear factor erythroid related factor2 (NRF2) signaling cascades play an important role in liver diseases. In HFD fed mice resveratrol led to hepatic hypomethylation of Nrf2 promoter which correlated with reduction in triglyceride levels [171]. Thus, consumption of polyphenols and anti-oxidants like resveratrol with potent anti-oxidant effects could be beneficial in NAFLD. Lyal et al., showed that diet induced hepatic steatosis in mice was linked to reversible 5-hmC changes in functionally essential genes, hence 5-hmC profile could be a potential marker for NAFLD state [172]. In mice fed on lipogenic, methyl donor deficient diet caused liver injury similar to human NASH. In this model, deviations in the expression of DNMT1 and de novo DNMT3a proteins were observed linking epigenetic alterations to hepatic steatotic pathogenesis [173].

A sedentary lifestyle is an important risk factor for NAFLD, obesity and T2DM [174]. Exercise training noticeably impacts DNA methylation in a tissue and locus specific manner [175]. A recent study by Wu et al., low-carbohydrate diet (LCD), and exercise plus low carbohydrate diet (ELCD) intervention, in both humans and mice NAFLD models showed GAB2 promoter hypermethylation thereby repressing mRNA expression.
GAB2 plays an important role in differentiation, proliferation and cell migration by recruitment of factors like Ras, p85 and Shp2. This study concluded ELCD intervention as an effective approach for the management of NASH through epigenetic modulation by environmental factors [176]. In an investigation assessing the role of low-carbohydrate/Mediterranean and low-fat diets with or without physical activity in 120 human participants, differential DNA methylation patterns and increased intrahepatic fat accumulation was observed (measured by MRI) after 18 months of lifestyle intervention. At the baseline, specific CpG methylation of AC074286.1, calcium release activated channel regulator2A (CRACR2A), alpha-2-macroglobulin pseudogene1 (A2M1P), ARH/RhoGEF and pleckstrin domain protein1 (FARP1) loci were inversely correlated with intrahepatic fat where FARP1 methylation achieved similar predictive value for NAFLD as obtained by MRI based-diagnosis. After 18 months of dietary intervention an altered pattern of DNA methylation in A2M1P was observed whereas CpGs of AC074286.1, CRACR2A and FARP1 were altered in different physical activity groups [177]. Thus, this study demonstrates a putative role of epigenetic remodeling subsequent to lifestyle interventions. Zhou et al., demonstrated that fast-food diet in mice elevated liver epigenome susceptibility to metabolic disorders concomitant with genome-wide differential DNA methylation. Physical exercise was observed confer protection against this fast food diet induced reprogramming of the epigenome and DNA hypermethylation [178]. Role of physical exercise in liver health emphasizing the role of epigenetics has been extensively reviewed by Stevanovic et al. [179]. Ahrens et al., in an intraindividual comparison of liver biopsies pre and post bariatric surgery observed NAFLD-linked methylation signatures to be distinct and partially reversible, providing an example of treatment induced epigenetic organ remodeling [96].

5. Conclusion

In the present review we have summarized the recent studies on the effects of DNA methylation patterns in NAFLD pathogenesis. Altered CpG methylation patterns known till now may be a snapshot of a highly active state that may be more dynamic than earlier hypothesized, encompassing an amalgamation of genetic, epigenetic, inflammatory, environmental and nutritional effects. There may be more multilayered alterations in the global and gene specific methylation patterns in NAFLD. Numerous studies in human and animal models suggest altered DNA methylation patterns at global and locus specific levels leading to hepatic lipid accumulation, steatosis, inflammation, and injury responsible for establishment and progression of NAFLD. Altered DNA hyper and hypo methylation patterns are correlated with altered expression of specific genes. The changes that lead to alterations in DNA methylation and expression of enzymes regulating these are beginning to get unraveled. DNA methylation status seems to be modifiable particularly with methyl donor supplementation in diet. Lifestyle variations like exercise, losing weight and switching from high fat/sugar diet to low carbohydrate diet has the potential to reverse the altered liver methylome in NAFLD. Further studies are required to decipher the associated mechanisms and to comprehend the complex epigenetic alterations in NAFLD establishment and progression. Thus, DNA methylation holds promise in understanding the interplay of genetic, lifestyle and metabolic factors in NAFLD development and progression. The augmented understanding would help in developing new diagnostic and therapeutic strategies for the timely management and risk stratification of NAFLD and NASH.

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