DNA methylation and hepatic insulin resistance and steatosis

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Purpose of review
In this review, we show novel evidence about the role of the liver in the development of insulin resistance and suggest that abnormal hepatic triglyceride accumulation is not an innocent bystander comorbidity but adversely affects the peripheral insulin sensitivity.

Recent findings
The core of this review is built up around the concept that liver DNA methylation of the peroxisome proliferative activated receptor gamma coactivator one alpha gene promoter modulates the status of peripheral insulin resistance and is strongly associated with plasma fasting insulin levels. We discuss about other mechanisms associated with peroxisome proliferative activated receptor gamma coactivator one alpha regulation, such as an acetylation and deacetylation switch and how these events impact on the liver metabolic function. We suggest a mitochondrial-centric approach to understand the connection between nonalcoholic fatty liver disease and insulin resistance. We finally show new data about how the liver epigenome is modulated by nutritional cues and introduce the role of epigenetics in liver metabolic programming.

Summary
The implications of these findings for clinical practice are promising, as the inherent plasticity of epigenetic modifications, produced either physiologically or pathologically, suggests that early therapeutic intervention in patients with fatty liver can potentially revert the systemic phenotype associated with insulin resistance.

Keywords
acetylation, deacetylation, DNA methylation, epigenetics, fatty liver, histones, mitochondria, nonalcoholic steatohepatitis, PGC1a, PPARGC1A

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD), a highly prevalent disease that results from excessive fat accumulation in the liver, is regarded as the hepatic manifestation of the metabolic syndrome [1] and is independently associated with an increased cardiovascular risk [2]. In a recent meta-analysis, we showed that NAFLD patients carry an increase of 13% of carotid intima-media thickness [3]. In addition, patients with NAFLD not only have increased circulating levels of biomarkers of atherosclerosis [4], but also show abnormal liver expression of mediators of atherogenesis and endothelial damage [5]. As insulin resistance is the hallmark feature of the metabolic syndrome, clinical and epidemiological evidence has suggested it as a major contributor to the pathogenesis and disease progression of NAFLD [1]. Nevertheless, there is scarce data about the molecular mechanisms by which insulin resistance and NAFLD are biologically linked. In addition, there is still a debate about whether insulin resistance is the triggering physiopathological mechanism of NAFLD, or conversely, NAFLD initiates the metabolic events associated with insulin resistance and metabolic syndrome-related phenotypes.

In this review, we describe novel evidence on the role of the liver in the development of insulin resistance and suggest that the abnormal hepatic triglycerides accumulation is not a bystander

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KEY POINTS

- Liver DNA methylation of *PPARGC1A* gene promoter might be the triggering event and perhaps an important causative factor of insulin resistance.
- Adaptation of body metabolic demands and variable environmental conditions is achieved through regulation of the liver *PPARGC1A* activity by mechanisms such as acetylation and deacetylation.
- Changes in liver mitochondrial DNA may explain the molecular events associated with the 'dyad' NAFLD-IR.
- Nutritional, pharmacological, and chemical cues can modify the metabolic capacity of the liver by epigenetic mechanisms and may imply a target for preventive or therapeutic intervention.
- Exposure to a nutritional insult in early life modulates the functionality of the liver during adulthood, and 'liver metabolic imprinting' during fetal life may further contribute to the pathogenesis of adult complications such as insulin resistance and NAFLD.

comorbidity but adversely affects the peripheral insulin sensitivity. This review is built up around the hypothesis that local epigenetic changes occurring in the liver critically modulate insulin resistance. We additionally suggest a mitochondrial-centric approach to understand the mechanisms associated with the 'dyad' NAFLD-IR. We also discuss new data about how the liver epigenome is modulated by nutritional cues; the list below summarizes schematically why we considered epigenetic factors as modifiers of NAFLD and insulin resistance.

(1) What we know about NAFLD and insulin resistance?
   (a) Insulin resistance and NAFLD result from a complex interplay between genes and environment.
   (b) Genetic risk factors seem to explain a small portion of the heritability of metabolic syndrome components.
   (c) Environmental factors, such as decreased physical activity and overnutrition, play an important role in the development of metabolic disturbances associated with the modern epidemic of metabolic syndrome-related phenotypes.

(2) Why epigenetics explains the pathogenesis of NAFLD and insulin resistance?
   (a) Epigenetic gene regulation is a key factor in the pathogenesis of complex disorders.
   (b) Epigenetic modifications can explain the mechanisms involved in the gene–environment interaction, sexual dimorphism, and metabolic programming during fetal development.
   (c) Epigenetic regulation is dynamic and is subject to both internal and external influences.
   (d) Epigenetic marks like DNA methylation can permanently modify the phenotype and be propagated during cell division and even transmitted to the next organismal generation.

(3) Implications for clinical practice or research
   (a) Epigenetic marks could be modified by diet, habits, and drugs.

LIVER DNA METHYLATION OF THE PPARGC1A PROMOTER LINKS FATTY LIVER WITH INSULIN RESISTANCE

Metabolic disturbances in the hepatic tissue, such as those found in the fatty liver, may be the triggering events and perhaps the causative factors of insulin resistance [6]. Furthermore, it also was suggested that fatty liver could be directly involved in the pathogenesis of obesity-associated dyslipidemia [6].

There are several candidates that can provide a direct link between external physiological stimuli and the metabolic disorders associated with NAFLD and insulin resistance. Perhaps, the main one that can orchestrate the regulation of genes involved in energy metabolism, response to starvation, positive regulation of gluconeogenesis and cell glucose homeostasis, positive regulation of fatty-acid beta oxidation, brown fat cell differentiation and adaptive thermogenesis, and mitochondrial biogenesis is the transcriptional coactivator *PPARGC1A*, also known as *PGC1α*. *PPARGC1A* also is putatively involved in the regulation of physiological processes, such as blood pressure [7] and cellular cholesterol homoeostasis, and clinical phenotypes, such as obesity and type 2 diabetes (T2D) [8].

Hence, we focused on the methylation of 5-methylcytosine in dinucleotides CpG, which is generally associated with gene silencing, and measured the level of DNA methylation of three putative methylation target sites in the promoter of the *PPARGC1A* (located at positions −513, −519, and −615 relative to the transcriptional start site) [9▪]. Interestingly, we demonstrated that the methylation status of the *PPARGC1A* promoter in the liver of patients with NAFLD is significantly associated with plasma fasting insulin levels and homeostasis model assessment of insulin resistance (HOMA-IR), regardless of the liver disease severity [9▪]. As expected, we observed that the methylation status of the *PPARGC1A* promoter was significantly

associated with fatty liver as disease trait, showing that a higher proportion of the alleles were methylated in NAFLD patients in comparison with that in the liver of control individuals [9▪]. In addition, we also observed that liver *PPARGC1A* mRNA abundance was inversely correlated with the methylation levels of *PPARGC1A* promoter CpGs, suggesting that the methylation of at least the three explored sites in the promoter efficiently repressed the transcriptional gene activity [9▪].

One may wonder whether this is a 'tissue-specific' event or if it is part of a global phenomena occurring in all of the metabolic syndrome target tissues. Actually, just few human studies have explored the DNA methylation status of candidate genes in tissues from affected patients. Thus, the evidence is still inconclusive, partly because of the limitations in sampling human tissues. Nevertheless, the published evidence confirms that the DNA methylation of the *PPARGC1A* promoter is critically involved in highly active metabolic tissues such as skeletal muscle and pancreas. For instance, Barres *et al.* [10] observed that in skeletal muscle biopsies of patients with T2D, the *PPARGC1A* promoter was hypermethylated, and most of the methylated cytosines were found within non-CpG dinucleotides, although surprisingly, the methylation status was neither related with peripheral insulin resistance nor influenced by glucose or insulin. In addition, DNA methylation of *PPARGC1A* promoter was explored *in vitro* in human isolated pancreatic islets of T2D patients, and methylation also was increased [11]. Despite *PPARGC1A* mRNA expression and insulin secretion being reduced in pancreatic islets, the methylation status was not correlated with insulin resistance [11]. In conclusion, altogether, these data support the 'liver-centric' hypothesis of the pathophysiology of insulin resistance and suggest the fatty liver as a modulator of the progressive impairment of insulin action in the liver, skeletal muscle, and adipose tissue, as shown by the clinical evidence [1,12,13]. This hypothesis supposes that liver epigenetic changes are not necessarily 'tissue specific', but their consequences on the systemic phenotype are 'organ specific'.

PPARGC1A IS HIGHLY REGULATED BY METABOLIC DEMANDS AND IS A TARGET FOR EPIGENETICALLY ACTIVE DRUGS

PPARGC1A activity is highly regulated by posttranslational modifications responding to metabolic stimuli. This premise supposes an important attribute of *PPARGC1A*: its capacity to adapt tissue metabolism to variable environmental conditions. The liver is particularly affected by nutritional factors, and the way the organ adapts to the metabolic demands is by a highly dynamic process that includes changes in metabolic enzymes and mitochondria. For example, a proteomic survey of mousse liver identified 388 acetylation sites in 195 proteins, being particularly abundant in the mitochondria [14].

In fact, nutritional signals modulate the effects of PPARGC1A on glycolytic genes in response to fasting, and this effect is regulated by sirtuin 1 (SIRT1) [15]. Recent data showed that AMP-activated protein kinase (AMPK) and SIRT1 directly affect PPARGC1A activity through phosphorylation and deacetylation, respectively [16]; actually, a previous report demonstrated that SIRT1 catalyzes PPARGC1A deacetylation both *in vitro* and *in vivo* [17]. Interestingly, these molecular events not only have a significant impact on energy expenditure, but also enhance mitochondrial biogenesis and oxidative phosphorylation (OXPHOS) capacity and indirectly modulate fatty acid oxidation and insulin resistance. The enzyme histone acetyltransferase GCN5 also acetylates PPARGC1A, a process that results in a transcriptional repression of the downstream *PPARGC1A*-regulated genes [18]; in-vitro functional evaluation showed that expression of GCN5 in mouse liver largely represses gluconeogenic enzyme gene transcription and decreases hepatic glucose production [18].

The coupled action of *PPARGC1A* and estrogen-related receptor alpha (*ERRα*) plays a central role in the transcriptional control of energy homeostasis, and the regulation of the ERRα activity also is under a dynamic acetylation and deacetylation switch [19]. Recent findings identified prospero homeobox 1 (Prox1), a genetic locus implicated in fasting glucose homeostasis and increased risk for T2D [20], as a negative regulator in the liver tissue of the ERRα–PPARGC1A axis [21].

A summary of functional protein interaction analysis around *PPARGC1A*-associated network using the above-mentioned proteins as template is shown in Figure 1; cellular response to hypoxia and histone modifications is highly predicted, and putative interesting new targets of epigenetic modifications are suggested. As a general conclusion, enzymes and complexes involved in epigenetic modifications of the chromatin structure also seem to play an important role in post-translational modifications of key proteins in metabolic pathways.

What may be the implications of these findings for clinical practice? As a possible answer, we highlight the concept of 'reversibility' supposing that these processes are plausible of intervention. For instance, acetylation–deacetylation of *PPARGC1A* was described to adapt mitochondrial energy

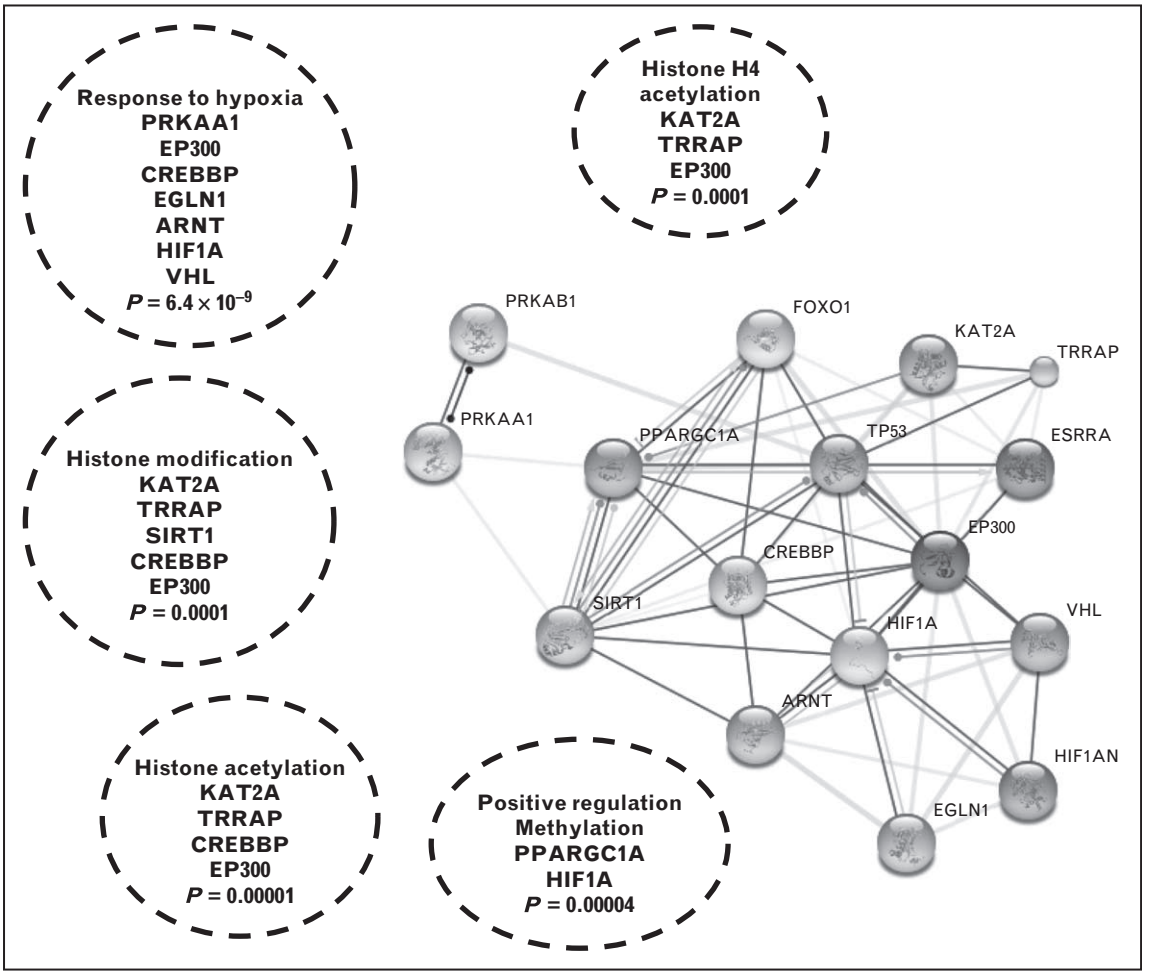

FIGURE 1. Network analysis of PPARGC1A protein interactions predicts new targets of epigenetic modifications. In-silico, protein-predicted interactions were performed by the STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) resource that includes direct (physical) and indirect (functional) associations; they are derived from four sources: genomic context, high-throughput experiments, conserved coexpression, and previous knowledge from literature. Network prediction shows putative functional links based on binding activity, post-translational modification, coexpression, or activation. Input genes: AMPK or PRKAB1, protein kinase, AMP-activated, beta 1 noncatalytic subunit-activated; ESRRA, estrogen-related receptor alpha; GCN5 or KAT2A, K (lysine) acetyltransferase 2A (functions as a histone acetyltransferase (HAT) to promote transcriptional activation; HIF1A, hypoxia-inducible factor 1, alpha subunit (basic helix -loop -helix transcription factor); PPARGC1A, peroxisome proliferator-activated receptor gamma, coactivator 1 alpha; and SIRT1, sirtuin (silent mating type information regulation 2 homolog) 1. New predicted targets: ARNT, aryl hydrocarbon receptor nuclear translocator; CREBBP, binding protein; EGLN1, egl nine homolog 1 (C. elegans); EP300, microRNA 1281 (functions as histone acetyltransferase and regulates transcription via chromatin); FOXO1, forkhead box O1; HIF1AN, hypoxia-inducible factor 1, alpha subunit inhibitor; PRKAA1, protein kinase; TP53, tumor protein p53; TRRAP, transformation/transcription domain-associated protein; VHL, von Hippel-Lindau tumor suppressor. Prediction of Go Biological processes are shown in dashed circles, which includes *P* values from predicted pathway.

demands [22▪▪]. Same concepts apply to histone methylation and demethylation [23].

As an example of the prospect of therapeutic intervention, several drugs or even natural compounds seem to act on histone deacetylases that decrease the acetylation of PPARGC1A and consequently increase its activity [24]. A small molecular activator of SIRT1, which is structurally unrelated to resveratrol but is a thousand-fold more potent, showed improvements in insulin sensitivity, plasma glucose levels, and increased mitochondrial capacity in an experimental model of insulin resistance [25].

In addition, a new concept is the 'mitochondrial epigenetics' as mitochondrial DNA (mtDNA) may be subject to methylation by a novel isoform of DNA methyltransferase 1 (DNMT1), which is upregulated by PPARGC1A [26▪]. We have found that the *ND6*

[NADH dehydrogenase, subunit 6 (complex I)] encoding by the mtDNA is hypermethylated in liver biopsies from NAFLD patients (unpublished data). This finding may be important to explain the following aspect.

INSULIN RESISTANCE AND NONALCOHOLIC FATTY LIVER DISEASE: A MITOCHONDRIAL-CENTRIC APPROACH TO EXPLAIN THE PATHOGENETIC CONNECTION

Mitochondrial dysfunction is largely recognized as being critically involved in the development of insulin resistance. In fact, normal activity of the mitochondria critically determines fatty acid beta-oxidation, OXPHOS, and insulin signaling. There is a close relation among metabolic stressors, mitochondrial biogenesis, and mtDNA copy number. We observed that mitochondrial biogenesis is reduced in the liver of NAFLD patients, and this reduction is associated with peripheral insulin resistance and *PPARGC1A* promoter methylation status [9▪]. This finding also was observed in other tissues, such as skeletal muscle [10]. Interestingly, in our study, mtDNA copy number was inversely correlated with HOMA-IR, serum fasting glucose, and plasma fasting insulin [9▪], suggesting that liver mtDNA is critically involved in the modulation of insulin resistance. The liver epigenome is perhaps more complex than expected, and other molecular mediators involved in the modulation of the mtDNA copy number, such as hypoxia-inducible factor 1 alpha (HIF1α) (Fig. 2) [27], are regulated by epigenetic modifications and also are substrate of histone deacetylases [28]. Remarkably, *PPARGC1A* is coupled to HIF1α signaling to modulate mitochondrial biogenesis [29].

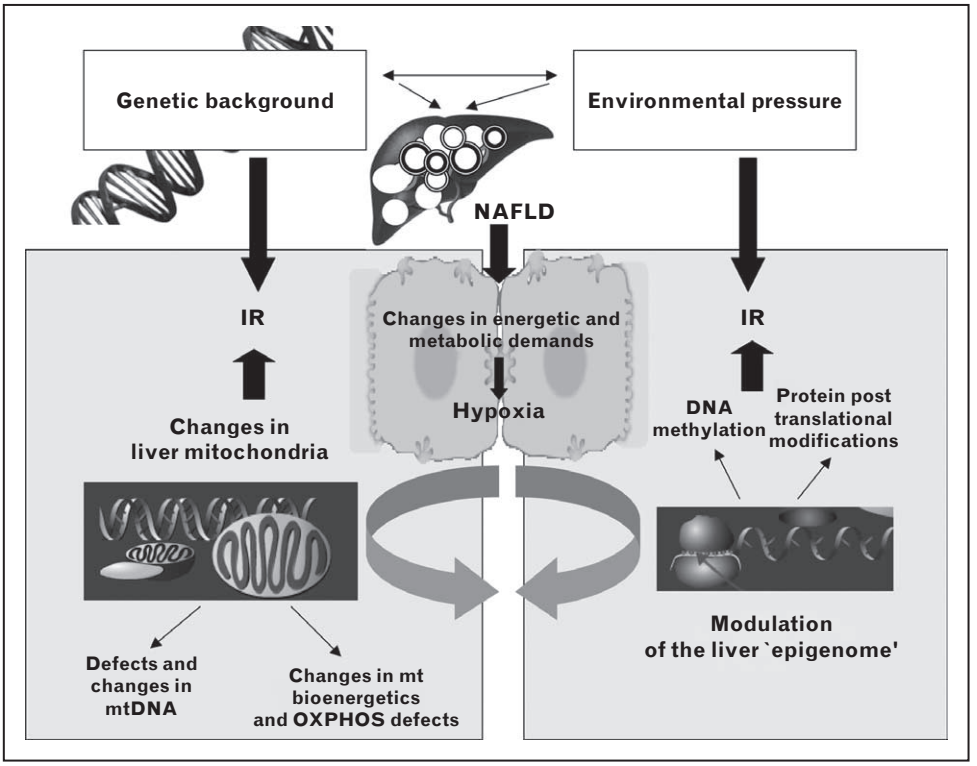

FIGURE 2. Mitochondrial-centric approach to explain the pathogenetic connection between fatty liver and insulin resistance. NAFLD and insulin resistance are complex disorders that develop from the interplay of genetic and environmental factors (high caloric intake, lifestyle, etc.). Fatty liver is associated with excessive cellular energetic demands to compensate the metabolic overload; in this scenario, hepatocyte hypoxia is a key molecular mediator, which may lead to the generation of reactive oxygen species, mitochondrial changes, and inflammation along with the associated insulin resistance state. The disease develops when adaptive mechanisms fail to keep the physiological functions, and both abnormal triglycerides accumulation and environmental pressure lead to changes in mitochondrial DNA (mtDNA) and mitochondrial bioenergetics, for instance, an increase in mtDNA copy number during initial disease state to adapt metabolic demands and a significant decrease when the disease progress and insulin resistance develops. In addition, NAFLD is associated with significant changes in the liver epigenome, which directly have an impact on transcriptional activity and protein function of target genes that modulate the mitochondrial function and physiology.

THE LIVER EPIGENOME IS MODULATED BY NUTRITIONAL CUES

Recent evidence from diet-induced NAFLD animal models showed that metabolic insults modify the DNA methylation of candidate gene promoters. For example, high-fat diet (HFD)-induced NAFLD is associated with markedly promoter hypermethylation of glycolytic genes, such as glucokinase (*Gck*) and L-type pyruvate kinase (*LPK*), that significantly correlated with the downregulation of their transcription and a profound impact on insulin sensitivity [30▪]. Conversely, high-sucrose diet was not associated with changes in liver DNA *Gck* methylation [31], suggesting that epigenetic changes in the liver might be influenced by some, but not all, nutritional factors.

Furthermore, mice fed a lipogenic methyl-deficient diet, which causes liver injury similar to that observed in human nonalcoholic steatohepatitis (NASH), showed aberrant histone modifications and alterations in the expression of Dnmt1 and Dnmt3a proteins in the liver [32].

Finally, recent data showed that under physiological conditions, protein acetylation is crucial in the regulation of liver gluconeogenesis. For example, the two key enzymes that catalyze the last and first step of glycolysis and gluconeogenesis, pyruvate kinase, and phosphoenolpyruvate carboxy kinase (PEPCK or PCK1), respectively, are both regulated by lysine acetylation [33,34].

METABOLIC PROGRAMMING OF THE LIVER EPIGENOME AND INSULIN RESISTANCE

Epigenetic changes, such as DNA methylation and histone modifications, also contribute to metabolic programming, and the most remarkable consequence is the transmission of the phenotype through generations. The concept of metabolic programming presumes a permanent change of the metabolism of the newborns exposed to an adverse intrauterine environment that continues to be expressed even without the original stimulus. Remarkably, epigenetic changes are the most attractive mechanisms to explain these events. This premise is well documented in rodents, and epigenetic changes in the liver tissue were demonstrated in models of maternal protein restriction and undernutrition. For example, maternal low-protein diet is associated with a significant increase in the hepatic expression of *Dnmt1* and *Dnmt3a* and methyl CpG-binding domain 2 (*Mbd2*) proteins, suggesting that maternal protein and folic acid restriction during gestation alters in the pups the global liver gene expression by regulating the genome-wide DNA methylation [35]. An interesting example was recently reported by Plosch *et al.* [36] showing that the liver X-receptor alpha (*Lxrα*, a nuclear receptor involved in control of cholesterol and fatty acid metabolism) promoter is hypermethylated in the fetal liver of protein-restricted pups, leading to a significant reduction of its mRNA.

Maternal overnutrition also causes a significant impact on the metabolic programming of the liver tissue. For instance, gestational HFD results in modifications of the hepatic Pck1 histone code in offspring livers, suggesting that in-utero exposure to HFD programs the gluconeogenic capacity of the offspring through epigenetic modifications leading to excessive glucose production and altered insulin sensitivity in adulthood [37].

In addition, HFD during pregnancy significantly causes transgenerational accumulation of epigenetic modifications leading to the upregulation of metabolic pathways in the liver [33].

Finally, reinforcing our mitochondrial-centric concept of the pathogenesis of insulin resistance, in a rodent model, we showed that maternal HFD feeding during pregnancy programs liver mtDNA content and the liver transcriptional activity of *Ppargc1a,* which strongly modulates, in a sex-specific manner, glucose homeostasis and organ fat accumulation in adult life, including the development of fatty liver [38]. Again, the association of changes in DNA methylation and mtDNA content also are seen in the abnormal extremes of fetal growth in human newborns [39,40].

In addition, gestational protein restriction that results in low birth weight was associated with significant changes in liver gene expression, specifically showing an upregulation of mitochondrial genes [41].

CONCLUSION

The liver is a critical metabolic sensor that tightly commands glucose and lipid metabolism. Phenotypic changes, such as fatty liver, are associated with tissue modifications at molecular level, particularly the liver epigenome. Hepatic DNA methylation of the promoter of master metabolic regulators, such as *PPARGC1A*, has a strong impact on peripheral insulin resistance and body insulin sensitivity and on liver mitochondrial biogenesis. Nutritional cues are powerful modulators of the liver epigenome and operate at different levels, including metabolic programming. It is still an open question to what extent the liver epigenetic marks are reversible by any intervention and if so, by modifying the epigenetic marks, we can operate efficiently on the phenotype. In addition, epigenetic marks are now found in

mtDNA; mitochondrial genomes should be included and further investigated. Finally, as DNA and histone-modifying enzymes are active on a wide array of cytosolic and mitochondrial proteins, the concept of the 'epiproteomics' may emerge.

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Conflicts of interest

There are no conflicts of interest.

REFERENCES AND RECOMMENDED READING

Papers of particular interest, published within the annual period of review, have been highlighted as:

- ■ of special interest
- ■■ of outstanding interest

Additional references related to this topic can also be found in the Current World Literature section in this issue (p. 404).

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