Chapter 12
Developmental Programming of Nonalcoholic Fatty Liver Disease (NAFLD)

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Abstract Nonalcoholic fatty liver disease (NAFLD) is currently the most common cause of chronic liver disease worldwide and is present in a third of the general population and the majority of individuals with obesity and type 2 diabetes. The less severe form of the disease is relatively common and can be somewhat benign. However, in certain individuals, the disease can progress to the more severe nonalcoholic steatohepatitis (NASH), resulting in a poor health, a poor prognosis, and a significant healthcare burden. In recent years, there has been a major research effort focused on identifying the factors that promote NALFD disease progression, and as a result there has been a significant advancement in our understanding of the interaction between nutrition and the molecular mechanisms that regulate hepatic lipid homeostasis. Nonetheless, the capacity of the maternal diet to alter these fundamental metabolic pathways and thus prime the development of severe fatty liver disease in the adult liver has proved to be one of the most striking findings from this body of research. Since the prudence of the maternal diet has wavered in recent years, this may explain why NAFLD—once commonly associated with older individuals—is now increasingly common in young adults, children, and adolescents. In the following chapter, we aim to review the current hypothesis surrounding the mechanisms that underlie the developmental priming of NAFLD. We will also explore how these novel insights have facilitated the emergence of promising new pharmacological and nutritional intervention strategies.

Keywords Nonalcoholic fatty liver disease (NAFLD) • Nonalcoholic steatohepatitis (NASH) high-fat diet • Pregnancy • Epigenetics • Circadian clock • Developmental priming

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12.1 The NALFD Spectrum

Nonalcoholic fatty liver disease (NAFLD), which was once thought of as a passive metabolic condition, describes a spectrum of disorders characterized by the accumulation of ectopic fat accumulation in the liver without significant alcohol use. At one end of the spectrum is simple steatosis, often termed NAFLD. Although many individuals with NAFLD remain stable, 25 % of these patients can progress to steatosis with inflammation, termed nonalcoholic steatohepatitis (NASH) [1]. This more severe form of the disease (NASH) can progress further still, with a significant proportion of individuals developing fibrosis (26–37 %) and cirrhosis [2]. NASH cirrhosis can eventually result in portal hypertension, liver failure, and ultimately death. Interestingly, a number of recent studies have shown that NASH cirrhosis is linked to hepatocellular carcinoma (HCC) [3]. Although the precise link between NAFLD and HCC is currently under investigation, early findings suggest that it involves alterations in major pathways that regulate hepatic metabolism, such as insulin resistance and cellular lipid metabolism. Since the development of NAFLD and HCC involves perturbations in the same molecular pathways, they are likely influenced by the same metabolic disorders such as obesity and type 2 diabetes (T2D). The rising prevalence of obesity-related disorders in many industrialized countries raises huge concerns regarding the concurrent rising incidence of NAFLD and HCC.

12.2 3-Hit Hypothesis

The precise interplay between factors that promote disease progression is still under investigation. Nonetheless, it is hypothesized that a 3-hit mechanism is involved in the pathogenesis and progression of NAFLD. The “1st hit” consists of hepatic triglyceride accumulation that may result simply from dietary or lifestyle factors. The “2nd hit” may include factors that promote disease progression such as pro-inflammatory cytokines, which in turn lead to steatohepatitis and/or fibrosis [4]. Recent work suggests that there are in fact a multitude of factors that may act as the 2nd hit to promote liver disease progression, including diets rich in saturated fat and cholesterol, diets low in polyunsaturated fat and fiber, diets during development, epigenetics, circadian rhythms, and disturbances in intestinal microbiota.

12.3 NAFLD Is the Hepatic Manifestation of the Metabolic Syndrome

The metabolic syndrome is a cluster of cardiometabolic conditions, which include obesity, insulin resistance, high blood pressure, and atherogenic dyslipidemia [5]. The present definition of metabolic syndrome does not include hepatic steatosis despite growing evidence suggesting that NAFLD is the hepatic manifestation of the metabolic syndrome [6]. While the existence of the metabolic syndrome remains controversial, features of the metabolic syndrome tend to aggregate in the same individuals, and over time the presence of multiple factors anticipates the onset of additional components [7]. Thus, the metabolic syndrome, and recognition of NAFLD as a primary feature, may serve an important utility in clinical practice as a predictor of progressive NASH and cardiometabolic disease.

12.4 The Incidence and Prevalence of NAFLD and NASH

The prevalence of NAFLD in the general population is variable and ranges from 9 to 37 % [8–10]. Current estimates state that NAFLD is the most common etiology of chronic liver disease in the USA and other developed countries [11, 12]. Specifically, in the USA recent estimates suggest that NAFLD affects 30 % of the general population, 58 % of overweight people, and 90 % of individuals who are considered morbidly obese [13]. As suggested by the natural history of NAFLD, the proportion of individuals with NASH is much lower and has been estimated to affect 5–7 % of the general population and as much as 34–40 % of patients who have elevated liver enzymes [14]. With the global rise of obesity, it is predicted that there will be greater rates of NAFLD progression and that NAFLD will be the most common etiology for liver transplantation in the twenty-first century [15].

12.5 Pediatric NAFLD

An increasing number of younger individuals are being diagnosed with NAFLD [16]. Recent estimates suggest that in Western societies, the number of children with NAFLD ranges from 3 to 10 % in the general population, and up to 70 % in children who are considered obese [17]. Alarmingly, the number of adolescents diagnosed with NAFLD has more than doubled in the last two decades [18], and like adults, pediatric NAFLD can also follow a severe disease progression to cirrhosis and end-stage liver disease [19], which is also predictive of features of the metabolic syndrome and intramyocellular lipid deposition [20]. Also similar to adults, both sex and race can be a risk factor for NAFLD onset, and its development appears to be more common in boys than girls [21]. However, unlike adults, in

pediatric NAFLD there is a unique deposition of fat in the periportal region [22]. While this difference is not well understood, it is clinically significant since periportal inflammation is often associated with more severe liver disease [23].

There is now a significant body of research which suggests that features of the metabolic syndrome including NALFD may have their origins very early in life. In humans, the liver itself begins to develop in the fetus at 4 weeks of gestation with the formation of the hepatic bud from the ventral endoderm, and gross morphogenesis is completed by the end of the first trimester with refined cellular development continuing throughout gestation [24, 25]. A plethora of genes and their transcription factors are involved in the development of metabolic processes, including gluconeogenesis, glycogenolysis, lipid oxidation, and de novo lipogenesis, and are already expressed in the fetal liver although not highly expressed until after birth [26]. The liver is also primary location of hematopoietic development from week 6 to 21 of gestation, with hematopoietic stem cells accounting for 60 % of total liver mass during peak hematopoiesis followed by regression to the fetal bone marrow by term [27]. The developing liver is therefore in a constant flux throughout gestation and is susceptible to adverse environment during this critical period of development such that the growing organism may undergo changes in its fundamental metabolic pathways in an attempt to adapt to its environment. Many of these changes persist into adult life and can increase the susceptibility of developing metabolic disease in later life stages. Thus, the metabolic health of the mother, whether inherent or acquired through imbalanced diet, may lead to a transgenerational amplification of metabolic disease, including NAFLD.

12.6 Conundrum of NAFLD Susceptibility in the Offspring: Is the "1st Hit" Down to Maternal BMI or Maternal Diet During Pregnancy?

Studies conducted in various animal models, including rodents, sheep, and nonhuman primates, have reported that consumption of a Western-style diet, mostly high-fat diet (HFD) during pregnancy, significantly increase NAFLD susceptibility in the adult offspring [28]. This is of particular relevance to humans in today's society, where abundance of food high in fat and calories coincides with increasing obesity epidemic that is occurring at a younger age. It is no coincidence that this epidemic is correlated with increasing number of obese women becoming pregnant and the onset of obesity-associated morbidities [29–31]. Although there is clearly an association between maternal obesity and subsequent childhood adiposity [32–35], it remains uncertain whether it is the consumption of HFD or the resulting obesity that leads to the development of NAFLD in the offspring.

On one hand, chronic consumption of a HFD, independent of maternal obesity and gestational diabetes, has been suggested to significantly increase the risk of NAFLD in the offspring. In a study conducted in a nonhuman primate, the Japanese

macaque, fetal offspring from both lean and obese mothers chronically consuming a HFD had significantly elevated liver triglycerides (TGs), suggesting an increased maternal lipid transfer to the fetus regardless of maternal obesity [36]. This was further substantiated by their findings of no change in mRNA or protein expression of lipogenic enzymes involved in de novo lipogenesis. These results therefore suggest that a developing fetus is highly vulnerable to excess lipids, independent of maternal obesity, increasing offspring risk to NAFLD. It was suggested that the increased lipid buildup in the fetal liver can cause lipotoxicity leading to increased macrophage infiltration and inflammatory cytokine production, the result of which causes premature gluconeogenic gene expression, steatosis, elevated triglyceride content, and oxidative stress that could persist into the postnatal period [37]. In another study, it was also suggested that maternal HFD feeding could increase apoptosis in the developing fetal liver contributing to the priming of the liver to NAFLD in later life [38]. However, others have countered that it is maternal obesity, and not the consumption of the HFD per se, which is the primary mechanism driving NAFLD susceptibility in the offspring. In a study conducted in rats, females were subjected to total enteral nutrition-based overfeeding to bypass the satiety response that limits ad libitum food intake, causing them to become obese prior to mating and this resulted in their offspring to be more prone to becoming obese when fed postnatally with a HFD [39]. In another study, rats dams fed a HFD but restricted to the caloric intake of pair-fed low-fat diet (LFD) mothers failed to become obese, and this prevention of maternal obesity resulted in normal body weight in the adult offspring [40]. Conversely, ad libitum maternal HFD feeding resulted in obese dams whose offspring were heavier in adulthood than offspring of non-obese dams. Although these studies show that maternal obesity rather than the HFD itself increased offspring body weight, it remains to be determined how this may lead to increased lipid accumulation in the offspring liver.

It is difficult to investigate NAFLD in neonatal studies due to the invasive nature of its definitive diagnosis. Thus, these kinds of studies have mainly been conducted in animal models, where maternal obesity is associated with NAFLD even before birth [28, 41, 42]. Evidence for a direct association between maternal obesity and offspring hepatic lipid accumulation in humans only recently came to light with the use of imaging technologies as a noninvasive means to screen for steatosis in newborn infants [43–45]. Maternal obesity is not only associated with greater morbidities in the mother but may also be responsible for accelerated hepatic fat accumulation in the offspring during early-life development. Interestingly, the aforementioned studies in the newborns found that neonatal hepatic fat did not correlate with newborn adiposity, suggesting that the drivers for hepatic fat storage and subcutaneous fat may be different and that factors associated with maternal obesity, such as excess serum lipids, could be associated with newborn hepatic fat accumulation [44, 45].

Pregnancies complicated by maternal obesity are often associated with gestational diabetes, which could serve as the catabolic switch that increases serum lipid levels and enhances placental lipid transport [46–48]. This excess lipid exposure may therefore utilize the fetal liver as ectopic sites of fat deposition and could

promote metabolic and cellular stress and inflammation in an organ not yet competent in handling such substrate overload.

12.7 The Role of Mitochondrial Dysfunction in Developmentally Primed NAFLD

Mitochondria are essential organelles that process glycolysis and lipolysis products to generate the cellular energy carrier ATP. They are the main energy source in hepatocytes and play a major role in oxidative metabolism and normal function of the liver. Mitochondria regulate cellular lipid metabolism, amino acid metabolism, cell proliferation, ion homeostasis, and even cell death pathways via reactive oxygen species (ROS) production. Therefore, it is no surprise that suboptimal mitochondrial function has been implicated in the development of chronic liver diseases including HCC and the NAFLD spectra. The mechanisms leading to altered mitochondrial energy metabolism and characterization of the transcriptional pathways that regulate mitochondrial biogenesis and function have been the subject of intense research focus. Recent findings have advanced our understanding and may offer important insights into possible therapeutic interventions aimed at improving hepatic pathophysiology.

In the fed state, food-derived NADH or flavin adenine dinucleotide (FADH2) acts as a hydrogen or electron donor and transfers the hydrogen/electron to an O_2 molecule, via redox components in the electron transport chain (ETC) complexes. This "oxidative phosphorylation" occurs in the inner mitochondrial membrane, where the majority of electron donors and acceptors are found, including cytochrome b, cytochrome b562 and b566, in ETC complex III [49]. In times of increased energy intake and metabolic demands, increased mitochondrial β-oxidation enhances the formation of NADH and FADH2 and increases the delivery of electrons to the ETC. Such an increase in electron flow through the ETC causes the buildup and leakage of electrons and ROS production [50]. Overproduction of ROS is considered as a major pathogenic agent of many metabolic diseases, including NAFLD.

The current hypothesis regarding the pathogenesis of NASH suggests that multiple "hits" are required for the disease to progress. While the "1st hit" may involve accumulation of fat in the liver, a growing body of evidence suggests that the second hit involves oxidative stress, lipid peroxidation, the production of malondialdehyde, 4-hydroxynonenal, pro-inflammatory cytokines, stellate cell activation, and fibrogenesis [51]. It is now fairly well established that mitochondrial dysfunction may be involved in at least one of these hits, due to their central role in the β-oxidation of free fatty acids (FFAs), ROS production, and lipid peroxidation [52]. In fact, a number of studies have reported defects in mitochondrial ETC enzymes in individuals diagnosed with NASH. Specifically, in patients with NASH, the activity of the ETC enzymes is markedly reduced and correlates

significantly with pro-inflammatory markers [53]. In addition, NAFLD is associated with ultrastructural mitochondrial abnormalities and depletion of mitochondrial DNA. Such changes in mitochondrial DNA have been shown to further suppress the expression of mitochondrial respiratory complexes I, III, IV, and V and exacerbate mitochondrial dysfunction [54]. While there are clear associations between mitochondrial dysfunction and NALFD pathogenesis, the mechanisms leading to improperly functioning mitochondria are not fully understood. It is plausible to suggest that nutrient excess may lead to increased ROS-mediated lipid peroxidation and mitochondrial dysfunction. In support of this hypothesis, NAFLD has been observed in the liver of obese sedentary and hyperphagic rats, characterized by reduced fatty acid oxidation, decreased cytochrome c protein content, and decreased carnitine palmitoyl-CoA transferase (CPT-1) activity [55]. Thus, it is thought that positive energy balance and nutrient excess may play a key role in NAFLD onset and progression.

Curiously, mitochondrial respiratory chain disorders are an established cause of liver failure in early childhood but have been underdiagnosed, partly due to underrecognition and partly due to the invasive nature of the investigations [56]. Since hepatic mitochondria are of maternal origin, they are a likely candidate vector for maternally inherited metabolic stress. Thus, mitochondria may be considered an important conduit for metabolic disease and a target for investigations into metabolic perturbations in offspring of obese mothers. Indeed, in recent years there has been a plethora of studies highlighting the role of mitochondrial dysfunctional in the molecular pathogenesis of developmentally primed NAFLD. Studies in rats have shown that adult offspring of mothers exposed to a HFD prior to conception, and throughout gestation and lactation, develop insulin resistance and features of NAFLD [57]. Similarly in mice, offspring of mothers fed a HFD, who are also fed a HFD in postnatal life, develop a more severe liver phenotype akin to human NASH [28]. In these studies, maternal HFDs have been linked to reduced ETC activity, which when coupled with further HFD challenge exceeds the liver's oxidative capacity, resulting in excessive fat accumulation and increased de novo lipogenesis, reduced β-oxidation, and inflammation [28].

These initial studies initiated an intensive research effort aiming to understand how maternal diets interact with mitochondrial function to reduce oxidative capacity. A number of studies have highlighted the role of mitochondrial Sirtuins and the acetylation of mitochondrial proteins in metabolic disease and aging. Mitochondrial protein acetylation regulates a number of enzymes involved in the TCA cycle, gluconeogenesis, and β-oxidation and is regulated (at least in part) by the mitochondrial class III NAD+-dependent deacetylate Sirtuin 3 (SIRT3) [58]. Since no significant changes in mitochondrial acetylation are observed in mice lacking both SIRT4 and SIRT5, SIRT3 is thought to be the primary mediator of mitochondrial protein acetylation [59]. Since mitochondrial acetylation is sensitive to nutrient status, and can be modulated in times of caloric restriction [60], SIRT3 seems a likely mediator of nutrient-derived mitochondrial stress. In fact, chronic (up to 16 weeks) feeding of a HFD has been reported to reduce SIRT3 activity and cause a threefold decrease in hepatic NAD+ levels. Chronic HFD feeding results in

hyperacetylation of mitochondrial proteins, which is associated with reduced protein activity and mitochondrial function. Interestingly, mice lacking SIRT3 demonstrate even greater hyperacetylation of mitochondrial proteins under HFD conditions and show a marked disruption in mitochondrial oxidative phosphorylation and ETC complex activity [60]. Importantly, this reduction in SIRT3 activity and abundance can be passed to the subsequent generation. For example, offspring of obese mothers have significantly reduced SIRT3 gene and protein expression [61]. One of the major consequences of reduced SIRT3 expression is reduced mitochondrial β-oxidation [61]. It is possible that impaired mitochondrial oxidative capacity creates a shunt of intermediary metabolites toward lipid storage and/or de novo lipogenesis, thus contributing toward the development of NASH in the offspring of obese mothers.

While the mechanisms leading to reduced SIRT3 activity are currently under investigation, a plausible explanation involves an altered availability of the essential cofactor NAD+, which in turn is able to directly affect SIRT3 function and abundance. This has implications not only for SIRT3 but for other NAD+-dependent Sirtuins, such as SIRT1, a protein long associated with metabolic health and longevity. During fasting, there is an increase in pyruvate and NAD+ levels that is able to facilitate SIRT1 activity and increase protein levels and (PMID: 15744310). Although it plays a number of intracellular roles, SIRT1 is also associated with mitochondrial function. When NAD+ levels are favorable, SIRT1 deacetylates and activates peroxisome proliferator-activated receptor alpha (PPAR-α), which in turn transcriptionally activates a number of genes associated with mitochondrial biogenesis, such as peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PCG1α) [62], and mitochondrial oxidative metabolism [63]. Although the mechanism is not fully understood, it appears that PPAR-α and SIRT1 act upstream of a number of factors that are heavily associated with the onset of hepatic steatosis. Interestingly, mice that lack PPAR-α develop severe hepatic steatosis during fasting, an observation which is consistent with reduced mitochondrial capacity [64]. On the other hand, viral-mediated overexpression of SIRT1 is able to induce a gene cassette associated with a healthy, non-fatty liver. This included downregulated expression in a number of "lipogenic" genes associated with increased lipid accumulation, such as sterol regulatory element-binding protein 1c (SREBP-1c), fatty acid synthase (FASN), and the elongation of very long chain fatty acids protein 6 (ELOLV-6) [65].

Several studies have shown that a maternal HFD feeding and maternal obesity are both able to downregulate SIRT1, thus preventing the antagonism of lipogenic transcription factors and contributing to the developmental priming of fatty liver. In a recent study, in utero exposure to a maternal HFD, but not obesity per se, was linked to a decrease in SIRT1 gene expression and in vitro protein deacetylase activity in the offspring liver [66]. Moreover, a maternal HFD was associated with altered expression of SIRT1-regulated downstream lipogenic effectors, such PPAR-α, PPAR-γ, sterol regulatory element-binding protein F1 (SREBF1), cholesterol 7alpha-hydroxylase (CYP7A1), FASN, and stearoyl-CoA desaturase (SCD) in the offspring liver [66]. On the other hand, recent studies using models of maternal

obesity have reported that SIRT1 mRNA is unchanged in the livers of offspring of obese dams compared to offspring of normal weight dams. However, the downstream PPAR-α and SIRT1 gene cassette still becomes dysregulated, including blunted PCG-1α expression, which may prevent the mitochondrial biogenesis that necessitates HF catabolism, resulting in increased hepatic accumulation susceptibility to develop NAFLD [61].

12.8 Epigenetic Modifications Underlying NAFLD Development

Epigenetics refers to the heritable changes in gene expression that do not involve changes to the underlying genome, i.e., a change in the observable physical traits or biochemical characteristics of an individual (phenotype) without a change in its genetic makeup or genotype [67]. Conrad Hal Waddington first coined the term epigenetics in 1942, which was derived from the Greek word "epigenesis" to mean the influence of genetic processes on development [68]. Since then, research efforts have focused on unraveling epigenetic mechanisms involved in the regulation of gene expression. Although epigenetic change can occur naturally, it can also be influenced by several factors including age and environment factors including diet [69, 70]. Epigenetic aberrations are generally transient and non-heritable, but some are transmitted from one generation to the next (transgenerational), thus affecting the traits of the offspring without altering their DNA structure [71].

The process of regulating the expression of genes involves modification of chromatin structure, initiation and processing of transcription to generate messenger RNA (mRNA), and the translation of the mRNA into sequences of amino acids, which defines the protein [72]. Epigenetic mechanisms thus regulate the modification of the chromatin structure and the initiation of transcription to alter availability of genes to transcription factors required for their expression [73]. These epigenetic mechanisms include DNA methylation, posttranslational modification of histones, chromatin remodeling, and RNA-based mechanisms such as microRNA [74]. Recent studies have demonstrated that metabolic pathways perturbed by diets rich in saturated fat and cholesterol can trigger epigenetic changes, thereby modifying gene expression [75–77]. These epigenetic effects are increasingly recognized as crucial factors in the pathophysiology of NAFLD, and there are now a plethora of epigenetic changes associated with genes involved in NAFLD, in both animals and humans (Tables 12.1 and 12.2). Earlier studies in animal models have investigated the effects of maternal undernutrition on the epigenotype and metabolically perturbed phenotype of the offspring. The focus has now shifted to maternal obesity and its consequential effect on the offspring epigenome. One of the very early studies using an obese Agouti mouse have shown that genetic tendency towards obesity was progressively exacerbated when the Agouti allele was passed along successive generations [78].

Table 12.1 DNA methylation and histone modification in genes linked to development of NAFLD

Epigenetic mechanisms	Species	Target genes	References
DNA methylation	Mouse	MMTP, PPAR-α, INSIG, FASN	[79, 80]
	Rat	SREBPF2, AGPAT3, ESR1, FASN, CDKN1α, leptin, PPAR-α	[81–85]
	Humans	PGC1α, TFAM, MT-ND6, PC, ACLY, PGCG1, IGF1, IGFBP1, PRKCE, GALNTL4, GRID1, IP6K3	[86–88]
Histone modification	Mouse	ChREBP, CYP8B1, TNFα, CCL2, PPAR-α, ERO1α, LXRα, SIRT1, SIRT3, RORα	[89–96]
	Macaques	GPT2, DNAJA2, RDH12, NPAS2	[97, 98]
	Human	NER	[99]

ACLY ATP citrate lyase, *AGPAT3* 1-acylglycerol-3-phosphate O-acyltransferase 3, *CCL2* chemokine C–C motif ligand 2, *CDKN1a* cyclin-dependent kinase inhibitor 1a, *ChREBP* carbohydrate-responsive element-binding protein, *CYP8B1* sterol 12α-hydroxylase, *DNAJA2* DnaJ (Hsp40) homolog, subfamily A, member 2, *ERO1α* oxireductase endoplasmic reticulum oxidoreductin1α, *ESR1* estrogen receptor 1, *FASN* fatty acid synthase, *GALNTL4* putative polypeptide N-acetylgalactosaminyltransferase-like protein 4, *GPT2* glutamic pyruvate transaminase 2, *GRID1* glutamate receptor δ-1 IP6K3 Inositol hexaphosphate kinase 3, *IGF1* insulin-like growth factor 1, *IGFBP2* insulin-like growth factor binding protein 2, *INSIG* insulin-induced gene, *LXRα* liver X receptor α, *MT-ND6* mitochondrially encoded NADH dehydrogenase 6, *MTTP* microsomal triglyceride transfer protein, *NER* nucleotide excision repair, *NPAS2* neuronal PAS domain-containing protein 2, *SREBPF2* sterol regulatory element-binding transcription factor 2, *PC* pyruvate carboxylase, *PGC1α* peroxisome proliferator-activated receptor gamma coactivator 1-alpha, *PLCG1* phospholipase C-gamma-1, *PPARα* peroxisome proliferator-activated receptors α, *PRKCE* protein kinase C, epsilon, *RDH12* retinol dehydrogenase 12, *RORα* retinoic acid-related orphan receptor α, *SIRT1* sirtuin 1, *SIRT3* sirtuin 3, *TFAM* mitochondrial transcription factor A, *TNFα* tumor necrosis factor α

Table 12.2 MiRNA changes in NAFLD

Species	Upregulated MiR	Downregulated MiR	References
Mouse	miRNA-24, miRNA-33a, miRNA-34a, miRNA-122, miRNA-155, miRNA-181a, miRNA-182, miRNA-183, miRNA-192, miRNA-199a-3p/5p, miRNA-200b, miRNA-705, miRNA-1224	miRNA-92b-3p, miRNA-216, miRNA-302a, miRNA-328-3p, miRNA-467b, miRNA-484, miRNA-574-5p, miRNA-615-3p	[128–137]
Rat	miRNA-15b, miR-155, miRNA-200a/b, miRNA-429	miRNA-27, miRNA-122, miRNA-451	[124, 138–140]
Humans	miRNA-10b, miRNA-16, miRNA19a/b, miRNA-21, miRNA-27b-3p, miRNA-34a, miRNA-122, miRNA125b, miRNA-192-5p, miRNA-451, miRNA-1290	miRNA-28-3p, miRNA-99a, miRNA-132, miRNA-146b, miRNA-150, miRNA-181d, miRNA-197, miRNA-296-5p, miRNA-433, miRNA-511, miRNA-517a, miRNA-671	[118, 121, 141–149]

12.8.1 DNA Methylation in NAFLD

Epigenetic changes through DNA methylation refers to the addition of methyl groups to cytosine residues of DNA. In mammals, DNA methylation mainly occurs in regions of DNA where a cytosine nucleotide occurs next to a guanine nucleotide, or so-called CpG sites. These CpG sites tend to cluster together to form CpG islands. When a CpG island in the promoter region of a particular gene is methylated, expression of the gene is repressed or is turned off (Table 12.1). In livers of patients with NAFLD, there is evidence for increased methylation of the CpG island in the promoter region of PGC1α, a key transcription factor involved in mitochondrial biogenesis, fatty acid oxidation, gluconeogenesis, and lipogenesis [87]. Moreover, it has been shown that there is an inverse correlation between mitochondrial DNA (mtDNA) content and the methylation levels of the PGC1α promoter. Thus, the finding of a reduction in mtDNA content in livers of these NAFLD patients suggests that mitochondrial dysfunction associated with hepatic steatosis is due to liver DNA methylation of PGC1α. The mitochondria are also a major source and target of reactive oxygen species (ROS). The mtDNA-encoded NADH dehydrogenase 6 (MT-ND6) gene is a target of methylation in NAFLD [86]. In patients with NASH, it has been reported that MT-ND6 is highly methylated and the MT-ND6 gene is considerably reduced in their livers. Thus, DNA methylation of the mitochondrial gene may play an important role in the development and pathogenesis of NAFLD.

Differential methylation has also been identified in genes involved in metabolism and in insulin signaling when liver samples from NAFLD patients were analyzed by array-based DNA methylation and mRNA expression profiling [88]. The former includes pyruvate carboxylase (PC), ATP citrate lyase (ACLY), and Phospholipase C-gamma-1 (PLCG1), while the latter includes Insulin-like growth factor 1 (IGF1), Insulin-like growth factor binding protein 2 (IGFBP1), and Protein kinase C, epsilon (PRKCE). On the other hand, global hepatic DNA methylation can become progressively demethylated in mice that develop a fatty liver phenotype similar to human NASH induced by feeding a lipogenic methyl-deficient diet [100]. Thus, DNA methylation is particularly affected by the availability of *S*-Adenosyl-*L*-methionine (SAMe) and the dietary methyl donors including folate, betaine, and choline, which are associated with SAMe synthesis [101, 102]. SAMe influences the pathogenesis of NAFLD as a methyl donor in the synthesis of phosphatidylcholine, which is required for very-low-density lipoprotein (VLDL) assembly and hepatic triglyceride export. Evidence of a role for SAMe in NAFLD development has largely been based on animal studies. Methyl-deficient diets have been reported to result in the development of NAFLD in mice [103, 104]. These mice were found to have reduced concentration of hepatic SAMe and CpG island methylation of genes involved in DNA damage and repair, lipid and glucose metabolism, and the progression of fibrosis in their livers [105]. Inducing hepatic fat accumulation by feeding a HFD can be reversed by supplementation with methyl donors containing folic acid, choline, betaine, and vitamin B12. These

methyl donors reduced hepatic global DNA methylation and changed the methylation levels of CpG sites in the sterol regulatory element-binding transcription factor 2 (SREBF2), 1-acylglycerol-3-phosphate O-acyltransferase 3 (AGPAT3), and estrogen receptor 1 (ESR1) promoter regions [81]. Furthermore, methyl donor supplementation resulted in fatty acid synthase (FASN) DNA hypermethylation, leading to improvement of HFD-induced NAFLD [82]. Betaine supplementation was also found to restore methylation capacity by increasing SAMe concentration and genomic methylation level and the reduction in the methylation of the microsomal triglyceride transfer protein (MTTP) promoter [79]. This promotes hepatic triglyceride export and attenuates fat accumulation.

Feeding pregnant mothers a HFD can result in increased NAFLD susceptibility in the offspring in mice [28, 41], and DNA methylation plays an important role in this process. DNA methylation can be inherited from parents and passed to the next generation [106]. In rat offspring of HFD-fed mothers that develop NAFLD, it was suggested that hypomethylation of cyclin-dependent kinase inhibitor 1A (CDKN1a), an inhibitor of the hepatic cell cycle, and increased hepatic expression of the CDKN1a gene in early postnatal life contribute to predisposition to NAFLD in later life [83]. Interestingly, the hormone melatonin, which regulates the body's 24-h "clock," has been reported to reverse the methylation of leptin and prevent glucocorticoid-induced hepatic steatosis [84].

12.8.2 Histone Modifications in NAFLD

Evidence is now accumulating that histone modifications are also involved in transmitting an epigenotype with increased NAFLD risk (Table 12.1). Histone modifications mainly consist of acetylation, methylation, phosphorylation, and ubiquitylation. Histones are proteins that organize DNA strands into nucleosomes by forming molecular complexes around which the DNA winds, and modification of histone proteins can impact gene regulation by altering chromatin structure or recruiting histone modifiers. Most of the current evidence points to changes in histone acetylation in the development of NAFLD [97]. The modifying enzymes involved in histone acetylation are called histone acetyltransferases (HATs) and histone deacetylases (HDACs), and they play an important role in controlling histone H3 and H4 acetylation [107]. Histone H3 is primarily acetylated at lysines 9, 14, 18, 23, and 56 (denoted at H3K9, H3K14, H3K18, H3K23, and H3K56), while HDACs catalyze the hydrolytic removal of acetyl groups from histone lysine residues. There are four classes of HDACs, with. for example. HDAC1, HDAC2, HDAC3, and HDAC8 grouped as class I HDACs. In primates, hyperacetylation of H3K14 has been reported in the fetal hepatic tissue and this was accompanied by upregulated acetylation at H3K9 and H3K18 [97]. The same study also showed that the feeding the pregnant mother with a HFD can result in the depletion of HDAC1 protein in the fetal liver. These findings indicate that maternal obesity due to HFD feeding can already change fetal chromatin structure via histone modifications.

NAFLD development is also regulated by carbohydrate-responsive element-binding protein (ChREBP) by acting as transcriptional activator of lipogenic and glycolytic genes. A reduction in the activity of the HAT activator p300 was found to attenuate ChREBP-mediated hepatic steatosis in mice [89]. Furthermore, histone modification of genes that regulate bile acid synthesis and dietary cholesterol absorption was found to be linked to NAFLD development. Sterol 12-α-hydroxylase (CYP8B1) regulates bile acid synthesis and intestinal cholesterol absorption, and that histone acetylation of the gene promoter CYP8B1 was found to be induced following recruitment of cAMP response element-binding protein-binding protein (CBP) by the cholesterol-activated nuclear receptor and clock-controlled gene retinoic acid-related orphan receptor α (RORα) [90]. Thus, modifying RORα activity could potentially attenuate NALFD progression by histone modification. The link between histone modification and 24 h or circadian rhythms will be discussed further in the proceeding section of this chapter.

In mice, hepatic lipid accumulation due to a HFD was also reported to alter histone H3K4 and H3K9 trimethylation in PPARα and lipid catabolism-related genes increasing their expression levels and thus perpetuating further lipid buildup leading to hepatic steatosis and NAFLD progression [92]. The modifying effect of a HFD on histone has been reported to occur over generations. Offspring from pregnant mice with a HFD was found to have altered expression of genes involved in the upregulation of lipogenesis and ER stress due to reduced accumulation of methylated histones in liver X receptor α (LXRα) and oxireductase endoplasmic reticulum oxidoreductin1α (ERO1α) gene promoters [93]. In another study, maternal HFD feeding resulted in increased fetal hepatic acetylation of histone H3K14 and decreased SIRT1 expression [66]. Deacetylation by SIRT1 is responsible for the regulation of various proteins that are involved in the pathophysiology of NAFLD [108]. In the liver, SIRT1 is also reported to interact with the protein MENIN, and a reduction in MENIN gene expression particularly in aging accelerated hepatic steatosis following HFD feeding by recruiting SIRT1 to regulate CD36 expression and triglyceride accumulation via histone deacetylation [94]. Feeding a HFD in mice also induces hepatic mitochondrial protein hyperacetylation and downregulation of the major mitochondrial protein deacetylase of another sirtuin SIRT3, which resides at the mitochondria and modulates fatty acid oxidation [95, 109]. Hence feeding a HFD alters both SIRT1 and SIRT 3 expression via histone modification, and this impacts on lipid metabolism that is associated with NAFLD development.

12.8.3 MicroRNA Changes in NAFLD

Another epigenetic modification that is linked to NAFLD development is the alteration of microRNAs (miRNAs) (Table 12.2). MiRNAs are short, single-stranded RNA molecules approximately 22 nucleotides in length that can negatively modulate post-transcriptionally around 30 % of all mammalian protein-

encoding genes [110]. They induce gene silencing by binding to target sites found within the 3′UTR of the targeted mRNA, thus preventing protein production by suppressing protein synthesis and/or by initiating mRNA degradation [111]. MiRNAs play a key role in many important physiological processes such as cell proliferation, differentiation, apoptosis, and embryonic development, and that altered miRNA expression has been implicated in obesity, insulin resistance, T2D, and fatty liver disease [112, 113]. In patients with NASH, about 100 miRNAs that are involved in the pathogenesis of steatohepatitis, including the regulation of lipid and glucose metabolisms, oxidative stress, cellular differentiation, inflammation, and cell survival pathways, are differentially expressed [114, 115]. The most abundant miRNA in the liver is miRNA-122, which is a key regulator of glucose and lipid metabolism in adult livers [116, 117]. Serum miRNA-22 levels, which mainly circulate in argonaute 2-free forms, are significantly higher in mice with NASH [118]. In NAFLD patients, early studies found this miRNA to be significantly underexpressed in their livers [119, 120]. Further studies have shown that reduction in hepatic miRNA-122 was much lower in NAFLD patients with mild steatosis compared to those with severe steatosis, while patients with mild fibrosis showed higher serum and hepatic miRNA-122 levels than those with severe fibrosis [121]. Genetic deletions of miRNA-122 in mice also resulted in hepatic steatosis and inflammation [122, 123]. Besides miRNA-122, other miRNAs are reported to be involved in NAFLD development, including miRNA-21, miRNA-23a, miRNA-34a, miRNA-143, and miRNA-146b, which were found to be overexpressed in human NAFLD and NASH [120].

Diet fed to rats can cause considerable dysregulation of miRNAs and their target genes. In a study done in rats, HFD feeding was found to cause marked reduction in hepatic miRNA-122, miRNA-451, and miRNA-27 expression and increased expression of miRNA-200a, miRNA-200b, and miRNA-429 [124]. This study also showed changes in expression levels of proteins involved in regulating lipid and carbohydrate metabolism and signal transduction that are being regulated by these miRNAs in livers from the HFD-fed rats. These finding demonstrate that a HFD can alter the expression levels of miRNAs and some of their targets, contributing to the development of fatty liver and progression of nutritional steatohepatitis. Nevertheless, there is a paucity of information on whether maternal nutrition during pregnancy impacts on the hepatic miRNA status in their offspring. Our own study in mice shows that in livers of offspring mothers fed a HFD during pregnancy had markedly increased hepatic expression of key genes including those regulating fetal growth, such as insulin-like growth factor-2, and fat metabolism, including peroxisome proliferator-activated receptor-alpha and carnitine palmitoyl transferase-1a [125]. These changes were accompanied by reduced expression of miRNAs involved in developmental timing (let-7c) and fat oxidation (miRNA-122). More recently, it has been reported that in livers of weaned offspring of mouse dams fed a HFD during pregnancy and lactation, the expression of miRNA-122 was reduced but that of miRNA-370 was increased [126]. Moreover, miRNA-370 is involved in metabolism by activating lipogenic genes indirectly through miRNA-122 [127]. Thus, changes in key metabolic genes and miRNAs in the liver of offspring

from dams fed a HFD may alter early fetal growth and fat metabolism increasing offspring NAFLD susceptibility in later life.

12.9 Disruption of the Circadian Clock and NAFLD Development

A wide array of physiological processes is expressed in a rhythmic pattern with duration of about 24 h, coinciding with the day–night cycle. These 24 h rhythms are termed "circadian" and are regulated by an endogenous circadian clock network composed of key "clock" genes. Circadian rhythms are entrained by the light–dark cycle but can also be influenced by environmental temperature and food availability. The central circadian clock network is found in the hypothalamic region of the brain called the suprachiasmatic nuclei (SCN). It is now well established that clock genes are found and rhythmically expressed in most organs and tissues, including those involved in metabolism such as the liver, muscle, and adipose tissues. The generation of circadian rhythms is through a series of autoregulatory transcriptional and translational interactions [150, 151]. The key clock genes are circadian locomotor output cycle kaput (CLOCK) and brain and muscle aryl 1-hydrocarbon receptor nuclear translocator-like 1 (BMAL1), which form a heterodimer complex that activates transcription of other clock genes, including Period (PER1, PER2, PER3) and Cryptochrome (CRY1 and CRY2). The translated PER and CRY proteins form complexes and translocate back to the nucleus where they then negatively regulate CLOCK and BMAL1 activity. Though the central circadian clock network regulates circadian processes such as the sleep/wake cycle, body temperature, blood pressure, and hormone secretion, at the whole body level, it is the intrinsic clock gene network in the liver that determines hepatic clock function. Nevertheless, the systemic cues, such as light–dark cycles, fine-tune hepatic rhythms.

The circadian clock network in the liver regulates a plethora of genes and nuclear receptors that are important in several metabolic pathways, such as the metabolism of glucose, fatty acids, cholesterol, and amino acids [152–156], and in the detoxification of xenobiotics [157]. Thus, an intact circadian clock is essential for the maintenance of body homeostasis, and disruption of the clock network at the central and organ level leads to desynchronization of metabolism and consequently the development of obesity and fatty liver disease. In healthy individuals, there is a nycthemeral rhythm in de novo lipogenesis associated with the sleep–wake cycle and the feeding–fasting cycle [158]. At night when individuals are normally asleep and are therefore in the fasted state, de novo lipogenesis supplies less than 5 % of fatty acids to the hepatocyte. During the day when individuals are normally in the feeding state, characterized by high insulin levels, insulin stimulates de novo lipogenesis, supplying approximately a quarter of the free fatty acids to

hepatocytes. This nycthemeral rhythm in de novo lipogenesis is absent in NAFLD patients [158].

Early studies have suggested mutations in the core clock genes are linked to NAFLD development. Mice with mutations in clock genes have provided key insights into the interdependence between the circadian clock and metabolism. Altering key components of the clock network, for example, in the CLOCK mutant mice, give rise to the development of metabolic pathologies including obesity and hepatic steatosis [159]. Moreover, mice deficient in CLOCK and BMAL1 exhibit suppressed diurnal variations in glucose and triglyceride levels, which were amplified by feeding a HFD [153]. This observation has been extended to findings in humans, where common genetic variations of the CLOCK gene are reported to be linked to susceptibility to NAFLD [160, 161]. These studies show that CLOCK variant haplotype frequencies significantly differ between NAFLD patients and controls.

The epigenetic modifications associated with NAFLD development involve the circadian clock network. CLOCK itself possesses histone acetyltransferase (HAT) activity, and this HAT activity is necessary for CLOCK-BMAL1-dependent transactivation of clock-controlled genes and, therefore, downstream circadian clock function [162, 163]. In addition, it has been shown that activation of several CLOCK-BMAL1 target genes involves changes in histone H3 acetylation in the PER1, PER2, and CRY1 promoter regions [164]. Thus, clock-mediated epigenetic processing is upstream of several cellular metabolic cascades associated with hepatic liver accumulation. PPAR-α, for example, is a nuclear receptor that regulates the transcription of genes involved in lipid and glucose metabolism following binding of endogenous nonesterified free fatty acids (NEFAs). The CLOCK-BMAL1 heterodimer mediates transcription of the PPAR-α gene and an increase in PPAR-α protein, which subsequently binds to the PPAR response element (PPRE) and activates the transcription and translation of BMAL1, demonstrating the reciprocal link between circadian and lipid metabolic processes [165, 166]. Studies have also shown that there is a daily whole-genome cycling of the activating chromatin mark H3K4me3 (histone H3 trimethylated at lysine 4) and the inhibitory chromatin mark H3K9me3 (histone H3 trimethylated at lysine 9) in the mouse liver [167], suggesting that these activation marks are regulated in a circadian manner at thousands of gene loci. In the same study, the histone-remodeling enzyme mixed lineage leukemia 3 (MLL3) was also found to modulate hundreds of epigenetically targeted liver circadian output genes, especially those in the one-carbon metabolism pathway [167]. This suggests that MLL3 is a clock-controlled factor that could potentially regulate circadian epigenomic profiles and is thus a good candidate linking the circadian clock and liver diseases.

HDAC3 occupancy on genes involved in lipid metabolism in the mouse liver was also shown to have a pronounced circadian pattern, which peaks during the day and is at its nadir at night [168]. This circadian pattern was found to be inversely associated with the genome-wide histone acetylation and RNA polymerase II recruitment at the same sites, suggesting that HDAC3 is involved in circadian epigenomic remodeling that leads to transcriptional repression of hepatic lipogenic

genes during the day but allows transcriptional activation of these genes at night. The genomic binding sites of REV-ERBα were also found to significantly overlap with those of HDAC3 and its binding partner, the nuclear receptor corepressor (NCoR), especially on genes involved in fatty acid synthesis, and that there is a close correlation between signal intensities of REV-ERBα binding and those of HDAC3-NCoR at the same sites. In the HDAC3 liver-specific knockout mice, depletion of HDAC3 in the liver switches metabolic precursors for lipid synthesis and storage within lipid droplets and away from hepatic glucose production by sequestration of lipids in perilipin 2-coated droplets and this contributes to the development of steatosis [169]. Thus, a loss in the circadian rhythm of REV-ERBα binding should result in de novo lipogenesis and development of hepatic steatosis in a similar manner as was found in the HDAC3 liver-specific knockout mice. This was indeed the case [168] and implies that circadian epigenomic remodeling controlled by HDAC3 is largely directed by REV-ERBα.

Disruption of circadian clock function caused by chronic lifestyle disturbances, such as professional jet lag (night workers) or long-term shift work, is also suggested to contribute to the manifestations of fatty liver disease [170, 171]. A mouse model of shift work appears to share the same mechanism in humans where timed sleep restriction resulted in disruption of circadian rhythms of genes in the liver that are involved in glucose and lipid metabolism, including BMAL1, PER1, REV-ERBα, and the D site of albumin promoter binding protein (DBP) [172]. It is interesting to note that timed food access was able to restore molecular rhythms in the liver and metabolic function under sleep restriction conditions, suggesting that hepatic circadian desynchrony marks an early event in the metabolic disruption associated with chronic shift work. Thus, strengthening circadian clock network in the liver by minimizing food intake during night shifts may counteract the adverse physiological consequences frequently observed in human shift workers. In another mouse study, increased lipogenesis brought about by timed sleep restriction was found to be blunted in PER1/2 double mutant animals [173]. Although this was examined at the adipose tissue, it suggests that the absence of a functional clock in these double mutants may also protect these mice from sleep restriction-induced metabolic reprogramming that may include the development of NAFLD.

Altered nutrition during critical developmental periods could lead to disruption of the circadian clock network modulations in the rhythm of expression and increased NAFLD susceptibility in later life. This notion is now being supported by results of recent investigations. In utero exposure to maternal HFD has been shown to upregulate the expression of fetal hepatic circadian-associated neuronal PAS domain-containing protein 2 (NPAS2), at least in part, through hyperacetylation of histone H3 at lysine 14 [98]. In another study in mice, offspring exposed to HFD both in utero and in postnatal life develop NAFLD, and this was accompanied by the disruption of rhythmic pattern in expression of the key clock genes BMAL1, CLOCK, PER1, PER2, CRY1, and CRY2 in the offspring liver [174]. Hypermethylation of the promoter regions for BMAL1 and PER2 and altered 24-h rhythmicity of hepatic pro-inflammatory and fibrogenic mediators were also observed in these offspring. Thus, exposure to HFD in utero may alter the hepatic

circadian clock network during development, resulting in the disruption of rhythmic patterns in metabolic processes leading to NAFLD development. It will be of interest to examine whether the REV-ERBα/NCoR/HDAC3-mediated epigenomic remodeling is involved in the HFD-induced modulation of the activity of other transcription factors involved in lipogenesis such as the SREBPs and PPAR-γ.

12.10 Developmental Priming of NAFLD as a Marker of Premature Metabolic Decline

As previously described throughout this book, there is a wealth of data from both human and animal studies to suggest that poor nutritional exposures during early life increase the risk of developing features of the metabolic syndrome in later life. Collectively, these findings demonstrate that early dietary exposures can accelerate the onset of conditions traditionally associated with aging such as insulin resistance, type 2 diabetes, obesity, hypertension, CVD, and NAFLD [175]. This suggests that nutritional challenges that are imposed during critical periods of development and plasticity are able to set the trajectory of "metabolic aging" throughout the life course. While the mechanisms that link early nutrition to longevity are currently under investigation, preliminary findings highlight changes in cellular processes with established roles in aging, such as reduced longevity-associated Sirtuin proteins, altered epigenetic regulation of key metabolic genes, and maternally inherited mitochondrial dysfunction [175].

SIRT1 is a longevity-associated lysine deacetylase, a crucial sensor of cellular metabolism, and a central molecule connecting various metabolic processes in the liver. As the nexus of metabolism and aging, SIRT1 protects cells against oxidative stress, regulates glucose/lipid metabolism, and promotes DNA stability by binding to and deacetylating several substrates [176]. During aging and the onset of age-related disorders, including metabolic diseases, cancer, and neurodegenerative conditions, Sirtuin abundance and activity is reduced [177]. Thus, it has long been hypothesized that SIRT1 may play a role in the developmental priming of fatty liver. Indeed, in utero exposure to a maternal HFD has been shown to increase fetal histone acetylation with a concomitant decrease in SIRT1 expression and activity, implying that SIRT1 is a likely molecular mediator of the fetal epigenome and metabolome, and with additional implications for hepatic SIRT1 in premature aging of the liver [66].

There is also a strong association between SIRT3 and longevity [178]. As previously described, several studies have also shown that the mitochondrial Sirtuin SIRT3 may be also perturbed by maternal obesity, with detrimental consequences for offspring liver function. Recent human studies have shown that obese pregnant women display decreased skeletal muscle mitochondrial ETC activity and reduced mitochondrial antioxidant defense, concomitant with reduced SIRT3 activity, suggesting that reduced SIRT3 plays a role in the increased oxidative stress often

observed in pregnancies complicated by obesity and gestational diabetes [179]. Such a decrease in this antioxidant capacity is likely to impair the defense system in the offspring liver. A recent rodent model has also demonstrated that maternally derived SIRT3 aberrations in the liver may be a conduit for suboptimal liver function in the offspring. Specifically, offspring of HFD dams show reduced SIRT3 expression, which leads to impaired hepatic fatty acid oxidation [61]. These observations suggest that SIRT3-mediated mitochondrial dysfunction may be key underlying mechanism that reduces hepatic fatty acid oxidation and antioxidant defense system, contributing to the metabolic aging of the liver and the premature onset of severe fatty liver disease.

While further studies are needed to ascertain the effect of early diet exposure on liver function and ultimately life span, reduced Sirtuin abundance is a likely candidate that mediates detrimental effects on both metabolism and longevity. Much of the research aiming to understand the mechanisms by which the maternal diet can prime the development of fatty liver disease has focused on SIRT1 and SIRT3. However, recent data suggest that other longevity-associated Sirtuins such as SIRT6 and its cofactor FOXO3 are also involved in the pathophysiology of NAFLD. For example, SIRT6 and FOXO3 may transcriptionally and epigenetically regulate proprotein convertase subtilisin kexin type 9 (PCSK9) expression and LDL-cholesterol homeostasis [180]. In particular, hepatic SIRT6 deficiency leads to elevated PCSK9 gene expression and LDL cholesterol. Since the ability of monoclonal antibodies that inhibit PCSK9 and dramatically lower LDL cholesterol has received much attention of late, the role of SIRT6 in this process is an exciting research avenue. Thus, Sirtuin proteins present a promising target for pharmacological intervention to prevent the developmental priming of NAFLD, and further investigation is needed to determine the role of other Sirtuin proteins and their transcriptional cofactors.

12.11 Potential Strategies to Delay and Reverse the Developmental Priming of NAFLD

Current efforts to ameliorate NAFLD or T2D with pharmacologic agents have been met with limited success. It is likely due to the fact that many treatments have focused on treating the end-stage disease and not the mechanisms that are central to the disease pathogenesis itself. While interest in the “developmental priming” phenomena has led to important nutritional and education guideline reforms during pregnancy and early life, arguably some of the most important outcomes have been due to scientific findings using preclinical disease that have provided unrivaled insight into the molecular pathogenesis of disease. Research into the developmental priming of NAFLD has been a particularly intensive and has highlighted a number of key mechanisms that are critical in the molecular pathogenesis of the disease, namely mitochondrial dysfunction, oxidative stress, lipid peroxidation, and de novo

lipogenesis and epigenetics. We are now seeing a new wave of innovative interventions that target these key pathways to prevent, delay, and reserve the onset of NAFLD.

12.11.1 Enhancing Mitochondrial Metabolism

As previously described, a number of studies have highlighted the role of suboptimal mitochondria in developmentally primed NASH. It is therefore interesting that a number of proof-of-concept studies have explicitly shown that increased mitochondrial efficiency can promote NAFLD reversal. In a recent study, a liver-targeted derivative of mitochondrial protonophore 2,4-dinitrophenol (DNP) was shown to enhance hepatic mitochondrial uncoupling and ameliorate NAFLD and T2D in the rat [181]. The thermogenic effect of this drug has long been known, and DNP has been investigated since the early twentieth century for its ability to promote weight loss. However, production of the drug ceased in the USA in the late 1930s following numerous reports of deaths in individuals taking DNP. In the aforementioned studies, the molecule used was targeted specifically to the liver, significantly reducing its toxicity, while retaining its potent mitochondrial uncoupling effects in the liver [181]. Subsequently, Perry et al. further improved the safety and efficacy of DNP by developing a version of the drug with lower peak plasma concentrations and sustained-release pharmacokinetics called CRMP (controlled-release mitochondrial protonophore) [181]. In rat models, CRMP produces mild hepatic mitochondrial uncoupling and reduced hypertriglyceridemia, insulin resistance, hepatic steatosis, and diabetes [182]. These data support the notion that mild hepatic mitochondrial uncoupling may be a safe and effective therapy for the related epidemics of metabolic syndrome, T2D and NASH. Whether this strategy could be employed in models of maternally inherited stress remains to be seen. One problem may lie within the already damaged mitochondrial pool in the livers of offspring from obese mothers. While mitochondrial uncoupling may increase oxidative metabolism in a healthy mitochondrial pool, CRMP may not have the desired effect in the dysfunctional mitochondrial pool in the developmentally primed liver and may in fact exacerbate oxidative stress. Needless to say, further research is needed to ascertain the effect of mitochondrial uncouplers on already suboptimal mitochondria.

12.11.2 PUFA Supplementation

The presence of inflammation within the liver is a key marker of NAFLD progression and NASH onset. In light of this, interventions that promote an anti-inflammatory state would be a suitable strategy to limit disease severity. Although a number of studies have shown that maternal obesity can cause inflammation in the

offspring, our understanding of the effectiveness of anti-inflammatory agents administered during pregnancy is limited. Nonetheless, preliminary studies have shown that dietary supplementation with polyunsaturated fatty acids (PUFAs) may have an anti-inflammatory effect. PUFAs exist as either omega 6 [n-6; linoleic acid (LA)] or omega 3 [n-3; alpha linolenic acid (ALA), eicosapentanoic acid (EPA), and docosahexanoic acid (DHA)] fatty acids [183]. It is generally accepted that eicosanoid signaling molecules derived from n-6 PUFAs are more immune-reactive than eicosanoids derived from n-3 PUFAs, considered to be anti-inflammatory [184]. Importantly, HFDs are predominantly n-6 PUFA rich and are relatively deficient in n-3 PUFAs, thus being a potential contributing factor to the pro-inflammatory state of obesity. Studies have shown that both EPA and DHA may have anti-obesogenic effects and may be able to prevent diet-induced obesity (DIO) [185]. Moreover, in a rat model of HFD feeding, dietary supplementation with krill-derived oils (KO) rich in EPA and DHA was able to increase fatty acid oxidation and inhibit lipogenesis in the liver, preventing hepatic steatosis. It is noteworthy that these effects may have mitochondrial origins since KO supplementation was associated with a significant increase in the activity of CPT-I, suggesting that the flux of fatty acids entering the mitochondria for oxidation may be enhanced by EPA and DHA [186]. Interestingly, CPT-I is known to be transcriptionally regulated by the member of the PPAR nuclear receptor family [187], of which n-3 PUFAs are a known agonist. It is therefore likely that PPAR signaling is an important mechanism in the insulin-sensitizing effects of n-3 PUFAs. In support of this notion, the hepatic insulin-sensitizing effects of n-3 PUFA supplementation were absent in PPAR-alpha knockout mice compared to wild-type controls [188].

The evidence from animal models regarding the potential of PUFAs as therapeutic agent to treat NAFLD is quite convincing; however, there is less data to support the notion that PUFA supplementation during pregnancy may have positive outcome on liver function in the resulting offspring. However, studies using Fat-1 transgenic mice, which are able to covert endogenous n-6 PUFA to n-3 PUFA, have shown that offspring of HFD-fed mothers who possessed the Fat-1 transgene were protected against hepatic fat accumulation, suggesting that increased relative n-3 fatty acids can ameliorate the developmental priming of fatty liver disease [189]. This lack in our understanding strongly suggests that further studies are warranted that specifically determine the effect of n-3 PUFA supplementation during development on hepatic fat accumulation and disease progression in offspring.

12.11.3 Sirtuin Activators

There has been considerable attention focused on the Sirtuin proteins that have been repeatedly implicated in the benefits to health and longevity associated with fasting and caloric restriction. It is therefore unsurprising that in models of maternal

obesity, a state of nutritional excess, reduced Sirtuin abundance and activity has been repeatedly observed in the developmental priming of NAFLD. In response, a number of studies have assessed the role of Sirtuin activators, such as resveratrol, in animal models of maternal obesity and maternal HFD feeding. For example, in a nonhuman primate model, resveratrol supplementation was able to improve both maternal and fetal hepatic fat accumulation [190]. While these proof-of-concept studies are promising, further studies in humans that better determine safety and efficacy are much needed.

12.11.4 Metformin

Metformin is a commonly used as an insulin-sensitizing agent that is able to suppress hepatic gluconeogenesis. Although still subject of intense investigation, current thinking suggests that the molecular mechanism of metformin involves inhibition of the mitochondrial respiratory chain (complex I), inhibition of glucagon-induced elevation of cyclic adenosine monophosphate (cAMP), activation of protein kinase A (PKA), inhibition of mitochondrial glycerophosphate dehydrogenase, and activation of AMP-activated protein kinase (AMPK) [191]. Probably one of the best-studied effects is AMPK activation, which is thought to stimulate ATP-producing catabolic pathways and to inhibit ATP-consuming anabolic processes such as gluconeogenesis. Indeed in the liver, activated AMPK reduces hepatic gluconeogenesis via the phosphorylation of CREB-binding protein (CBP) and the dissociation of the gluconeogenic transcriptional complex CREB–CBP–TORC2 [192]. Metformin-induced activation of AMPK decreases fatty acid and cholesterol synthesis at least in part by reducing acetyl-CoA carboxylase (ACC), 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase, fatty acid synthase (FASN), and inhibiting SREBP-1c [193]. In support, in the obese leptin-deficient Ob/Ob mice, a proxy model for hepatic steatosis, metformin treatment was able to reverse hepatomegaly, hepatic fat accumulation, and ALT abnormalities [194]. Clearly, metformin administration may be a suitable intervention to ameliorate the increased fat accumulation and gluconeogenesis that occurs during the developmental priming of NAFLD.

Metformin has been given to pregnant women since the 1970s [195] and is increasingly used as an alternative treatment of infertility and gestational diabetes [196–198]. However, its effect on offspring metabolic health is the current focus of research. In a study conducted in rats, diet-induced obesity during pregnancy enhanced fetal and placental cytokine production, which was reduced by maternal metformin treatment [199]. It remains to be determined whether this reduction in maternal and fetal inflammation impacts on NAFLD susceptibility of the adult offspring. In another study in mice, maternal metformin treatment was found to significantly improve glucose tolerance in HFD-fed offspring [200]. Nevertheless, it still remains to be determined if the improved metabolic profile in the metformin-exposed offspring also protects them from developing NAFLD. Several clinical

trials are currently under way to examine the effect of maternal metformin treatment during gestational diabetes in the offspring. In an earlier trial conducted in women with gestational diabetes, Rowan et al. have shown that children exposed to metformin had more subcutaneous fat at 2 years of age without the expense of the total amount of fat compared to those exposed only to insulin [201]. These changes in the fat distribution were suggested to provide a protection against later accumulation of ectopic fat, but can only be validated when these children have become adults. Findings from the recent Efficacy of Metformin in Pregnant Obese Women, a Randomised controlled (EMPOWaR) clinical trial, however, were discouraging and showed that metformin did not affect birth weight percentile in obese pregnant women and suggested that metformin should not be used to improve pregnancy outcomes in obese women without diabetes [202]. However, it is important to remember that birth weight is not the only important marker for long-term health in the offspring, but liver fat accumulation and function should also be considered.

In summary, while there are a number of strategies showing promising clinical outcomes, the capacity to reverse the developmental priming of NAFLD has not been demonstrated. Several studies have shown that proof-of-concept interventions during critical periods of development or plasticity may be able to ameliorate or reverse the effects of maternal obesity. However, their specific effect on liver function requires further investigation. It is important to note that while many of the interventions described target common metabolic pathways, the exact mechanisms are distinct (i.e., promotion of mitochondrial uncoupling via DNP, versus mitochondrial complex inhibition via metformin). A therapy that is beneficial for one individual may exacerbate the condition in another. Thus, the patient should be metabolically assessed as thoroughly as is reasonably possible before a pharmacological intervention during pregnancy is recommended.

As a group, pregnant women are extremely compliant to healthcare recommendations in order to do the very best for their developing baby and thus are likely to strongly adhere to suggested lifestyle and nutritional regimes during pregnancy. Therefore, identification of suitable intervention, or indeed preventative, strategies during pregnancy has huge clinical potential for both the current and the future generations.

12.12 Conclusion

The prevalence of maternal obesity is rapidly increasing worldwide, and as a consequence of developmental priming, the features of the NAFLD are also increasing in the next generation. The exact pathogenesis of NAFLD is likely multifactorial and adverse in utero events very likely play a role. Exposure to excess maternal lipids during pregnancy can already promote fetal mitochondrial dysfunction, oxidative stress, a disrupted circadian clock network, and premature gluconeogenesis, glycogenolysis, lipid oxidation, and de novo lipogenesis, thus priming the offspring liver to increase susceptibility to postnatal nutritional insults

resulting in NAFLD development. Thus, reduced oxidative capacity of the liver not only contributes to liver disease progression but also to whole-body hyperlipidemia, insulin resistance, and consequent metabolic syndrome and T2D.

Although further studies are still needed in both human and animal models to better understand the role of prenatal events in the pathogenesis of NAFLD, potential treatments are already emerging that benefit not only the obese pregnant mothers but also the future metabolic health of the offspring.

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