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Review

Insulin resistance and epigenetic regulation: insights from human studies and prospects for future research

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Abstract

In this study, we review the current knowledge and recent insights on the role of epigenetic factors in the development of human insulin resistance (IR)- and metabolic syndrome (MS)-related phenotypes, and attempt to lay a framework to consider IR as a potentially reversible incapacity to control metabolic homeostasis that is strongly influenced by the interplay between external and internal cues. We summarize the evidence on how tissue-specific epigenetic markers participate either by activating or repressing the gene expression programs to modulate IR- and MS-associated traits. Some additional data are provided about how the exploration of DNA methylation markers in peripheral blood mononuclear cells potentially offers appealing information about the impact of epigenetics in the pathogenesis of IR. Clues about the relation between IR and impaired intrauterine growth explained by fetal metabolic programming and epigenetic modifications are shown, including novel findings about the impact of histone modifications. For instance, we observed that specific epigenetic factors in genes associated with mitochondrial biogenesis may be associated with birth weight. Furthermore, some prospective ideas about the functional consequences of genetic variation modulated by allele-specific epigenetic markers and its impact on MS susceptibility are also illustrated. Finally, we summarize the current knowledge of epigenetics as the biological rationale for potential therapeutic intervention in IR and MS.

Keywords: allele-specific epigenetic marks; DNA methylation; epigenetics; fatty liver; fetal metabolic programming; histone deacetylases; histone modifications; insulin resistance; intrauterine growth; large for gestational age; metabolic syndrome; mtDNA copy number; PGC1a; *PPARGC1A*; small for gestational age.

Introduction

Metabolic syndrome (MS) is associated with several metabolic disturbances, including insulin resistance (IR) in several tissues. Indeed, IR is considered as the main link among all the clinical disorders clustered in MS, namely type 2 diabetes (T2D), dyslipidemias, central obesity, arterial hypertension, prothrombotic and proinflammatory states, ovarian polycystosis, and non-alcoholic fatty liver disease (NAFLD). From the perspective of clinical importance, MS has two prominent features: its worldwide prevalence that is dramatically increasing and its strong association with cardiovascular disease, as initially described by Reaven (1).

It is broadly accepted that IR results from a complex interplay between genes and environment; however, despite significant efforts, the understanding of its pathogenesis remains a major challenge. In fact, while both genome-wide and candidate gene association studies have identified several loci that influence the susceptibility of all the clustering traits of MS, they have also demonstrated that the identified loci only explain <10% of the population variance (2, 3), owing to the fact that the reported effect size of the gene variants is modest, as shown, for example, for T2D and other components of MS (2, 4).

Likewise, environmental factors, such as decreased physical activity, increased nutrient availability and overnutrition, play an important role in the development of metabolic disturbances associated with IR and are also largely recognized as being responsible for the modern epidemic of MS-related phenotypes. Nevertheless, they do not explain the pathophysiology of IR and MS. This is, in part, because of the fact that the environmental factors operate at different levels, and its influence is even more significant in a genetically predisposed background because of an important individual susceptibility as observed from twins studies, although this concept has been recently challenged (5, 6). Hence, it is reasonable to speculate that the gene-environment interaction is a strong modifier of the IR state, and this interaction is modulated by epigenetic mechanisms.

Why is epigenetics important to understand the pathophysiology of IR? Not only because of the biological plausibility

but also because epigenetic modifications regulate gene transcription, regulate chromosome organization, are highly regulated by environmental stimuli, including nutritional status, are highly dynamic, may operate in a tissue-specific manner, and while epigenetic changes are heritable across cell division, they can also occur *de novo*.

Cytosine (C) methylation (5-methylcytosine) is a common epigenetic modification, where a C is adjacent to a guanine (G) nucleotide (CpG). Around 5% of cytosines are methylated, and nearly 50%–60% of genes have CpG-rich islands (regions of typically 300–3000 bp in length with a high content of CpG and C/G% in their five untranslated regions). During fetal development as well as in adult life, normal somatic cells (and also cancer cells) exhibit alterations in DNA methylation induced by environmental stimuli. As CpGs are paired by GC in the opposite strand, methylation in one strand is mirrored by methylation in the other. During replication, methylation in the parent strands directs methylation in the newly replicated DNA by recruiting DNA methyltransferases (DNMTs). Subsequently, stable transfer of gene methylation patterns to progeny lines is accomplished. CpG methylation is thought to constrain expansive regions of the genome by silencing repetitive sequences or repressing promoters by recruiting methyl-CpG binding proteins (MBD protein family). Although methylation is associated with repressed promoters, transcriptional repression *via* histone methylation and acetylation precedes DNA methylation. Interested readers can find several excellent reviews on this issue in the literature; owing to space constraints, we have just mentioned two in this study (7, 8). A short overview on the main features of epigenetic modifications is shown in Table 1.

Table 1 Epigenetic modifications: a brief overview.

Epigenetic modification	Main outcome and effect
1. DNA methylation Methylation in CpG rich regions or CpG islands Regions are usually longer than 500 bp GC base content >55% Located in the promoter regions and at the end of the 5′ region Types of promoters: rich and poor in CpG islands Methylated sites are distributed globally on approximately 80% of CpGs Enzymes involved in this process: DNA methyltransferases (DNMTs) Dnmt1, Dnmt3a, Dnmt3b, Dnmt2 and accessory proteins, such as Dnmt3L Stable in somatic cells, but modifiable by environmental factors Methylation levels may show inter-individual heterogeneity Different tissues are able to show local differences in DNA methylation	• Modulates gene transcription • Methylation of CpG islands of promoter region is mostly associated with silencing of gene expression • Methylation of CpG dinucleotides located in the gene coding sequence is weakly associated with gene silencing
Non-CpG methylation Location in the promoter remains controversial The functional significance of non-CpG methylation in early development is uncertain May be modulated by DNMT3 activity (70) Can occur in CpA, CpC and CpT nucleotides situated in the DNA of embryonic stem cells and episomal DNA (70)	Inducible by environmental factors?
2. Histone post-translational modifications Modifications: Acetylation, methylation, ubiquitination and SUMOylation of lysine residues Phosphorylation of serine residues Methylation of arginines Most frequent histone lysine modifications (71): methylation of histone H3 at Lys9 (H3-K9) or Lys27 (H3-K27): associated with gene silencing and methylation or acetylation of histone H3 at Lys4 (H3-K4) or acetylation of H3 at Lys27 (H3-K27): associated transcriptional activation	• Implicated in the *de novo* methylation of DNA • Histone acetylation: associated with more open chromatin and transcriptional activation • Histones hypoacetylation: associated with condensed chromatin structure and repression of gene transcription
Enzymes involved in these processes: Histone acetyltransferases (HATs) Histone deacetylases (HDACs) Histone methyltransferases Methyl-binding domain protein MECP2	• HATs can be divided into several families, including the PCAF/ Gcn5, p300/CBP, MYST, SRC, TAFII250, HAT1, and ATF-2 families • HDACs are classified into four groups (I–IV)

In this study, we review the current knowledge and recent insights on the role of epigenetic factors in the development of human IR- and MS-related phenotypes, and attempt to lay a framework to consider that IR is a potentially reversible incapacity to control the metabolic homeostasis that is strongly influenced by the interplay between external and internal cues.

Peroxisome proliferator-activated receptor γ coactivator 1 α (*PPARGC1A*) is a target gene of epigenetic modifications in MS-related phenotypes

The transcriptional coactivator, peroxisome proliferator-activated receptor γ coactivator 1 α (*PPARGC1A*) coordinates the regulation of genes involved in energy metabolism by controlling transcriptional programs of mitochondrial biogenesis, adaptive thermogenesis and fatty acid β oxidation. In fact, its tissue specificity pattern of expression is mainly located in the heart, skeletal muscle, liver and kidney (9). Interestingly, the protein encoded by this gene is involved in controlling blood pressure, regulating cellular cholesterol homoeostasis and development of obesity, and altered signaling of *PPARGC1A* contributes to glucose intolerance, IR and T2D (10). Not surprisingly, this gene has been the most chosen candidate gene for the evaluation of epigenetic modifications in human phenotypes associated with IR and MS (Figure 1). For instance, we had demonstrated for the first time that in the liver of patients with NAFLD, the methylation status of the *PPARGC1A* promoter is significantly associated with plasma fasting insulin levels and homeostasis model assessment of insulin resistance (HOMA-IR) (11). In addition, in the pancreatic

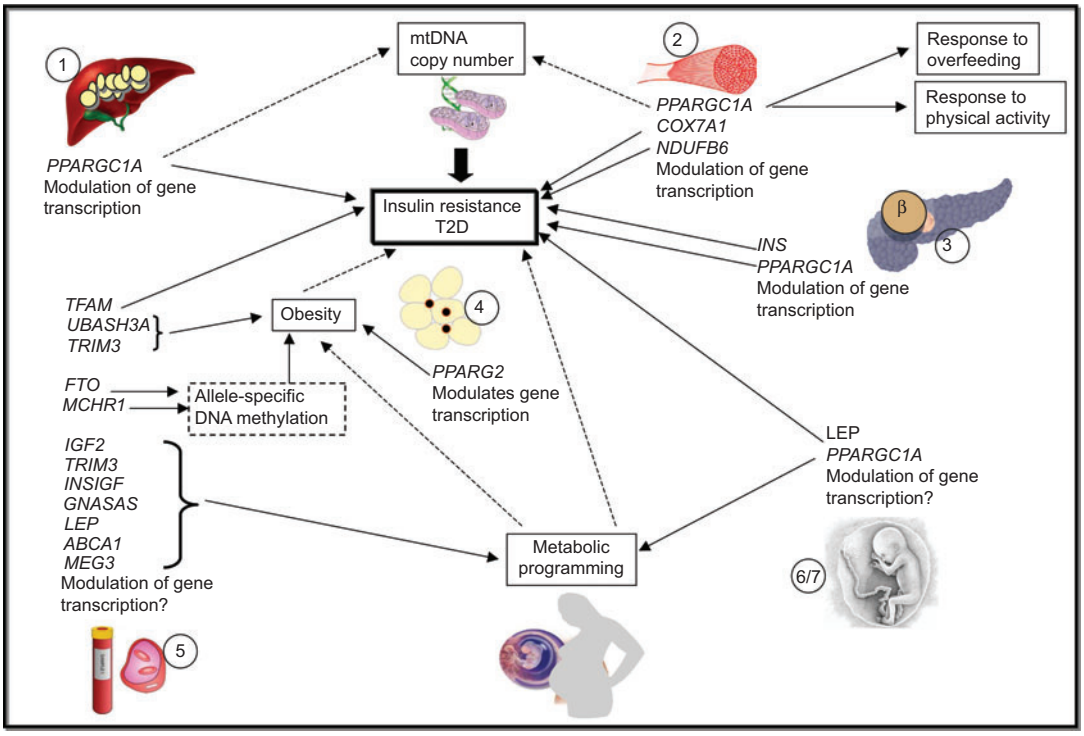

Figure 1 Role of DNA methylation status of target genes in the development of human IR- and MS-related phenotypes: summary of current knowledge.

Several tissues are shown, such as liver (1), skeletal muscle (2), pancreatic islets (3), adipose tissue (4), white cells of the blood (5), umbilical cord (6) and placenta (7) where the status of promoter DNA methylation of some candidate genes was explored in humans. The effect of DNA methylation in some tissues significantly impacts on modulation of gene transcription, for example, liver, skeletal muscle, pancreatic islets and adipose tissue. In some other tissues or cells, the consequences of DNA methylation of candidate genes remain unexplored, for example, white cells of the blood, umbilical cord and placenta. The resulting phenotypes substantially vary according to the target tissue and the target gene, for instance, DNA methylation of the *PPARGC1A* promoter in the liver, skeletal muscle and pancreas directly impacts on modulation of the status of insulin resistance (solid arrows) or indirectly by modulation of mitochondrial biogenesis (dashed arrows) or obesity. Some evidence on modulation of the promoter activity of *PPARGC1A* in skeletal muscle by lifestyle modifications, such as physical activity or weight reduction programs, is also shown. Finally, evidence on allele-specific DNA methylation of selected genes, such as FTO and MCHR1, and the role of DNA methylation of candidate genes in metabolic programming is also provided. Citations of each tissue or cell are shown as follows: fatty liver (11), skeletal muscle (13, 15, 72, 73), pancreatic islets (12, 18), adipose tissue (16), peripheral white blood cells (22, 23, 25, 26, 33, 74), blood umbilical cord and placenta (34, 39).

islets of patients with T2D, Ling et al. observed high levels of DNA methylation of the *PPARGC1A* promoter (12) (Figure 1).

Interestingly, Barres et al. observed that in vastus lateralis muscle biopsies of patients with T2D, the promoter of *PPARGC1A* was hypermethylated, but surprisingly, most of the methylated cytosines were found within the non-CpG dinucleotides (13). In fact, by whole-genome methylation using luminometric methylation assays, the authors found that global CpA and CpT methylation was increased, although CpG methylation was unaltered (13). If replicated in other tissues, this finding might have important biological implications because cytosine-5 methylation at CpA and, to a lesser extent, at CpT, is almost confined to embryonic stem cells, but not somatic tissues (14).

An important finding to highlight is that in addition to an impact on the phenotype, the methylation status of the *PPARGC1A* promoter was observed to be inversely correlated with the expression of the mRNA in all the above-mentioned tissues (11–13). Perhaps, the most remarkable observation is that in the liver of NALFD patients and in the muscle of T2D patients, a tight interaction between the transcriptional activity of *PPARGC1A* and the mitochondrial DNA copy number was noted, which had a direct impact on the status of IR (11, 13) (Figure 1).

Finally, a remarkable finding about the potential modulation of the *PPARGC1A* gene by external cues was reported by Alibegovic et al., who showed that IR induced by physical inactivity is associated with multiple transcriptional changes in the skeletal muscle in young men, which had a direct impact on mitochondrial function (15). The authors also showed a trend towards increased *PPARGC1A* DNA methylation after bed rest (15) (Figure 1).

Epigenetic markers encompass tissue-specific effects that independently modulate the status of insulin resistance

The metabolic state known as IR is actually a systemic failure to respond to physiological levels of insulin, leading to a global dysregulation in the ability of the target tissues to maintain the equilibrium among storage, mobilization and utilization of energy fuels, such as glucose and free fatty acids. Thus, at least three tissues are involved, namely, muscle, liver and adipose tissue. Nevertheless, although the ultimate consequence is the loss of insulin capability to maintain normal glucose metabolism, each tissue has its own profile of activated or repressed genes, because each tissue needs to adapt to its physiological functions. Hence, epigenetic markers are either cell- or tissue-specific to activate or repress their own gene expression programs.

A clear example of this observation is the report by Fujiki et al., who demonstrated that in 3T3-L1 preadipocytes, the promoter of the proliferator-activated receptor γ 2 (*PPARG2*) is hypermethylated, but this state changes progressively and becomes demethylated upon induction of differentiation, which is accompanied by an increase in *PPARG2* mRNA expression (16). Interestingly, the expression of the *PPARG2* isoform in the adipose tissue was found to be regulated at the transcriptional level by nutrition (17).

Kuroda et al. evaluated the role of epigenetic regulation in human pancreas and examined the DNA methylation pattern of the insulin gene (*INS*) in human β and non-β cells, observing that the *INS* promoter, CpG demethylation, plays a crucial role in β cell maturation and tissue-specific *INS* gene expression, because methylation suppresses *INS* expression (Figure 1) (18).

Maternal IR is an important player in the development of gestational diabetes, and most of the cellular mediators of IR in this condition are placental-derived hormones. Bouchard et al. recently reported an interesting association between leptin gene DNA methylation in placenta and gestational impaired glucose tolerance (19). Despite this significant association between placental DNA methylation and the mother's glycemia during pregnancy, it requires further investigation; the finding opens an interesting question on the role of epigenetic modulation of leptin levels during pregnancy by a tissue that normally produces high levels of leptin, which is also highly associated with newborn birth weight, and is involved in human perinatal morbidity and mortality.

Another interesting topic for discussion is the fact that patterns of DNA methylation are variable between individuals and 'epigenotypes' may contribute to the susceptibility of complex diseases (20). A recent report on whole-genome DNA methylation analysis (methylome) in human peripheral blood mononuclear cells (PBMCs) demonstrated that this methylome is rich in biological information (21). Li et al. also observed that 68.4% of CpG sites and 0.2% of non-CpG sites were methylated, demonstrating that non-CpG cytosine methylation is minor in human PBMCs (21). Based on these findings, the analysis of the methylation pattern in PBMCs of candidate genes could be very informative. Moreover, PBMCs are readily accessible and most of the Biobanks around the world might have access to them. A clear example of the potentially appealing information on DNA methylation markers in PBMCs and MS-related phenotypes is given in the following paragraph.

We evaluated the DNA methylation status of the transcription factor A mitochondrial (*TFAM*) promoter in PBMCs of adolescents with features of MS, and observed an inverse correlation with IR, considering either metabolic quantitative traits (fasting insulin and glucose levels and HOMA index) or IR as a dichotomous condition (22). In fact, the ratio of the promoter methylated DNA/unmethylated DNA was 0.012±0.0009 (1.2% of alleles) and inversely correlated with the biochemical features of IR (plasma fasting insulin: R=-0.26, $p<0.004$; HOMA index: R=-0.27, $p<0.002$) and obesity (R=-0.27, $p<0.002$) (22).

Another study provided evidence that obesity is associated with methylation changes in two immune-related genes, *UBASH3A* and *TRIM3*, in blood leukocyte DNA and suggested an interesting role of obesity-induced immune dysfunction (23).

Epigenetics and genetic variation: how are epigenomics and genomics integrated to understand the susceptibility to MS-related phenotypes?

Epigenetic markers can also give us a mechanistic explanation about how single nucleotide polymorphisms (SNPs) may influence the genetic susceptibility of common diseases, because it was shown that differential DNA methylation between alleles is not restricted to imprinted genes or the female X chromosome (24). In fact, recent evidence showed that approximately 10% of autosome human genes may be affected by allele-specific methylation (24). This finding is also relevant because allele-specific gene expression contributes not only to common diseases but also to inter-individual differences in disease susceptibility.

Robust examples of two candidate loci involved in MS-related diseases, such as fat mass and obesity associated (*FTO*) and melanin-concentrating hormone receptor 1 (*MCHR1*), which show allele-specific DNA methylation, are given in the following paragraphs.

For instance, by evaluating the density of methylated CpGs in the whole blood genomic DNA from Caucasian individuals, Bell et al. recently reported an increased DNA methylation on the obesity susceptibility *FTO* haplotype, tagged by the rs8050136 risk allele A (25).

Stepanow et al. performed methylation analysis of a CpG island in the first exon of the *MCHR1* encompassing the rs133072 and rs133073, a gene involved in the control of energy metabolism and linked to obesity, and found that *MCHR1* methylation is allele-specific, age-dependent, BMI-associated and affects gene expression (26).

IR and impaired intrauterine growth: fetal metabolic programming and epigenetic modifications

A growing body of evidence supports the notion that epigenetic alterations, such as DNA methylation and histone modifications, which mediate phenomena, such as genomic imprinting, may also contribute to metabolic programming (27). Perhaps, the most important consequence of metabolic programming is the transmission of the phenotype from mother to the progeny and even through generations (27). This observation has a direct impact on public health as epidemiological data show that impaired intrauterine growth and adult metabolic and cardiovascular disorders, including T2D and IR, are strongly associated (28–30).

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. Although this premise has been well documented in rodents (27, 31), few human studies have examined the interplay between an adverse *in utero* environment and epigenetic modifications as a potential mechanism to explain the later development of MS-related phenotypes.

The most robust observation about the influence of the prenatal environment on DNA methylation came from epigenetic studies that examined individuals who were exposed to famine during gestation, such as the Dutch famine at the end of World War II, which affected the western part of The Netherlands from 1944 to 1945 (32). Heijmans et al. evaluated 60 individuals from the Hunger Winter Families Study and demonstrated that those who were prenatally exposed to the Dutch famine had, six decades later, less DNA methylation of the imprinted insulin-like growth factor 2 (*IGF2*) gene, when compared with their unexposed, same-sex siblings (33). In fact, Heijmans et al. showed that periconceptional exposure to starvation was associated with a 5.2% lower methylation in the promoter of *IGF2*, evaluated in the genomic DNA from whole blood (33) (Figure 1). *IGF2* encodes a member of the insulin family of polypeptide growth factors that are involved in development and growth; it is an imprinted gene, expressed only from the paternal allele. To further explore the associations between prenatal famine (as low as 500 kcal/day) and DNA methylation of non-essential imprinted genes, in the Hunger Winter Families Study, Tobi et al. assessed the methylation state in the regions of 15 candidate genes involved in metabolic and cardiovascular disease, and observed that six loci showed significant differences in DNA methylation after famine exposure during periconception (*INSIGF*, *GNASAS*, *MEG3*, *IL10*, *LEP* and *ABCA1*); interestingly, three of the loci (*INSIGF*, *GNASAS* and *LEP*) differed by sex (34) (Figure 1). The methylation status of just one locus, *GNASAS*, was associated with exposure to famine late in gestation (34); this gene produces a paternally imprinted antisense RNA transcript that helps to regulate the *GNAS* complex locus that encodes the α subunit of the stimulatory G protein.

Likewise, infants who are born either with low birth weight (29) (small for gestational age, SGA) or are large for gestational age (LGA) (35) are at an increased risk of developing MS and its complications (36).

This association between birth weight and adult chronic diseases also demonstrates the concept of metabolic programming. In fact, the epidemiological evidence on the relationship between abnormal fetal growth and adult disease replicated all around the world (37, 38) illustrates the importance of the *in utero* nutritional environment in the modulation of the adult phenotype, and suggests the induction of epigenetic markers that reprogram the metabolic machinery of the offspring.

An interesting premise to follow-up is how and why both extremes of fetal growth are connected by a common outcome. In this context, a reasonable question arises.

Does DNA methylation connect both extremes of abnormal fetal growth with metabolic programming?

To answer this question, we recently examined the status of differential DNA methylation in master genes that control either mitochondrial number – such as *TFAM* – or mitochondrial function, adaptive thermogenesis, glucose and fat oxidation in muscle and fat tissue, and gluconeogenesis in liver – such

as *PPARGC1A* – or adipogenesis and insulin signaling, such as *PPARG* in umbilical cord of newborns between the extremes of abnormal fetal growth, namely SGA and LGA (39). We also evaluated the relationship between the methylation status of the promoter of these genes and the mother's pre-pregnant characteristics (39). Interestingly, we observed a positive correlation between maternal body mass index (BMI), which suggests a potential role of *PPARGC1A* methylation in the metabolic programming of the fetus (39). In addition, a novel aspect of our findings is the evaluation of epigenetic markers in the DNA of the umbilical cord, which is a crucial link between the mother and the fetus during pregnancy. An interesting aspect worth mentioning is that the *PPARGC1A* promoter methylation in the umbilical cord is modified by the presence of homozygosity to the rs8050136 risk allele A of the *FTO*, in particular, in the LGA offspring, which indicates that *FTO* may act by altering DNA methylation of metabolic relevant genes. FTO is a nuclear protein of the AlkB-related non-heme iron and 2-oxoglutarate-dependent oxygenase superfamily, but the exact physiological function of this gene is unknown. As other non-heme iron enzymes function to reverse alkylated DNA and RNA damage by oxidative demethylation, it is rational to hypothesize that FTO, with other proteins of the TET (ten-eleven-translocation) oncogene family and Jumonji C domain-containing proteins, may participate in the metabolism of another recently described epigenetic marker, 5-hydroxymethyl-cytosine, which may be an intermediate in the demethylation process and may be altered in malignancies (40, 41).

Under the same hypothesis that individuals born with low birth weight (LBW) are at increased risk of developing IR, T2D and MS later in life, Brons et al. hypothesized that young and healthy LBW subjects show altered DNA methylation in the promoter region of *PPARGC1A* (42) (Figure 1). Thus, the authors performed an evaluation of the status of *PPARGC1A* methylation in the skeletal muscle in subjects during a control diet as well as after the exposure to a diet high in fat and calories for a 5-day period (42). The results of this study showed that LBW subjects had elevated DNA methylation of the *PPARGC1A* promoter at baseline, and this status modulated *PPARGC1A* mRNA expression when challenged by a high-fat overfeeding diet (42).

The evidence showing that the maternal environment alters the fetus growth also suggests the concept of 'parental effect' that is defined as the effect on the phenotype of the offspring, which is determined by the genotype or environment experience of the parents (43). The most clear example of this concept is the influence of maternal environment on the development of fetus adiposity, which shows that underweight mothers are more likely to give birth to lower weight infants and overweight women are more likely to give birth to larger infants (44). We also observed that maternal pre-gestational BMI is associated with the weight of their offspring, and SGA babies had lean mothers (BMI=21.4±0.7) and LGA babies had overweight mothers (BMI=26.7±1.4), when compared with babies with appropriate weight for gestational age (BMI=23.0±0.7; $p<0.003$) (45). Furthermore, weight of newborns may be predicted from the weight of previous offspring.

After reviewing the current data about the association between fetal programming, adult disease, and changes in the status of DNA methylation in candidate genes, another interesting question arises.

Do post-transcriptional histone modifications play a role in human fetal programming?

Around 10 years ago, Jenuwein and Allis postulated the 'histone code' hypothesis (46). According to the hypothesis, chromatin structure is regulated by a specific combination of histone modifications. Currently, histone modifications are considered to be central epigenetic markers. In fact, several dynamic post-translational covalent reactions (acetylation, methylation, phosphorylation, ubiquitylation, SUMOylation, ADP ribosylation, deamination and proline isomerization) have been reported in the histone tails, the histone N-terminals that protrude from the nucleosome, a portion of 166 bp DNA wrapping of histone octamers (two molecules of histones 2a, 2b, 3 and 4). The acetylation of histone Lys (K) is associated with activation of adjacent chromatin regions, whereas the opposite is closely related to inactivated chromatin states.

Previously, histone acetylation was considered to be a hallmark of transcriptional activation. However, detailed investigation has revealed that histone methylation at specific amino acid residues, mainly K, in histone N-terminal tails is an important modification defining chromatin state (47). Methylations at H3 lysine 4 (H3K4) and lysine 36 (H3K36) appear to be signals of chromatin activation, whereas methylation of H3K9 and H3K27 seems to be related to chromatin condensation. A further complexity is added by the fact that one to three methyl radicals could be added (Me1, Me2 or Me3).

Unfortunately, how histone modifications impact the human fetal programming remains poorly understood and, to the best of our knowledge, the only evidence about a possible association comes from animal studies (48).

Therefore, to investigate the patterns of histone methylations across the promoter of *PPARGC1A* (from -320 to -700 bp from the transcription start site) and *TFAM* (from -512 to -930 bp from the transcription start site) genes in newborns exposed to different prenatal environments, we extracted DNA with or without chromatin enrichment in specific histone modifications by chromatin immunoprecipitation (CHIP) from the cell nuclei of the umbilical cord. In this analysis, we evaluated 50 newborns, including 16 with appropriate weight for gestational age and 34 representing both the extremes of abnormal fetal growth: SGA (n=17) and LGA (n=17). We used antibodies specific for modified H3, such as H3K4Me3 and H3K9Me3 (Active Motif, Carlsbad, CA, USA). Specific gene abundance in the immunoprecipitated chromatin against the input, non-enriched chromatin, was evaluated by real-time qPCR. Interestingly, we observed that the H3K4Me3-related *TFAM* promoter is associated with birth weight (Wilks=0.60, $p<0.014$); two different segments of the promoter were perfectly correlated (Spearman R=0.89, $p<0.0000001$; unpublished data).

Previous evidence from our group (49) and others (50) showed that the missense Gly482 Ser (rs8192678) variant in *PPARGC1A* is associated with features of MS. These findings prompted us to explore a potential relationship between the status of histone methylation in the *PPARGC1A* promoter and the Gly482Ser genotypes. Using antibodies specific to H3K9 Me3, we observed significant differences between alleles showing that carriers of the 482Ser allele had 50% (p<0.02) less H3K9 Me3-associated *PPARGC1A* promoter regions than the non-carriers. This novel finding suggests an interesting interaction between post-transcriptional histone modifications and Gly482 Ser polymorphism in *PPARGC1A*, because H3K9 trimethylation is usually associated with gene repression. Hence, our data might indicate an allele-specific chromatin modification. Although these data must be investigated further, they suggest that potential phosphorylation of the *PPARGC1A* 482Ser (as predicted *in silico*) by kinases, such as CKII, may modulate its own expression by inducing chromatin modifications. In fact, PPARGC1A is one of the best-characterized nuclear receptor coactivators by docking histone acetylases or other coregulators (51). Although the majority of these regulatory subunits remain to be characterized, *in silico* analysis (52) showed that *PPARGC1A* is a central regulator of chromatin modifications, exhibiting important interactions with other nuclear receptors, DNA methylases, histone acetylases and deacetylases (Figure 2).

Expert opinion

Do tissue specific or global deviations from the physiological epigenome explain IR?

The pathogenesis of peripheral IR is strongly associated with the ability of the liver to suppress endogenous glucose production, suggesting that this organ is a key player in this scenario. Some metabolic disturbances in the hepatic tissue, such as abnormal triglycerides accumulation observed in fatty liver, have been suggested as the trigger events and perhaps the causative factors of IR (53–55). In addition, NAFLD represents the hepatic component of the MS, and IR is a hallmark feature in the pathophysiology of NAFLD. Despite all the

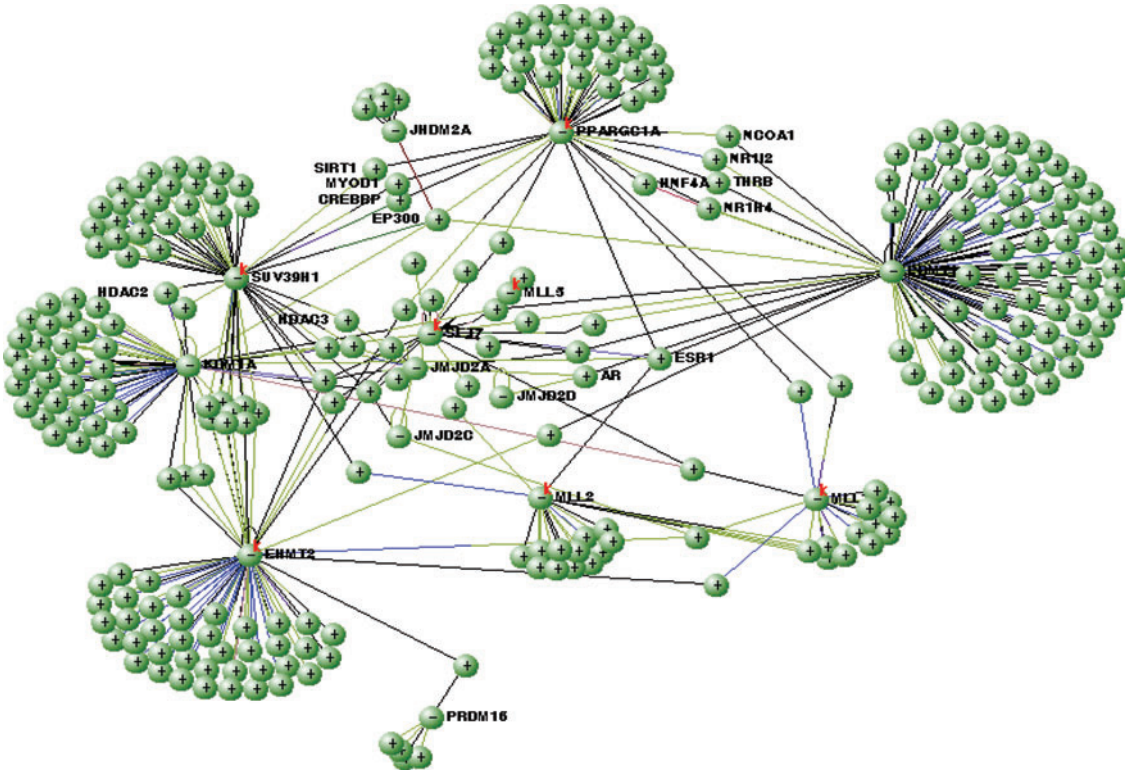

Figure 2 Network predicted by VisANT web-based software (52) using *PPARGC1A* and putative histone methylases and demethylases listed in Table 1 of Ref. (51).

Other DNA-binding transcriptional regulatory factors, nuclear receptors, such as androgen receptor (AR), estrogen receptors (ESRs), PPARs, etc., and coregulators, such as PPARGC1A, for ligand-dependent and -independent gene regulation require basic transcription factors and RNA polymerases. In addition, they must interact with histone acetylases, methylases and demethylases to modify chromatin structure to modulate gene transcription. DNA methylation is another epigenetic marker that plays a role in this process through the action of DNA methylases (DNMTs) and demethylases (TET and Jumonji-domain protein families). Most of the names of the nodes have been removed for the sake of simplicity and just to show that in the past two decades, more than 300 nuclear receptors and their coregulators have been described being PPARGC1A, one of the central ones in connecting gene transcription with metabolic status.

evidence, how IR and NAFLD are connected remains inconclusive. In this context, we hypothesized that NAFLD and IR are strongly linked by epigenetic modifications that act as a potential modifier of both conditions, and observed that methylation levels of the *PPARGC1A* promoter in the liver tissue of NAFLD patients are correlated with HOMA-IR and plasma fasting insulin levels (11). Interestingly, we also observed that liver abundance of *PPARGC1A* mRNA is inversely correlated with the methylation levels of *PPARGC1A* promoter CpGs, and also with the status of peripheral IR, suggesting that methylation of, at least, three explored CpG sites in the gene promoter efficiently repressed its transcriptional activity (11). Finally, we were able to show that mitochondrial biogenesis is reduced in the liver of NAFLD patients and is associated with peripheral IR and *PPARGC1A* promoter methylation status.

Do targeting epigenetic modifications have any potential as a therapeutic intervention in IR?

Epigenetics has emerged as an important field in the therapeutic intervention of chronic human diseases because it offers a unique framework of potentially heritable, but reversible mechanisms that modulate cellular transcriptional machinery and then command signatures of tissue gene expression.

There are currently two classes of compounds that interfere with the epigenetic process, DNA methyltransferase (DNMTs) and histone deacetylase (HDAC) inhibitors, but both approaches are currently directed to the treatment of cancer. A major question in this field is whether targeting epigenetics does not compromise, or alter, the transcription program of non-targeted tissues. For example, targeting epigenetic modifications using DNMT inhibitors is probably hampered by the potential inherent toxicity of inhibiting global genomic DNA methylation at 'non-affected cells'. Similar concern is attributed to many HDAC inhibitors as they can disrupt the function of healthy cells.

Nevertheless, targeting epigenetics in the field of metabolic diseases, such as MS-related traits, has tremendous potential. In the previous sections of this review, we provided evidence that epigenetic markers associated with IR are characterized by global changes of a target gene: the transcriptional coactivator *PPARGC1A*. As shown in Figure 3, we speculate on the potential of *PPARGC1A* as the target for epigenetically active drugs by the modulation of the acetylation/deacetylation switch mediated by Class III HDACs of the NAD^+-dependent SIR family.

Post-translational modifications of PPARGC1A play a key role in the induction of the *PPARGC1A* promoter, either by direct phosphorylation mediated by AMPK or by deacetylation mediated by NAD-dependent deacetylase SIRT1 (56, 57); these events directly ameliorat IR, enhancing mitochondrial biogenesis and oxidative phosphorylation (OXPHOS) capacity, and modulating fatty acid oxidation. The group of Class III HDACs is composed of seven mammalian sirtuins (SIRT1–7), which are nicotinamide adenine dinucleotide (NAD^+)-dependent protein deacetylases; SIRT1 is the enzyme that mediates the (NAD^+)-dependent deacetylation of *PPARGC1A* (58). PPARGC1A is, in addition, directly acetylated by another acetyltransferase enzyme, such as General Control Nonderepressible 5 (GCN5) (59), a process that results in a transcriptional repression of the *PPARGC1A* (Figure 3). Moreover, there is evidence that some protein substrates of HDACs participate in the transcriptional machinery of several genes modulated by PPARGC1A; for example, glucocorticoid receptor (GR) is a substrate of HDAC2 (60) and HDAC8 and SIRT1 interact directly with estrogen-related receptor α (ERRα) *in vivo* and deacetylate and increase the DNA-binding affinity of ERRα *in vitro* (61). Hypoxia inducible factor 1 α (HIF1α) is also a protein substrate of HDACs (62), which takes part in a critical process in the mechanism underlying HIF1α stability; and *PPARGC1A* also indirectly modulates *HIF1α* signaling to adapt mitochondrial demands (63) (Figure 3).

Finally, there are several compounds that regulate the action of HDACs; among them, resveratrol (Figure 3), a natural compound mainly found in the skin of grapes with antioxidant properties, has been found to be associated with a decrease in PPARGC1A acetylation and an increase in PPARGC1A activity (64).

Outlook

Challenge for the future: modulation of IR in the central nervous system by epigenetic modifications

Examining epigenetic markers not only in the periphery but also in the central nervous system might draw the epidemiological link between fetal programming and MS in adulthood. We suggest that a putative mechanism through which fetal programming can contribute to the development of obesity and IR in adult life may stem from epigenetic modifications in tissues, which orchestrate the endocrine system, modify the energy balance and have a direct connection with the visceral organs. This concept places the hypothalamus and the diencephalon in a central position, as they are potentially able to sense nutrient status. Under this premise, in a rat model, we investigated the impact of developmental and long-term adult nutritional insult of a high-fat diet (HFD) on diencephalic DNA methylation of a selected gene, a member of the zinc finger family of proteins called zinc finger protein 91 homolog (*ZFP91*). We compared the male and female offspring of dams exposed to different nutrition treatments (HFD vs. standard chow diet), and observed that male offspring of HFD-fed dams, which were born large according to the mean weight of their littermates (above the 80 percentile), had higher levels of DNA methylation in the *ZFP91* promoter (unpublished data). Interestingly, the protein encoded by this gene is a potent survival factor for neurons and also acts as a regulator of the non-canonical NF-κB pathway, and is a critical regulator in *TNFSF14*. These pathways seem to be involved in the association of two principal components of the MS (65).

Systems biology analysis revealed a highly predicted interaction between *ZFP91* and *CDKN2A* (cyclin-dependent kinase inhibitor 2A) (Figure 4), a gene that is not only highly expressed in the pancreas and central nervous system but has also been found to be strongly associated with T2D in

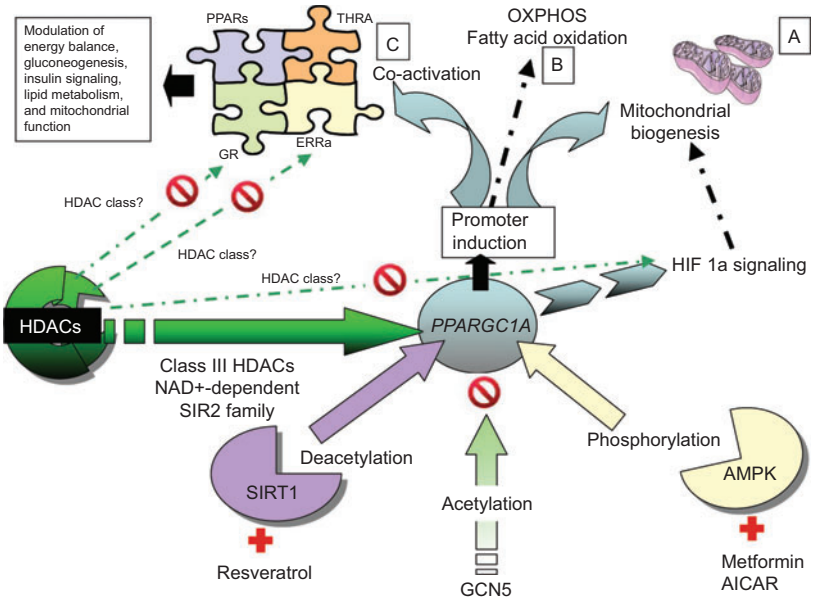

Figure 3 Biological rationale of targeting epigenetics by focusing on *PPARGC1A*: modulation of the acetylation-deacetylation switch. Induction of the *PPARGC1A* promoter results in modulation of mitochondrial biogenesis, OXPHOS activity and coactivation of nuclear receptors involved in the regulation of metabolic functions (A, B and C). But the activity of the *PPARGC1A* promoter can be epigenetically regulated resulting in either repression or activation of gene transcription. For example, *PPARGC1A* is a putative target for epigenetically active drugs by the modulation of the acetylation-deacetylation switch mediated by Class III HDACs of the NAD^+-dependent SIR family, which can repress the promoter activity. This repression significantly impacts on the transcriptional machinery of several other genes modulated by *PPARGC1A*, for example, glucocorticoid receptor, *ERRα* and *HIF1α* that also results in modulation of the mitochondrial biogenesis. PPARGC1A is, in addition, directly acetylated by another acetyltransferase enzyme, such as General Control Nonderepressible 5 (GCN5), a process that also results in a transcriptional repression of the *PPARGC1A*. On the contrary, post-translational modifications can positively regulate the activity of the *PPARGC1A* protein, either by direct phosphorylation mediated by AMPK or by deacetylation mediated by NAD-dependent deacetylase SIRT1. Some drugs and natural compounds can activate these two post-translational modifications, such as metformin and AICAR or resveratrol, respectively. *GR*, glucocorticoid receptor; *THRA*, thyroid hormone receptor α; *PPARS*, peroxisome proliferator-activated receptors; *ERRα*, estrogen-related receptor α; *PPARGC1A*, peroxisome proliferator-activated receptor γ, coactivator 1 α; *HIF1α*, hypoxia inducible factor 1, α subunit (basic helix-loop-helix transcription factor); *SIRT1*, sirtuin 1; AMPK, 5′ AMP-activated protein kinase; OXPHOS, oxidative phosphorylation; GCN5, General Control Nonderepressible 5; HDACs, histone deacetylases; Sir2, silent information regulator-2.

several genome-wide association studies (66, 67). The aberrant methylation of *CDKN2A* has been described in tumors (68), but its role in IR and T2D has not been reported. Hence, our finding may serve as a proof-of-concept on a putative role of the central nervous system, specifically, hypothalamic neurons, in the modulation of fetal programming of metabolism. In addition, our data may give some clues about the role of sexual dimorphism and fetal programming, because overnutrition during fetal life may interfere in a sex-specific manner and control mechanisms associated with metabolic regulation (69).

Highlights

Key conclusions

- Epigenetic modifications, such as hypermethylation of the *PPARGC1A* promoter, are consistently observed in the target tissue that modulates IR; local tissue DNA methylation status is significantly associated with repressed gene transcription.
- Methylation status of the *PPARGC1A* promoter has an impact on the mitochondrial DNA copy number regulation, which has a direct influence on the modulation of IR.
- Exploration of DNA methylation markers in PBMCs may offer potentially appealing information on how epigenetics contributes to the susceptibility of IR.
- Epigenetic markers modulate fetal metabolic programming and strongly modify metabolic performance in adult life after exposure to metabolic insults.
- Epialleles might have a place in the susceptibility to IR.
- Epigenetic modifications can act as therapeutic targets in MS and IR; for instance, targeting *PPARGC1A* by Class III HDACs.

Unresolved issues and important areas for future study

- Do prospective ideas about functional consequences of genetic variation modulated by allele-specific epigenetic markers explain IR susceptibility?
- Can SNPs-modified histone markers influence MS-related phenotypes?

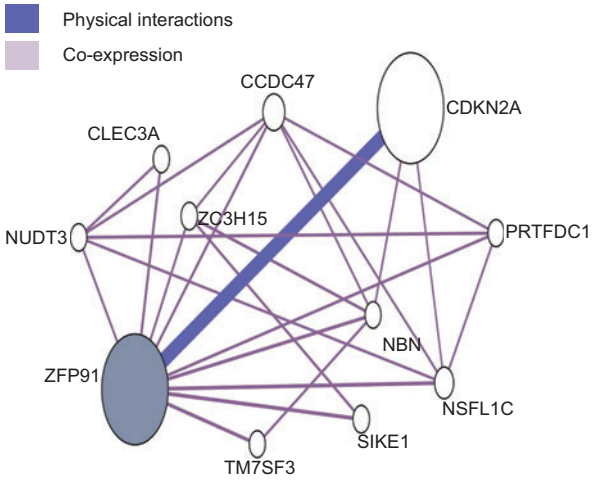

Figure 4 Functional pathway analysis of genes closely related to *ZFP91* (physical interaction and co-expression).
Graph obtained from the Genemania application (www.genemania.org) for 10 predicted related genes (empty circles). Candidate gene (*ZFP91*) is shown in gray circle. Among the predicted genes, *CDKN2A* is predicted to have physical interaction (blue line) with the candidate gene, whereas the remaining nine predicted genes are predicted as co-expressed (purple lines) with ZFP91. GeneMANIA searches many large, publicly available biological datasets to find related genes. These include protein-protein, protein-DNA and genetic interactions, pathways, reactions, gene and protein expression data, protein domains and phenotypic screening profiles. Data on physical interaction reflects protein-protein interaction data. These data are collected from primary studies found in protein interaction databases, including BioGRID and Pathway Commons. Data on co-expression reflects gene expression data, and two genes are linked if their expression levels are similar across conditions in a gene expression study. Most of these data are collected from the Gene Expression Omnibus (GEO); we only collect data associated with a publication. ZFP91, zinc finger protein 91 homolog (mouse); CDKN2A, cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4); CCDC47, coiled-coil domain containing 47; NSFL1C, NSFL1 (p97) cofactor (p47); SIKE1, suppressor of IKBKE 1; ZC3H15, zinc finger CCCH-type containing 15; NUDT3, Nudix (nucleoside diphosphate linked moiety X)-type motif 3; CLEC3A, C-type lectin domain family 3, member A; PRTFDC1, phosphoribosyl transferase domain containing 1; TM7SF3, transmembrane 7 superfamily member 3; NBN, nibrin.

- To what extent are the epigenetic markers reversible by environmental intervention, such as nutrigenomics?
- Is there any potential for sequence-specific targeted therapeutics?

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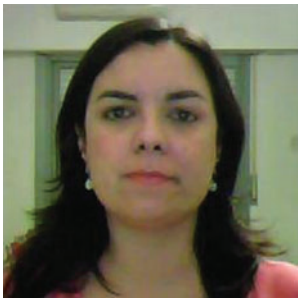

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