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REVIEW ARTICLE

DNA methylation in obesity and type 2 diabetes

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To elucidate the mechanisms related to the development of type 2 diabetes (T2D) and other degenerative diseases at a molecular level, a better understanding of the changes in the chromatin structure and the corresponding functional changes in molecular pathways is still needed. For example, persons with low birth weight are at a high risk for development of T2D later in life, suggesting that the intrauterine environment contributes to the disease. One of the hypotheses is that epigenetic regulation, including changes in DNA methylation leading to modifications in chromatin structure, are behind metabolic alterations, e.g. leading to the phenomenon termed metabolic memory. Altered DNA methylation has been shown to affect healthy aging and also to promote age-related health problems. There is suggestive evidence that lifestyle changes including weight loss can have an impact on DNA methylation and consequently gene expression. In this review we provide an overview of human studies investigating DNA methylation in obesity and T2D and associated risk factors behind these diseases.

Key words: Diabetes mellitus type 2, DNA methylation, epigenomics, obesity

Key messages

- Current data support the view that changes in DNA methylation might play a role in the pathogenesis of obesity and type 2 diabetes.
- Lifestyle changes that reduce the risk of developing type 2 diabetes and improve known risk factors seem to be associated with DNA methylation modification.

Introduction

The prevalence of obesity and type 2 diabetes (T2D) is increasing rapidly worldwide. Both obesity and T2D are conditions caused by an interplay between environmental and genetic factors. Despite intensive genetic research including genome-wide association studies the risk variants identified so far explain only a small portion of the heritability of obesity and T2D (1,2). Furthermore, it is well known that physical inactivity and unhealthy dietary patterns have a major impact on the current epidemic of both obesity and T2D, and lifestyle changes, if successful in the long term, can also overcome the genetic and familial risk of T2D (3).

Changes in lifestyles have beneficial effects on many of the known risk factors of obesity and T2D, in particular insulin sensitivity, but the basic mechanisms are poorly understood (4,5). Moreover, recent studies have shown that changes in body weight and increased physical activity seem to have an impact on DNA methylation of certain genes related to obesity and T2D (6–8). Early nutrition may have an effect on the later risk of chronic diseases, possibly due to genetic imprinting *in utero*, and methylation of DNA may play a role in this process (9).

Therefore, there has been an increasing interest in the possible role of epigenetic changes in the field of obesity and T2D, and in how lifestyle by means of diet and physical activity could be related to these changes. In this review we aim to present data on human studies relating epigenetic changes to obesity and T2D and risk factors behind these diseases. We will focus on epigenetic changes induced by DNA methylation.

Metabolic memory

In diabetes, the term 'metabolic memory' or 'legacy effect' has been suggested based on results from the effect of intensive diabetes treatment with the aim of achieving blood glucose and glycosylated hemoglobin concentrations as close to the normal range as possible (tight glycemic control) versus conventional diabetes treatment in individuals with both type 1 and T2D at early stages of the disease on diabetic complications. In both the Diabetes Control and Complications Trial (DCCT) and the United Kingdom Prospective Diabetes Study (UKPDS), tight glycemic control achieved in the intensive diabetes treatment resulted in substantial reduction in the risk of microvascular complications, which persisted with time despite an early post-intervention loss

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of difference in glycemic control between the intervention group and the control group—the 'legacy effect' (10,11). The pathophysiological mechanisms responsible for this long-lasting effect are unclear. Epigenetic regulation of gene expression has been suggested as one of the hypotheses that may explain this effect based on recent studies using experimental models (12–14).

Interestingly, it has been recently shown that lifestyle intervention lasting for a median of 4 years resulted in long-term protection (over 13 years) against T2D, conferring a 39% reduction in the risk of developing T2D. The authors speculated that the results may be due to sustained lifestyle changes as well as to the legacy effect of early improvements in glycemia, which occurred during the first year of the study followed by a gradual and more or less parallel increase in both groups during the rest of the follow-up (5). One could hypothesize that the lifestyle benefit on postponing T2D onset could also be explained by modulation of DNA methylation in pancreatic cells, for example, through its benefit on glycemic control (15,16).

Fetal programming

Epidemiological studies in humans have shown that an adverse maternal environment during embryonic development is associated with an increased risk of developing T2D in the offspring during adulthood. Studies performed in the United Kingdom by David Barker and colleagues found an inverse relationship of low birth weight, a crude marker of an adverse intrauterine environment, with T2D and impaired glucose tolerance later in life (17). The authors then proposed the thrifty phenotype hypothesis where they suggest that these associations result from the effects of poor nutrition in early life, which produces permanent changes in glucose-insulin metabolism. Later on, several studies confirmed these observations (18–20). Therefore, it was postulated that fetal malnutrition, which leads to impaired fetal growth and thinness at birth, favors later development of impaired glucose metabolism and T2D (21), the thrifty phenotype hypothesis.

Other retrospective epidemiological studies have shown that perinatal exposure to maternal diet is also associated with low birth weight, increased adiposity during childhood, and increased risk of obesity and T2D later in life (22–24). This 'metabolic memory' during 'fetal programming' *in utero* seems to have an impact in adult life. In the Dutch Hunger Families Study, a famine which occurred in the Netherlands during the winter of 1944 (25), *in utero* exposure to the famine (exposed group) was associated with low birth weight and increased incidence of insulin resistance later in life (25). However, over-nutrition in early life or high birth weight is also associated with an increased susceptibility to metabolic disease, which may account for the U-shaped or J-shaped relationships observed in some studies (26,27). Moreover, low birth weight coupled with rapid postnatal weight gain could also influence the development of T2D and disturbances in insulin and glucose metabolism (28–30).

In mammals, maternal exposure such as nutritional status influences metabolic phenotypes through epigenetic changes. Therefore, epigenetic regulation, including changes in DNA methylation, might explain these phenomena in humans described above (31,32). For example, further studies conducted in the Dutch Hunger Winter cohort, where whole blood samples were collected for DNA methylation studies, showed that, six decades later, the exposed group had lower levels of DNA methylation at the insulin-like growth factor 2 (*IGF2*) imprinted gene, indicating a link between prenatal nutrition and DNA methylation (33). Imprinted genes are the genes whose expression is determined by only one parental allele. Of note, genomic imprinting is an epigenetic process that allows the gene to be expressed this way and has an essential role in normal growth and development (34). In a subsequent study, using a candidate gene (loci) approach, the authors also reported that DNA in another six genes of relevance to metabolic and cardiovascular disorders (*INSIGF*: insulin-IGF2 read-through, *GNASAS*: guanine nucleotide alpha subunit antisense RNA 1 gene, *MEG3*: maternally expressed 3, *IL10*: interleukin 10, *LEP*: leptin, and *ABCA1*: adenosine triphosphate-binding cassette, sub-family A, member 1) were differently methylated in the exposed group compared to the unexposed group, indicating that persistent changes in DNA methylation later in life might occur due to transient environmental conditions during human gestation. They also found that these differences in DNA methylation can depend on gestational timing of exposure (e.g. first weeks of gestation or later pregnancy) and sex (35).

Epigenetic mechanisms: focus on DNA methylation

Epigenetics is a term used to describe the study of stable alterations to the DNA and histone proteins that alter gene expression but do not involve changes in the DNA sequence. Epigenetic effects, which can be inherited meiotically or mitotically through somatic or germline pathways, also have an impact on phenotype induction during early development (36–39). Epigenetic processes are essential for cell development and differentiation, but they can also arise in mature animals and humans, either by random change or under the influence of the environment (37). Epigenetic mechanisms, or epigenetic marks, involved in the control of gene expression include, among others, DNA methylation, chromatin remodeling to some extent, and several types of post-translational histone modifications, such as methylation, acetylation, phosphorylation, and ubiquitination (40,41). DNA methylation is perhaps the best studied epigenetic marker.

DNA methylation in mammals is a post-replication modification within the genome that is predominantly found in cytosine of the dinucleotide sequence cytosine-guanine (CpG) (p denotes the intervening phosphate group). Methylation of the 5' position of a cytosine is mediated by the enzymatic family of DNA methyltransferases (DNMTs) to form 5-methylcytosine (5mC). In mammals, DNA methyltransferases use S-adenosyl methionine as a methyl group donor to methylate the carbon in position 5 of cytosine residues of a CpG dinucleotide (CG) context. Methyl group supply for DNA methylation is determined largely by two factors: dietary methyl intake and methyl group utilization. The major dietary methyl donors are methionine, choline, and betaine (42). Dietary factors that influence the availability of S-adenosyl methionine and are likely to have an impact on DNA methylation include folate, vitamin B12 (cobalamin), vitamin B6 (pyridoxine), and vitamin B2 (riboflavin) (43).

DNA methylation is important for asymmetrical silencing of imprinted genes, X-chromosome inactivation, and silencing of retrotransposons (44–46). DNA methylation is also critical for cell differentiation by silencing the expression of specific genes during the development and differentiation of individual tissues (47,48). As presented below, proper DNA methylation seems to be essential for cell differentiation and embryonic development. However, aberrant methylation is related to disease, as will be discussed in this review.

The change in DNA methylation during development starts with demethylation during cleavage, followed by *de novo* methylation after implantation (37). These sequences of demethylation and methylation or reprogramming during development can be seen as the reason why the intrauterine period can be considered as a critical period in long-term health and disease risk (49). The

de novo DNA methyltransferases DNMT3A and DNMT3B are the enzymes responsible for the establishment of the DNA patterns in early development, which are passed on from the parental strand of DNA to the daughter cells during cellular replication. During cell division, maintenance methyltransferase DNMT1 is the enzyme responsible for the maintenance of DNA methylation pattern that will remain through cellular generations (50). Another important DNMT in mammals is DNMT3L, which is essential in enhancing *de novo* methylation and increasing binding of methyl donors in germ cells (51).

With respect to DNA demethylation, 5mC can be converted back to cytosine when the maintenance methylation that usually follows DNA replication is inhibited (52), or through removal of a methyl group resulting from mechanisms that include active removal of oxidation products from 5mC by specific enzymes through base incision repair mechanisms (53,54). It has been shown that oxidation of 5mC to hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC), and 5-carboxylcytosine (5caC) is catalyzed by a family of proteins called TETs (ten-eleven translocation) (53,55,56). This demethylation process is defined as the main pathway of global demethylation during preimplantation (57). Alternatively, 5fC and 5caC can be removed by thymine DNA glycosylase (TDG) through base excision repair (BER), resulting in an active demethylation of 5mC, which seems to occur extensively in the mammalian genome (54). Recently, it has been proposed that demethylation, through these intermediates derived from oxidized 5mC, could have an impact on gene expression (58).

Approximately 70% of CpG pairs in the mammalian genome have been considered to be constitutively methylated (59), and the degree of methylation varies between different genomic regions (60). However, recent studies that have used modern tools to analyze the global degree of DNA methylation have shown that the degree of DNA methylation is in fact much lower than 70% in some genomic regions (61–63). Moreover, non-CpG methylation also occurs through the genome, which seems to be a characteristic of an embryonic stem cell state (61).

When these CpG dinucleotides are clustered and form a dense repeat sequence in the genome, they are known as CpG islands and are located especially at promoter regions (37,40). However, they can also be found, for instance, at intragenic (64,65) and enhancer (66) regions. As a rule, the majority of CpG islands in the promoter remain unmethylated in normal cells. By the time *de novo* methylation occurs after implantation, these unmethylated CpG islands seem to be protected by a particular mechanism (67). However, particular subgroups of promoter CpGs are methylated, such as tissue- and germline-specific genes. This methylated state is often associated with gene silencing (40,41,68). The mechanisms proposed are either that DNA methylation would prevent the binding of certain transcription factors that require contact with cytosine in the major DNA groove, or that DNA methylation would affect chromatin states indirectly through the recruitment of methyl-CpG-binding proteins (69).

In a tissue-specific manner, the degree of methylation of these CpG islands located in the promoter region of a gene is inversely related to the transcription of that particular gene (70). The general rule is that hypermethylation is associated with transcriptional repression and hypomethylation with transcriptional activation (68). However, some evidence shows that methylation of CpG sites in regulatory regions outside of the promoter is also involved in gene transcription in some specific tissues (71). For example, the majority of tissue-specific expression and cancer-induced aberrant expression is governed by variations in the shore regions, which are about 2 kb from the CpG island, called the 'CpG island shore' (65,71). A high prevalence of CpG-poor regions near genes with tissue-specific expression in humans has also been reported (72). Moreover, methylation of intragenic regions is involved in gene transcription (64,65). Therefore, regulation of gene expression by DNA methylation could be far more complex and beyond what is mostly studied, which is usually methylation in promoter regions.

Both DNA and histone (the protein component of chromatin, which is part of the nucleosome) modifications do not just have an impact on gene expression, but also seem to interact through cross-talk between histone and DNMTs. Histone modifications could precede or succeed DNA methylation. In some cases, transcriptional repression may occur through the action of methyl-CpG binding proteins that are capable of recruiting histone deacetylases to the methylated region of the DNA (73,74). Conversely, unmethylated DNA does not attract deacetylating enzymes to the histones, allowing them to remain acetylated and more mobile, usually associated with an opened chromatin structure, thus promoting gene transcription (75–77). In other situations, this interaction between some DNMTs with histone methyltransferases and also deacetylases may allow genes which are already silenced by co-repressors to become permanently silenced by *de novo* methylation (78–81). This topic has been previously reviewed (37,67), and it is not our aim to discuss all these mechanisms in depth.

Factors influencing DNA methylation

Not only environmental factors, but also genetic background may have an effect on DNA methylation. One example was illustrated by Coolen et al. in a monozygotic (MZ) and dizygotic twin study (82) where the authors show that the gains or losses of methylation within individual twin pairs were more common in MZ than in dizygotic twins. These results indicated that maintenance of the methylation is influenced by the underlying DNA sequence (82). Another study later showed that the genetic background can also be additive to the environmental contribution on DNA methylation (83). In this study, periconceptional famine exposure and genetic variation were both independently associated with DNA methylation at the IGF2/H19 DMRs within the *INS* (insulin gene) and *IGF2*-transcribed region. Furthermore, it has been shown more recently that the role of DNA methylation on gene regulation can be either active, by being a likely cause of gene expression variation levels, or passive, by being a consequence of the change in gene expression as a result of genetic variation or an independent mark of gene expression levels when the SNP independently affects gene expression and DNA methylation (84). These relationships can be tissue-specific and depend on the genomic region.

Strong evidence also indicates that drifts in DNA methylation occur with increasing age. For example, in studies conducted in skeletal muscle the association of non-genetic variables including age with changes in DNA methylation of genes related to T2D has been studied (85,86). In these studies, which were both conducted in the same population of young and elderly twins, genes regulating oxidative phosphorylation were examined. It was shown that DNA methylation in the promoter region of *NDUFB6*, a gene encoding a subunit of complex 1 of the respiratory chain, is increased in elderly compared with young twins carrying the rs629566 G/G genotype. Interestingly, this explained the finding that this single nucleotide polymorphism (SNP), which creates the possibility for the DNA methylation, was associated with higher *NDUFB6* mRNA expression in young and lower mRNA expression in elderly twins (85). Moreover, in the other study (86), DNA methylation was also increased in the promoter

region of another gene involved in the respiratory chain, named *COX7A1*, in elderly compared to young twins. The inverse was seen for *COX7A1* mRNA expression (86).

In searching for biomarkers, array-based genome-wide methylation analyses have the advantage of genome-wide testing, and the candidate-gene approach could be underpowered in this sense. However, for this purpose, a combination of both methods could be confirmative. Still, a candidate gene approach (site-specific DNA methylation) is useful when the aim is to target a certain gene which is already known in terms of its biological function in that particular tissue in which it is being studied. Nowadays, the best way to identify methylation at a base-nucleotide resolution is to use a next-generation genome-wide sequencing approach (87). Moreover, methylation of repetitive elements may provide a global picture of genomic stability (88). Measurement of long interspersed nucleotide elements (LINE)-1, ALU (Alu repetitive elements), and satellite-repeat 2 (Sat-2) DNA methylation can be used as a surrogate marker of genome-wide methylation changes (88). However, global methylation provides no information on the genomic positions at which nucleotide methylation is altered. In this sense, locus-specific quantification of methylation would be more informative, either by genome-wide or candidate-gene approaches (89).

DNA methylation in obesity, weight reduction, and overfeeding

Obesity is defined as an accumulation of excess body fat as a result of the storage of energy as triglyceride in adipose tissue and involves an increase in both the number and size of adipocytes (90). Adipose tissue in obese persons develops a state of chronic low-grade inflammation which ultimately leads to insulin resistance (91–93). Obesity is also associated with alterations in immune function (94).

As earlier mentioned, low birth weight and maternal nutrition are among the risk factors for developing obesity later in life. Therefore, obesity is a multifactorial condition involving genetic and environmental factors. Once established, overweight and obesity substantially increase the risk of morbidity from, among other diseases, T2D. A higher body mass index (BMI) is also associated with an increase in all-cause mortality (95,96). Management of obesity consists of weight loss and correcting metabolic risk factors. Weight loss along with healthy diet and physical activity is also a strategy for treating these metabolic risk factors (97).

The central nervous system (CNS) influences energy balance and body weight through its effects on behavior, autonomic nervous system activity, and the neuroendocrine system (90). Several studies have established the existence of several CNS ligand-receptor pathways, including serotonin, capable of modifying energy intake, energy balance, and metabolic status (90). It is firmly established that the fat mass obesity gene (*FTO*), a gene predicted to be involved in the central control of food intake (98,99), is a strong candidate gene predisposing to obesity (2). Moreover, 31 other loci have been associated with BMI, but still, fixed genomic variations have only been able to explain a small fraction of the risk of obesity (2). Among these loci, loci near genes such as the melanocortin 4 receptor (*MC4R*), proopiomelanocortin (*POMC*), and brain-derived neurotrophic factor (*BDNF*) have been confirmed or identified (2).

However, gene versus lifestyle interaction has been found to influence the outcomes. For example, physical activity and perhaps low-fat and high-carbohydrate diets seem to weaken the influence of *FTO* on obesity risk (100,101). In this sense, both missing heritability and the underlying mechanisms of this interaction are still to be revealed. For instance, it is possible that different transcription rates among genotypes and differences in epigenetic regulation ultimately leading to gene expression are emerging candidates.

Studies examining intrauterine environment and obesity

Obesity has been associated with changes in DNA methylation of individual candidate genes and to some extent also with changes in global DNA methylation pattern. A study investigating DNA methylation in fetal cord blood from newborns has suggested a mechanistic epigenetic link between *in utero* and later phenotype (Supplementary Table I to be found online at http://informahealthcare.com/doi/abs/10.3109/07853890.2013.857259). The authors reported that DNA methylation levels of genes involved in development of nervous system, control of insulin signaling, cell cycle and proliferation, adipogenesis, and degradation of extracellular matrix were associated with altered gene expression and with body size and composition in later childhood (Supplementary Table I to be found online at http://informahealthcare.com/doi/abs/10.3109/07853890.2013.857259) (102). More specifically, another study has shown, in leukocytes from fetal umbilical cord, that aberrant DNA methylation at sequences regulating imprinted genes may be a useful marker for identification of children at risk for the development of early obesity (Supplementary Table I to be found online at http://informahealthcare.com/doi/abs/10.3109/07853890.2013.857259) (103). Of note, aberrant DNA methylation at differentially methylated regions (DMRs) of the imprinted gene *IGF2* has been associated with aberrant changes in its gene expression in certain types of cancer (104,105) and with clear phenotypes of altered growth (36).

In another study in which the global methylation status in blood leukocytes was evaluated, lower global DNA methylation of boys at school-age participating in a longitudinal study in Latin America was related to development of adiposity (Supplementary Table I to be found online at http://informahealthcare.com/doi/abs/10.3109/07853890.2013.857259) (106). Also in peripheral blood leukocytes, another study has shown that exon-3 hypermethylation of the *POMC* gene was significantly associated with obesity in cross-sectional and longitudinal analyses (Supplementary Table I to be found online at http://informahealthcare.com/doi/abs/10.3109/07853890.2013.857259) (107). The mechanism for this association could be that *POMC* hypermethylation at exon3 interferes with binding of the transcription factor enhancer P300 and reduces expression of the *POMC* transcript (107). It has been shown that mutations in the *POMC* gene that prevent production of *POMC* products, including α-MSH, cause obesity in mice and humans (108). Lack of production of α-MSH, for example, could have an adverse effect on food intake (109) and ultimately on weight gain.

In a study conducted by Godfrey et al. (110), epigenetic factors in early life were linked with clinically relevant phenotypic variation later in childhood (Supplementary Table I to be found online at http://informahealthcare.com/doi/abs/10.3109/07853890.2013.857259). Moreover, this study was the first one in humans directly to state that dietary factors could possibly affect a child's adiposity through the *RXRA* (retinol X receptor, alpha) metabolic pathway, since the carbohydrate intake of the mother during early gestation was negatively associated with DNA methylation of this gene in the newborn (110).

Cross-sectional and case-control studies in obesity

Using peripheral blood mononuclear cells as a model, Wang et al. conducted a pilot study in young males in order to explore if methylation pattern in obesity was associated with immune

dysfunction (111). The results revealed some evidence that in the presence of obesity, specific CpG sites that were methylated in the *UBASH3A* (ubiquitin-associated and SH3 domain-containing protein A) and in the *TRIM3* (tripartite motif-containing) genes, both of which are involved in immune responses, were not methylated in lean controls (111). However, these results are not easy to interpret, as the functional result of high methylation levels of these two genes, even though related to immune function, has not yet been demonstrated.

Studies using MZ twin pairs as a model in epigenetic studies in obesity are useful in order to control for confounding factors such as genotype and sex and many other unknown factors. In a cross-sectional study conducted in 84 male MZ twin pairs (Supplementary Table I to be found online at http://informahealthcare.com/doi/abs/10.3109/07853890.2013.857259), Zhao et al. have shown that promoter hypermethylation of the serotonin transporter gene (*SLC6A4*) in peripheral blood mononuclear cells (PBMCs) was significantly associated with an increased prevalence of obesity (112). The authors speculated that functionally this hypermethylation could reduce or silence gene expression, thereby lowering serotonin uptake and activity which has been associated with obesity (112). For example, depletion of serotonin results in hyperphagia and obesity (113). Moreover, a promoter gene polymorphism in the SLC6A4 has been previously shown to be associated with obesity (114,115).

DNA methylation and weight reduction

Lifestyle changes by means of diet-induced weight loss intervention studies have also been associated with changes in DNA methylation. Results coming from dietary intervention studies show that in obese and overweight individuals, differences in DNA methylation seem to be present between low and high responders to caloric restriction estimated by the amount of weight loss (6,116–119). Moreover, these differences seem to be corrected after weight loss (Supplementary Table I to be found online at http://informahealthcare.com/doi/abs/10.3109/07853890.2013.857259) (6,116). In most of these studies, low responders to caloric restriction were characterized as the participants who lost less than 5% of their initial body weight at the end of the intervention, while high responders were the ones who lost, among the studies, either at least 5% (118,119) or more than 10% (6,116) of their initial body weight at the end of the intervention. The only exception was the study conducted in adolescents (117), in which the low responders were those that did not achieve a weight loss of at least 0.4 BMI – standard deviation score, and high responders were those who lost more than 1.1 BMI – standard deviation score after the weight loss intervention period.

For example, in subcutaneous adipose tissue it has been shown that DNA methylation differences at baseline, before the intervention had started, between low and high responders to weight loss include biologically relevant genes such as the glis family zinc protein finger (*GLIS3*), insulinoma-associated 1 (*INSM1*), and the cholecystokinin B receptor (*CCKBR*) (6). Even though weight reduction altered the gene expression profile, no differences in gene expression level was found between low and high responders (6). As a result of weight loss, in adipose tissue the most relevant finding seems to have been the increase in DNA methylation and decrease in gene expression at the 5q13 locus in the genomic region to which ectodermal-neural cortex 1 gene (*ENC1*) belongs, after the intervention (Supplementary Table I to be found online at http://informahealthcare.com/doi/abs/10.3109/07853890.2013.857259) (6).

In PBMCs methylation at baseline in most of the CpG sites of both *ATP10A*, a gene encoding an aminophospholipid translocase related to lipid trafficking, and *CD44*, a gene that encodes a cell surface glycoprotein, correlated with the amount of weight loss and decrease in markers of adiposity after caloric restriction. Moreover, in PBMCs, one CpG site at *ATP10A* and one CpG site at *WT1* (Wilms tumor 1 gene) were shown to be hypermethylated as a result of the weight loss intervention. Altogether, the results coming from both studies suggest that epigenetic background may play a role in the success of weight management programs (6,116).

In obese children, it has been shown that even though correction for multiple testing did not confirm the initial findings in which 97 CpG sites were differently methylated in PBMCs between high and low responders to weight loss intervention (Supplementary Table I to be found online at http://informahealthcare.com/doi/abs/10.3109/07853890.2013.857259), selected regions located near three relevant genes which are involved either in lipogenesis (aquaporin 9: *AQP9*), protein phosphorylation (homeodomain interacting protein kinase 3: *HIPK3*), or are used as indicator of ketoacidosis (troponin I type 1: *TNNI1*) were differently methylated between low and high responders to the multidisciplinary weight loss program when DNA methylation status was assessed using a Sequenom EpiTyper-MassARRAY approach in a larger sample size (Supplementary Table I to be found online at http://informahealthcare.com/doi/abs/10.3109/07853890.2013.857259) (117). In addition, changes in body composition after the intervention were associated with baseline DNA methylation at these respective genes and also with a gene involved in inflammatory pathways (dual specificity phosphatase 22: *DUSP22*) and with a gene used as a 'cardiac' marker (troponin I type 3 (cardiac): *TNNI3*) (117).

Also, in both subcutaneous adipose tissue and PBMC samples, using a candidate gene approach, DNA promoter methylation at *TNF*, which encodes tumor necrosis factor alpha, has been shown to differ between successful and non-successful individuals in weight loss (118,119). In subcutaneous adipose tissue, the same was true when studying DNA methylation at the *LEP* gene (119). However, the authors could not demonstrate a difference for *TNF* or *LEP* at transcriptional levels between the responders and non-responders to weight loss (119).

Moreover, weight loss as a continuous variable has been associated with methylation levels of one CpG in the *CLOCK* gene, which encodes a basic helix-loop-helix transcription factor essential for circadian rhythm, and with two CpGs at *PER2*, a gene that encodes a functional component of the mammalian circadian clock (120) in blood leukocytes before a 4-month weight reduction program consisting of moderate-intensity physical activity and a Mediterranean-type diet (120).

A comprehensive study of DNA methylation in skeletal muscle of obese individuals who had undergone Roux-en-Y gastric bypass (RYGB) surgery-induced weight loss was recently conducted (121). While global DNA methylation analyses revealed that obesity was associated with hypermethylation at CpG shores and exonic regions close to transcription start sites, a candidate gene approach showed that promoter methylation at *PPARGC1A*, a gene encoding a transcriptional activator protein that regulates genes involved in energy metabolism, and *PDK4*, a gene encoding a mitochondrial protein involved in glucose metabolism, changed after RYGB, as did their respective transcription levels (Supplementary Table I to be found online at http://informahealthcare.com/doi/abs/10.3109/07853890.2013.857259). Moreover, in genome-wide microarray analysis, obesity was associated with altered gene expression of genes enriched in lipid metabolic process and mitochondrial function, including *PPARGC1A* and *PDK4*, which was normalized to levels observed in normal-weight

women (controls) after RYGB surgery-induced weight loss. Interestingly, among a set of metabolic genes selected based on the microarray results for DNA methylation analyses, 11 of them correlated with their respective gene expression levels (Supplementary Table I to be found online at http://informahealthcare.com/doi/abs/10.3109/07853890.2013.857259). In the genome-wide methylation analysis, the vast majority of the DMRs that responded to the surgery (Supplementary Table I to be found online at http://informahealthcare.com/doi/abs/10.3109/07853890.2013.857259) were specifically enriched within the proximity of transcription start sites, at CpG shores or at exonic regions.

Overall, the results coming from these studies suggest that there is evidence toward an involvement of DNA methylation in the etiology of obesity, but it is still unknown whether the associations found with obesity traits mean that changes in methylation are the cause or consequence of obesity, especially in those studies that measured DNA methylation and related phenotypes cross-sectionally and later in life. However, the studies which more firmly suggested a role of DNA methylation in late development of obesity were the ones conducted in relevant tissues taken at birth highlighting the importance of maternal exposure and consequent life *in utero* on the newborn epigenome. Still, it is important to evaluate whether changes in weight along time would be associated with changes in DNA methylation, and if these changes would occur with concomitant modulation of gene expression and other clinically relevant phenotypes related to obesity.

Based on the genome-wide studies reviewed in this section, after the validation stage no common gene was found to be related to the development of obesity later in life. The same was true concerning the weight loss intervention studies. The only gene which was differently methylated between high and low respondents to weight loss in two studies using a candidate-gene approach was *TNF*, from two distinct target tissues (118,119).

On the other hand, weight loss significantly affected some relevant genes at the DNA methylation level. One of the modulated genes, *WT1*, in which mutations can affect phenotypes related to obesity (122), also encodes a protein associated with hypertension (123). Surgery-induced weight loss reduced DNA methylation at *PPARGC1A*, which has been previously associated with T2D (124,125), and increased DNA methylation at *PDK4*, which encodes a protein that if inhibited seems to improve glucose metabolism (126). However, the significance of the differently methylated genes found in the weight loss intervention studies is still unknown. Still, DNA methylation at genes that responded to weight loss, that were predictive of better outcome after caloric restriction, and that were associated with measurement of adiposity later in life could give insights in the prevention of T2D. The differential DNA methylation found in adipose tissue between low and high responders to weight loss at *GLIS3* (6), a gene that has been previously associated with T2D (127), is an example. Therefore, lifestyle changes involving weight loss seem to be able to modulate DNA methylation, which may modulate gene expression. More studies are needed to evaluate if this could have an impact on clinically relevant phenotypes in the long term.

Overfeeding studies

In humans, very few studies have been carried out with the purpose of studying changes in DNA methylation following overfeeding (128,129). In one of these studies, DNA methylation in skeletal muscle revealed that overfeeding induces DNA methylation in a reversible manner at the *PPARGC1A* gene based on the fact that the observed increase in DNA methylation was shifted down after overfeeding had stopped (Supplementary Table I to be found online at http://informahealthcare.com/doi/abs/10.3109/07853890.2013.857259) (128). Later on, in a subsequent study, it was found that short-term high-fat overfeeding (HFO) in fact introduced changes in DNA methylation at 7909 CpG sites, corresponding to more than 6500 genes. In most of these CpG sites, HFO led to an increase in DNA methylation, among which almost all were classified before the intervention as hypomethylated (129). The most affected genes based on their largest increase (9.7% to 13%) or decrease (−11.2% to −12.9%) are displayed in Supplementary Table I to be found online at http://informahealthcare.com/doi/abs/10.3109/07853890.2013.857259. Moreover, 24 T2D-susceptible genes were affected by HFO when DNA methylation levels were measured using a candidate gene approach, from which 6 were also detected in the methylation array (Supplementary Table I to be found online at http://informahealthcare.com/doi/abs/10.3109/07853890.2013.857259). Pathway analyses revealed that pathways relevant to diseases such as cancer, the reproductive system, and inflammatory systems were mostly represented. Interestingly, the changes induced by the HFO diet were only slowly reversed, as observed in participants that started the study in the HFO group. These results underline the plasticity and reversibility of DNA methylation response to dietary interventions. Because these studies were conducted in skeletal muscle and DNA methylation pattern changed at sites of candidate genes for T2D and genes involved in pathways of common diseases, the findings coming from both studies (128,129) can be of relevance in the etiology of disease.

DNA methylation in type 2 diabetes and hyperglycemia

It is estimated that diabetes mellitus, including largely T2D, affects approximately 346 million people worldwide, and these numbers are projected to increase above 550 million by 2030 (130). Diabetes is a disease of metabolic dysregulation ultimately leading to reduced life expectancy due to its specific microvascular (retinopathy, nephropathy, neuropathy, impaired wound healing), and macrovascular (heart disease and stroke) complications (131). As with obesity, both genetic and environmental factors contribute to the development of T2D (1). For example, persons at high risk for developing T2D should benefit from lifestyle changes involving healthy diet, moderate weight loss, and increased physical activity as seen in the Finnish Diabetes Prevention Study and Diabetes Prevention Program (4,5). Moreover, as in obesity, gene versus lifestyle interaction has been found to influence the outcomes (132–134). However, despite the intensive research in this area including genome-wide association studies, the genetic basis of T2D has remained largely obscure, since all the risk variants identified so far explain only approximately 10% of the estimated heritability for T2D. Therefore, scientific researchers have begun to examine the role of epigenetic changes in the development of T2D.

DNA methylation, markers of T2D, and lifestyle changes

Insulin resistance, one of the hallmarks of T2D, has been associated with aberrant DNA methylation (135). In a study conducted in 84 MZ twin pairs, higher global DNA methylation was associated with higher insulin resistance estimated by the homeostasis model assessment, independently of other risk factors (135). Interestingly, a 6-month exercise intervention program has been associated with changes in DNA methylation at many genes related to T2D after applying a genome-wide DNA methylation approach (7). Among the differently methylated genes were: *THADA* (thyroid adenoma associated), a gene previously associated with T2D, and also *NDUFC2* (NADH dehydrogenase

(ubiquinone) 1, subcomplex unknown, 2, 14.5 kDa), which is part of the respiratory chain. Moreover, exercise intervention also changed DNA methylation and expression of metabolically relevant genes such as the ones encoding adiponectin receptors (*ADIPOR1* and *ADIPOR2*) and *BDKRB2*, which encodes for bradykinin receptor B2 (7). In this same study, before the intervention period, the authors observed epigenetic differences in skeletal muscle of individuals with a positive family history of T2D compared to those without a family history of T2D (7).

More recently, the same research group has also shown that in subcutaneous adipose tissue DNA methylation in many genes related to both obesity and T2D responded to this same 6-month exercise intervention program after applying a genome-wide DNA methylation approach (8). Two genes related to obesity were differently methylated and expressed in response to the exercise intervention (*CPEB4*, encoding a cytoplasmic polyadenylation element binding protein, and *SDCCAG8*, encoding for the serologically defined colon cancer antigen 8). Whereas DNA methylation levels increased, their respective mRNA expression levels decreased in response to exercise intervention. Even more interesting, among the 21 candidate genes for T2D that were differently methylated in the adipose tissue before versus after the intervention, ten CpG sites at *KCNQ1*, encoding a protein for a voltage-gated potassium channel, and six sites in *TCF7L2* (transcription factor 7-like 2) increased their methylation levels, with a simultaneous decrease in mRNA expression in the latter. Moreover, a total of 17,975 CpG sites in 7663 unique genes were differently methylated after exercise intervention and resulted in differential mRNA expression of one-third of these genes (8).

Therefore, lifestyle intervention by means of an exercise program including 3 hours of aerobic exercise followed by formerly sedentary otherwise healthy individuals (7,8) seem to modify DNA methylation at a genome-wide level, including important genes related to the development of T2D in two relevant tissues, adipose tissue and skeletal muscle. The concomitant change at the transcriptomic level might represent the functional consequence of DNA methylation and its link to an altered phenotype associated with the disease.

Case-control studies in T2D

There is some direct evidence that DNA methylation is altered in T2D. One of the first works involving measurement of DNA methylation in relation to T2D was conducted by Barrès et al., where a genome-wide promoter analysis of DNA methylation was performed in skeletal muscle (Supplementary Table I to be found online at http://informahealthcare.com/doi/abs/10.3109/07853890.2013.857259) (136). In this study, the interesting finding was that *PPARGC1A* promoter was hypermethylated in T2D patients compared with normal-glucose-tolerant individuals. Interestingly, mRNA expression of *PPARGC1A* was negatively correlated with the methylation at its promoter. These results were in line with previous findings in pancreatic islets from T2D patients (137).

Following this study, other case-control and functional studies in pancreatic islet cells have been conducted (15,16,138). Yang et al., using a candidate gene approach, have demonstrated that patients with T2D had increased DNA methylation at four CpG sites of the human insulin gene (*INS*) promoter and at ten CpG sites in the distal *PDX1* promoter, the gene encoding the transcriptional activator pancreatic and duodenal homeobox 1, and enhancer regions compared with non-diabetic donors (15,16). Moreover, DNA methylation at these genes was negatively correlated with their respective mRNA expression levels and positively correlated with the level of HbA_{1c}. The authors suggested that reduced expression of *PDX1* is a result of epigenetic modification which may lead to impaired *INS* gene expression and insulin secretion (16). Moreover, the results from these two studies suggest that hyperglycemia may affect the degree of DNA methylation. The authors speculated that reduced *INS* gene expression, for instance, could be the cause of hyperglycemia. In contrast, increased DNA methylation at *PDX1* and consequent decreased gene expression could be a result of hyperglycemia. In addition, the authors cultured clonal rat β-cells in high glucose concentration medium which resulted in elevation of DNA methylation at *PDX1* and *INS* promoter regions (15,16). However, although these functional studies help in trying to elucidate the differences in DNA methylation between diabetic and non-diabetic cases, it is still unknown whether these epigenetic changes in humans are causal or reaction of the islet cells to a diabetic environment.

In the study conducted by Volkmar et al. (138), a DNA methylation profiling was performed in pancreatic islet cells of 11 non-diabetic and 5 diabetic deceased donors using a methylation array covering more than 27,000 CpG sites as a screening tool before validation of the results. The authors found a prevalent promoter hypomethylation in T2D islets which probably indicated active biological processes involved in adaptation to the diabetic environment (metabolic stress) and associated with β-cell dysfunction and apoptosis (138). Comparison of data from microarray gene expression with DNA methylation profile revealed that at least for a subset of genes differently methylated in T2D, changes in the promoter methylation correlated inversely with its respective mRNA expression levels. Validation of the results confirmed the genes that were hypermethylated: *CDO1* (cysteine dioxygenase type 1) and *MADD* (MAP-kinase activating death domain), and the genes that were hypomethylated: *ALDH3B1* (aldehyde dehydrogenase 3 family, member B1), *CASP10* (caspase 10), *CHAC1* (ChaC, cation transport regulator homolog 1), *CDK5R1* (cyclin-dependent kinase 5, regulatory subunit 1 (p35)), *GABRB3* (gamma-aminobutyric acid (GABA) A receptor, beta 3), *GP1BB* (glycoprotein Ib (platelet), beta polypeptide), *GRB10* (growth factor receptor-bound protein 10), *LRRC15* (leucine rich repeat containing 15), *FAM129A* (family with sequence similarity 129, member A), *NR4A1* (nuclear receptor subfamily 4, group A, member 1), *PPP2R4* (protein phosphatase 2A activator, regulatory subunit 4), *SIRT6* (sirtuin 6), and *TPM3* (tropomyosin 3) (138). The different DNA methylation in T2D was, therefore, more gene-specific rather than global because LINE-1 DNA methylation was not different between cases and non-cases of T2D. In this study, the authors also observed that T2D-related methylated changes detected in pancreatic cells were absent in leukocyte blood cells. This result highlights the fact that DNA methylation measured in blood cells might not reflect DNA methylation at other tissues such as pancreatic tissue, adipose tissue, and skeletal muscle. Contrary to the studies conducted by Yang et al. (15,16), though, induced hyperglycemia in islet cells did not significantly affect DNA methylation at CpG sites of studied genes (138).

Toperoff and co-workers, using blood leukocyte cells as the target tissue, have found that significant hypomethylation of the earlier documented risk genes for T2D in young individuals is a strong risk factor for development of T2D later in life (Supplementary Table I to be found online at http://informahealthcare.com/doi/abs/10.3109/07853890.2013.857259) (139). For example, the first intron of *FTO*, one of the genes most strongly associated with obesity, found by genome-wide genetic analysis (140), was frequently hypomethylated in affected individuals when compared with controls, and the effect was found to be independent of the genetic variation in the *FTO* gene.

More recently, Ribel-Madsen et al. have performed genome-wide DNA methylation analysis in both skeletal muscle and subcutaneous adipose tissues from elderly MZ twin pairs discordant for T2D (Supplementary Table I to be found online at http://informahealthcare.com/doi/abs/10.3109/07853890.2013.857259) (141). Interestingly, LINE-1 repetitive DNA sequence methylation was not different between T2D and non-diabetic twins, but the variation in its methylation levels was largest in twin pairs being different in BMI and 2-hour plasma glucose after an oral glucose tolerance test, whereas methylation at CpG sites in the promoter region of genes measured by microarray approach did not associate with phenotypic differences between siblings (141). Moreover, in a candidate gene approach, from 17 known susceptibility genes for T2D represented also in the microarray (13 in adipose tissue and 8 skeletal muscle) (Supplementary Table I to be found online at http://informahealthcare.com/doi/abs/10.3109/07853890.2013.857259), 2 of them in adipose tissue (*CDKN2A*: cyclin-dependent kinase inhibitor 2A and *HNF4A*: hepatocyte nuclear factor 4, alpha) remained statistically significant after correction for multiple testing. Although these results should be interpreted cautiously, after applying pathway analyses, the genes differently methylated in muscle were found to be predominantly involved in inflammation (hepatic fibrosis and IL-6 pathways), lipid metabolism (peroxisome proliferator-activated receptors α and γ, and sphingolipids pathways), and carbohydrate metabolism (pyruvate and propionate pathways). In adipose tissue, in addition to one pathway related to carbohydrate metabolism, another pathway involved in circadian rhythm signaling pathways was differently methylated between T2D and non-diabetic twins (141).

Overall, the studies discussed in this section demonstrated differences in DNA methylation at susceptible genes for T2D in relevant metabolic tissues between T2D and non-diabetic cases. A few examples are: *CDKN2A*, *CDKN2B*, *JAZF1* (JAZF zinc finger 1), *KCNQ1*, *PDX1*, *PPARGC1A*, *SLC30A8* (solute carrier family 30 (zinc transporter), member 8), *TCF7L2*, and *THADA*, which in skeletal muscle were also reversibly modulated after overfeeding (129) and changed with lifestyle intervention (7). Of note, *TCF7L2*, the gene whose genotype has so far the greatest association with T2D in Europeans (1), was also modulated by lifestyle intervention involving increase in physical activity at both transcriptomic and epigenomic levels (8).

While studies conducted in blood leukocyte cells have shown that lower global methylation was associated with development of adiposity in children during a 2-year follow-up (106) and with lower insulin resistance in adults (135), studies conducted in the field of T2D and in relevant metabolic tissues such as pancreas, adipose tissue, and skeletal muscle could not observe any difference in DNA methylation status when assessing global DNA methylation between T2D cases and non-cases (138,141). Because DNA methylation varies among tissues, results coming from DNA methylation studies in immune cells might not reflect DNA methylation status at other tissues such as pancreatic cells, skeletal muscle, and adipose tissue. Moreover, although DNA methylation at repetitive elements that are used to estimate DNA global methylation make part of a substantial percentage of the genome (142), there seems to be no strong evidence of e.g. Alu or LINE-1 methylation and metabolic risk factors. Furthermore, because global methylation provides no information on the genomic positions at which nucleotide methylation is altered, no specific insights into locus-specific T2D or obesity risk loci can be retrieved from these kinds of studies.

It is important to point out that, in some of the studies discussed in this review (102,121,136–138,141), including the ones involving lifestyle changes (7), it was possible to observe that DNA methylation levels correlated with their respective gene expression levels. However, one has to keep in mind that gene expression and its respective DNA methylation do not always occur in parallel as it is generally assumed. This could have been partly because of methodology reasons, e.g. positioning of array probes or tissue under investigation. However, it is also known that the role of DNA methylation in gene regulation and its dependency on genomic sequence and environment are not fully understood (84). Moreover, it is important to mention that a role for an effect of DNA methylation on regulation of alternative splicing has been proposed (143). In the field of T2D some evidence comes from a recent study which suggested that certain T2D-associated SNPs that introduce or remove CpG sites (CpG-SNP) were associated with DNA methylation and alternative splicing events in their respective genes in islet pancreatic cells (144), indicating a possible role of DNA methylation in the regulation of alternative splicing.

Conclusions

Methodologies for detection of changes in DNA methylation have emerged during the last few years. New platforms for detection of genome-wide methylation profiles have been developed to receive high-throughput data. However, with the exception of *PPARGC1A* in skeletal muscle that seems to link insulin resistance (15,16), obesity (121), and T2D (136,141), and which was also reversibly modulated after overfeeding (129) and changed with lifestyle intervention, there is no other gene that has been consistently associated with obesity or T2D at the DNA methylation level.

Current data support the view that epigenetic changes, more specifically changes in DNA methylation, in relevant metabolic tissues such as adipose tissue, skeletal muscle, and pancreatic cells might play a role in the pathogenesis of obesity and T2D. It remains to be established to what extent DNA methylation could explain the current epidemic of obesity and T2D. Lifestyle changes that are associated with persistent lower risk of developing T2D and with improvement of known risk factors may modify DNA methylation and thus gene expression. Therefore, larger intervention studies examining the effect of increased physical activity, weight reduction, and healthy diet pattern, and their combination should be conducted to find out whether in the long term these lifestyle changes can restore the genome to the pattern of a healthy individual.

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Supplementary material available online

Supplementary Table I.