DNA methylation in the pathogenesis of type 2 diabetes in humans

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ABSTRACT

Background: Type 2 diabetes (T2D) is a multifactorial, polygenic disease caused by impaired insulin secretion and insulin resistance. Genome-wide association studies (GWAS) were expected to resolve a large part of the genetic component of diabetes; yet, the single nucleotide polymorphisms identified by GWAS explain less than 20% of the estimated heritability for T2D. There was subsequently a need to look elsewhere to find disease-causing factors. Mechanisms mediating the interaction between environmental factors and the genome, such as epigenetics, may be of particular importance in the pathogenesis of T2D.

Scope of Review: This review summarizes knowledge of the impact of epigenetics on the pathogenesis of T2D in humans. In particular, the review will focus on alterations in DNA methylation in four human tissues of importance for the disease; pancreatic islets, skeletal muscle, adipose tissue, and the liver. Case-control studies and studies examining the impact of non-genetic and genetic risk factors on DNA methylation in humans will be considered. These studies identified epigenetic changes in tissues from subjects with T2D versus non-diabetic controls. They also demonstrate that non-genetic factors associated with T2D such as age, obesity, energy rich diets, physical activity and the intrauterine environment impact the epigenome in humans. Additionally, interactions between genetics and epigenetics seem to influence the pathogenesis of T2D.

Conclusions: Overall, previous studies by our group and others support a key role for epigenetics in the growing incidence of T2D.

Keywords  Epigenetics; DNA methylation; Type 2 diabetes

1. INTRODUCTION

Genetic, epigenetic, and non-genetic factors are known to influence the pathogenesis of type 2 diabetes (T2D) [1]. Non-genetic risk factors for T2D include age, physical inactivity, and energy rich diets that result in obesity. Genome-wide association studies (GWAS) in large case-control cohorts and family-based studies demonstrate that the risk for T2D is influenced by genetics. Although well over a hundred genetic variants that associate with T2D risk have been identified, we can still only explain a modest portion of the T2D heritability [2]. Thus, other explanations for the T2D heritability have been posited, including alterations to epigenetic patterns. Epigenetic modifications include DNA methylation, non-coding RNA (ncRNA), and histone modifications. In differentiated mammalian cells, DNA methylation mainly takes place on so-called CpG sites, cytosines that are followed by a guanine. Recent studies have used Illumina DNA methylation arrays and RNA sequencing to identify differences in DNA methylation and ncRNA, respectively, in tissues from subjects with T2D and from non-diabetic controls. However, knowledge of alterations in histone modifications in human tissues from subjects with T2D remains limited. Epigenetic modifications may predispose to disease or they can occur once a disease has developed. Several human models are subsequently needed to dissect the role of epigenetics in the pathogenesis of T2D. These models may include case-control cohorts, prospective cohorts, intervention studies, and cultures of human cells exposed to environmental risk factors. Functional follow-up studies in cell lines and animals are also useful. Together, these models can help us understand how epigenetic modifications take place and how they influence the development of disease. Numerous enzymes are responsible for attaching and removing DNA methylation and histone modifications in the human genome. The activity and function of these...
enzymes may thereby affect T2D. Availability of the substrates needed for epigenetic modifications may also affect the development of disease. This review will focus on a body of literature that dissected whether DNA methylation in human tissues influences the pathogenesis of T2D.

2. PANCREATIC ISLETS

2.1. T2D and DNA methylation in human pancreatic islets

T2D develops due to insufficient release of insulin from pancreatic β-cells, while the pancreatic α-cells often exhibit an abnormally increased secretion of glucagon. Many studies have investigated the importance of DNA methylation and other epigenetic players in pancreatic islet function in different models [3]. The initial studies on human islets were candidate driven and investigated genes with known importance in β-cell function or cellular metabolism (Table 1 and Figure 1). These studies identified that the promoters of INS [4] (encoding insulin), PDX1 [5] (encoding a transcription factor important for both pancreatic development [6] and the function of mature β-cells [7]), PPARγ1A [8] (encoding the mitochondrial regulator PGC1α [9]), and GLP1R [10] (encoding the GLP1 receptor which stimulates insulin secretion and enhances/protects β-cell mass when activated [11]) were all hypermethylated in islets from donors with T2D compared to islets from non-diabetic donors. Additionally, a higher methylation level was associated with reduced mRNA expression of the respective gene in the diabetic islets and with higher glycated hemoglobin A1c (HbA1c, a measure of long-term plasma glucose levels), suggesting a possible role in β-cell perturbation in T2D. Interestingly, high levels of glucose were also found to directly increase DNA methylation of Pdx1 and Ins in clonal β-cells [4,5].

With the introduction of array based methods for analysis of DNA methylation, the focus turned to epigenome-wide association studies (EWAS) (Table 1). The very first study to utilize this methodology on islet DNA was by Volkmar et al. [12]. They used the Infinium HumanMethylation27 BeadChip [13] (27k array, probes for 27,578 methylation sites) to analyze DNA methylation in pancreatic islets from 11 non-diabetic donors and 5 donors with T2D. Islets from the two groups clustered separately based on the DNA methylome, indicating significant differences between non-diabetics and diabetics. Furthermore, some methylation differences associated with altered β-cell function and survival, both under normal and stress (metabolic stress or pharmacologically induced ER-stress) conditions. One drawback of the 27k array is that it is very “CpG island-centric” and covers fewer than 15,000 genes. The second version of methylation array, the Infinium HumanMethylation450K BeadChip [14] (450k array, probes for 485,577 methylation sites), covered >99% of all RefSeq genes and many more sites outside CpG islands. The 450k array was subsequently used to analyze DNA methylation in islets from 15 T2D and 34 non-diabetic donors [15] and identified significant methylation differences in 853 genes. Global expression data and experimental evidence showed that some of the methylation changes resulted in altered mRNA expression. One of the altered genes was HDAC7, which encodes a histone deacetylase (HDAC). HDAC7 was hypomethylated and overexpressed in islets from donors with T2D. This finding was followed up in a separate study which showed that increased Hdad7 expression in rat islets and clonal β-cells impaired mitochondrial function and insulin secretion [16]. This suggests that changes in HDAC7 methylation and expression may contribute to the perturbed β-cell function in T2D. Interestingly, HDAC inhibitors could restore these defects in Hdad7-overexpressing β-cells, proposing a potential novel therapy for T2D.

Although the methylation arrays have greatly improved the analysis of DNA methylation they have one major shortcoming; they still only analyze a small proportion of CpG sites. For example, the 450k array analyzes only ~1.5% of the CpG sites in the genome. One solution to this problem is to use next generation sequencing of bisulfite treated DNA, so-called whole-genome bisulfite sequencing (WGBS), and thereby move closer to truly genome-wide data. The deep sequencing needed when analyzing DNA methylation has until recently resulted in unreasonably high costs to run this kind of project. One of the few WGBS studies published so far analyzed DNA methylation in islets from a T2D case/control cohort [17]. This study identified close to 26,000 differentially methylated regions (DMRs, defined as ≥3 consecutive differentially methylated CpG sites, with an average absolute methylation difference of ≥5% with no more than 300 bp between adjacent CpG sites) in islets from donors with T2D compared to non-diabetic islets. The average DMR was 414 bp long (range 6–3411) and contained 8.7 CpG sites (range 3–164). These DMRs were located in many genes important for islet cell function and genes previously identified as modulators of T2D risk, such as TCF7L2 [18], THADA [19], and KCNQ1 [20]. They were also enriched for binding sites for islet specific transcription factors, further implying an important role in islet cell function. Many genes, such as NRA4, PARK2, P51D, and SOCS2, exhibited both DNA methylation and expression changes in T2D islets. When the expression of these genes was altered in a similar manner in clonal β-cells, glucose-stimulated insulin secretion was impaired, providing further support for altered methylation patterns having a role in T2D. In addition to this, Jeon et al. presented WGBS data on a 10 kb stretch in the MISO2 gene showing methylation differences in islet preparations from two donors with T2D and 16 non-diabetic donors [21]. The functional relevance of this finding was not tested, however.

2.2. Interactions between genetics and DNA methylation in human islets

Thanks to many GWAS and family-based studies, it has become clear that the risk for T2D is influenced by genetics. However, even though well over a hundred genetic variants that associate with altered T2D risk have been identified, we can still only explain a small portion of T2D heritability [2]. Due to this, alternate explanations for the T2D heritability have been put forward. One such explanation is an interaction between genetics and epigenetics. For example, as already mentioned, the methylation changes taking place in T2D islets overlap with candidate genes identified in GWAS. Additional support for this model comes from methylation quantitative trait loci (mQTL) studies, i.e. studies identifying genetic variants associated with DNA methylation. One such study investigated pancreatic islets from 89 non-diabetic donors and merged data on over half a million single nucleotide polymorphisms (SNPs) with methylation data from the 450k array [22]. The analysis identified more than 100,000 mQTLs, where more than 6% of the analyzed SNPs significantly associated with methylation at specific CpG sites. Interestingly, these mQTLs associated with gene expression and insulin secretion. Causal inference tests (CITs) further supported that SNPs can mediate their effects on islet gene expression via altered DNA methylation (Figure 1).

Another line of support for the genetics/epigenetics interaction model comes from the fact that ~25% of all SNPs in the genome either introduce or remove a CpG site. These so-called CpG-SNPs affect whether certain locations can be methylated or not. In a study investigating the 40 SNPs associated with T2D risk known at the time, 19 were indeed CpG-SNPs [23]. Interestingly, not only did all analyzed CpG-SNPs associate with differential DNA methylation of the CpG-SNP
Together these two studies show that SNPs that increase the T2D risk may do so via effects on DNA methylation (Figure 1).

2.3. Associations between non-genetic factors and DNA methylation in human islets
DNA methylation is not only influenced by genetic factors, there are also studies showing that risk factors for T2D associate with changes to the DNA methylation pattern in human pancreatic islets (Figure 1).
2.3.1. Sex
The risk for T2D is greater among men than women [24]. Studies have also identified sex differences in the DNA methylome of several tissues [25–33], including pancreatic islets from non-diabetic donors [34]. Islets from women exhibited greater in vitro glucose-stimulated insulin secretion when compared to islets isolated from men. Analysis of DNA methylation showed large methylation differences between the sexes, with >90% of significant CpG sites being on the X-chromosome. This may seem unsurprising considering that female cells have an inactivated X-chromosome [35] and that this inactivation is thought to be accomplished in part through extensive DNA methylation [36]. However, almost 40% of the significant X-chromosome CpG sites were more methylated in islets from male donors compared to female donors. The methylation differences in women and men were reflected in differential expression of both protein coding genes and miRNA. For example, APLN and NKAP were hypermethylated and repressed in islets from women. Interestingly, using siRNA to ‘feminize’ clonal β-cells by downregulating Apln and Nkap resulted in enhanced insulin secretion, recapitulating the secretory difference between islets isolated from women and men.

2.3.2. Age
Age is a strong risk factor for T2D [37]. Age increases the T2D risk by, among other factors, increased insulin resistance and impaired β-cell function [37]. Age is also associated with changes to the DNA methylation pattern in human tissues, and DNA methylation data can even be used to predict the age of a person (see Ref. [38] and papers cited within). These tissues include muscle [39–42], adipose tissue [43], and liver [44], i.e. tissues that play an important role in glucose homeostasis and T2D. Therefore, we previously hypothesized that age-associated changes to the methlyome also take place in pancreatic islets and may underlie the impaired insulin secretion seen in aged individuals. Indeed, 450k array analysis of an islet cohort in which the age of the donors ranged between 26 and 74 years identified 241 methylation sites associated with age, all positively [45]. We could further show that methylation and expression correlated for some genes, and functional analyses showed that the expression of these genes affects insulin secretion. Contrary to our original hypothesis, mimicking the age-associated changes resulted in increased insulin secretion in response to glucose. This suggests that the identified methylation changes may be part of a β-cell compensation process, which aims to overcome the increased demands for insulin due to age-related insulin resistance. Some of the CpG sites were also analyzed in blood samples in two prospective cohorts. The data showed similar age-related changes to methylation, suggesting that methylation in blood can be used as a biomarker for the methylation status in islets. Furthermore, we saw that methylation of some sites could predict not only future insulin secretion during oral glucose-tolerance tests (OGTT), but also future T2D. This strengthened the notion that the age-related methylation changes improve β-cell function and provided proof of principle that methylation markers can be used to predict disease. Similarly, predictive CpG sites were found in two other studies in both European and Indian populations [46,47]. However, although the predictive power for these markers is as strong as some of the top T2D SNPs identified by GWAS, even stronger methylation biomarkers need to be identified before this method will be useful for T2D prediction in the clinic.

2.3.3. Gluco- and lipotoxicity
Circulating nutrient levels are often increased in individuals with T2D or at high risk for the disease, and it is well established that prolonged exposure to elevated nutrient levels impairs islet cell survival and/or function. While proof for a negative effect of high concentrations of lipids (lipotoxicity) is mainly from in vitro studies, the case for high glucose levels (glucotoxicity) contributing to T2D is also supported by in vivo experiments [48]. The effects of glucosis and lipotoxicity on islets/β-cells are impaired insulin secretion and/or increased cell death. Several studies have shown that excess glucose or lipids induce transcriptomic changes in human islets [49–53]. Studies supporting a role for DNA methylation as a player in these changes have also been published [51,52]. In these studies, treatment of human islets with high glucose or high levels of the fatty acid palmitate resulted in altered expression and DNA methylation patterns. The altered genes included candidate genes for T2D, such as TCF7L2 [18], SLC30A8 [54], and GLIS3 [55]. There was also an enrichment of genes in pathways important for β-cell function, such as oxidative phosphorylation [56] and SNARE protein interactions [57]. Together, these studies support a role for epigenetics in the detrimental effects of excess nutrients.

3. ADIPOSE TISSUE
3.1. T2D and DNA methylation in human adipose tissue
Adipose tissue plays a critical role in controlling energy metabolism due to its capacity to store and release lipids. It also functions as an endocrine organ by secreting adipokines such as leptin, adiponectin, and tumor necrosis factor alpha (TNFα). Circulating adipokines regulate food intake, insulin sensitivity, and metabolism. Excess lipids will accumulate in tissues other than adipose tissue, such as the liver,
Among these, methylation in blood of two loci annotated to respective study [47]. In blood, higher methylation of PHOSPHO1 were also associated with future T2D in the Botnia prospective study [58,59] (Table 1). While both studies support altered DNA methylation in adipose tissue from subjects with T2D, the methylation differences between the discordant twins were modest, and the data support a strong heritable component of the methylation pattern. Importantly, when DNA methylation was analyzed in adipose tissue of a T2D case—control cohort from unrelated subjects, 15,627 CpG sites annotated to 7046 genes were found differentially methylated, supporting an important role of epigenetics in diabetes [59].

Candidate genes for T2D such as PPARG, IRS1, TCF7L2, and KCNQ1 were among those differentially methylated. Epigenetic differences identified in a case—control setting cannot tell us whether epigenetics cause disease or whether differences have occurred after the disease developed. Prospective cohorts are better models for studying causal changes. Using this approach, Chambers et al. identified five DNA methylation loci in blood that were associated with future T2D risk [46]. Among these, methylation in blood of two loci annotated to ABCG1 and PHOSPHO1 were also associated with future T2D in the Botnia prospective study [47]. In blood, higher methylation of ABCG1, which encodes a protein that regulates cellular lipid homeostasis and is involved in cholesterol and phospholipid transport, was associated with elevated risk of future T2D, and the same CpG site showed higher DNA methylation in adipose tissue of the diabetic twin among MZ twins discordant for T2D [47].

DNA methyltransferases (DNMTs) are the enzymes responsible for adding methyl groups to DNA. DNMT1 has been suggested to be responsible for copying the methylation pattern during cell replication, while DNMT3A and B have been suggested to be responsible for de novo methylation [1]. Interestingly, DNMT3A mediated insulin resistance in cultured human and mouse adipocytes [60]. Fgf21 was found to be a Dnmt3a target gene in adipocytes, and DNA methylation of FGF21 was elevated and correlated negatively with expression of FGF21 in adipose tissue of subjects with T2D. Increased Fgf21 could also rescue the insulin resistance induced by Dnmt3a. Future prospective studies performed in tissues would further support the causal role of methylation in T2D.

3.2. Associations between non-genetic and genetic factors and DNA methylation in human adipose tissue

3.2.1. Obesity

Obesity is an important risk factor for T2D. It is hence possible that DNA methylation associated with obesity contributes to the development of diabetes. A few studies have identified associations between obesity and DNA methylation in adipose tissue [43,61,62]. These studies found associations between obesity and DNA methylation of CpG sites annotated to HIF3A, which encodes a transcription factor that regulates several adaptive responses to low oxygen tension (hypoxia). Rönn et al. found associations between obesity and methylation of several candidate genes for T2D e.g. FTO, TCF7L2, and IRS1 [43]. Some of these genes also showed differential DNA methylation in adipose tissue from subjects with T2D [59]. It is possible that obesity drives these epigenetic differences before diabetes develops and that they influence the pathogenesis of T2D.

3.2.2. Exercise

A healthy lifestyle including exercise and healthy food reduces the risk for T2D [63,64]. Interestingly, a six-month exercise intervention altered the DNA methylation pattern of 17,975 CpG sites annotated to 7663 genes in adipose tissue of overweight and sedentary men [65]. Some of these genes also showed differential gene expression, e.g. HDAC4 and NCOR2, which both showed increased methylation and decreased expression after exercise. Hdac4 and Ncor2 were silenced in adipocytes to mirror the effect of exercise, and this resulted in increased lipogenesis. Moreover, methylation of sites annotated to 18 obesity and 21 T2D candidate genes, e.g. FTO, KCNQ1, and TCF7L2, changed after exercise.

3.2.3. Diet and weight-loss

The impact of diets on DNA methylation in human adipose tissue has also been investigated in a few studies [66–68]. In a study by Gillberg et al., young men were fed a high-fat diet (HFD) for 5 days. This resulted in elevated fasting glucose and insulin levels together with methylation changes at 652 CpG sites in adipose tissue [66]. Some of these CpG sites were annotated to CDK3, CIDEA, IGFBP5, and SLCA2, which were previously linked to adipose tissue metabolism and/or differentiation. This study also analyzed HFD-induced changes in gene expression, and these were merged with changes in methylation. Notably, the most significantly enriched KEGG pathways among genes with both methylation and expression changes after the HFD were mapped to oxidative phosphorylation (OXPHOS, 34 genes) and insulin signaling (36 genes). Not only HFD, but also the dietary fat composition may affect ectopic lipid accumulation and insulin resistance. Diets that are high in

![Figure 2: Genetic, non-genetic, and environmental factors alter the DNA methylome and transcriptome in adipose tissue, triggering changes in cellular function that may contribute to the development of type 2 diabetes. See main text for details.](imageurl)
saturated fatty acids (SFAs) or polyunsaturated fatty acids (PUFAs) elicit different metabolic responses. In a recent study, Perfiliev et al. performed a randomized control trial to study the effects of SFA versus PUFA on DNA methylation in adipose tissue of healthy young subjects [67]. Interestingly, DNA methylation of 4875 CpG sites in adipose tissue was affected differently depending on the diet. Fasting may also affect the DNA methylation pattern. Indeed, 36 h of fasting affected the methylation level of the genes encoding leptin and adiponectin in a birth weight dependent manner in adipose tissue [68]. Together, these studies show that overeating, nutritional content of the diet, and fasting affect the epigenome in human adipose tissue, including changes in genes known to regulate food intake and energy balance, and these changes may influence both whole body and adipocyte metabolism.

In 2010, Bouchard and colleagues tested whether DNA methylation and gene expression in adipose tissue of obese women influences their weight-loss in response to dieting [69]. Interestingly, they found 35 loci with DNA methylation differences between the high and low responders before dieting. These include sites annotated to genes regulating weight control (KCN3 and NFX1), diabetes (GLIS3), and growth regulation (IGF2-H19). After weight-loss, they only found three loci with differential methylation. Gastric bypass is an efficient way to treat T2D and is commonly used to help obese subjects lose weight. Benton et al. studied DNA methylation in adipose tissue of obese women before and after gastric bypass and found changes in methylation of 15 and 3601 CpG sites in omentum and subcutaneous adipose tissue, respectively [70]. These include sites annotated to HDAC4, DNMT3B, KCNQ1, and Hox genes. Together these studies support that weight-loss influences the epigenetic pattern in adipose tissue.

3.2.4. Age
Increased age also has a strong effect on the DNA methylation patterns in human adipose tissue. Ronn et al. found that methylation of numerous CpG sites increased with increased age [43]. Similar findings were also made in other tissues such as blood and human pancreatic islets [45,71]. CpG sites that showed increased methylation with age in several tissues include KLF14, FHL2, GLRA2, and ELOVL2.

3.2.5. Genetic variation
We and others have studied the interactions between genetic and epigenetic variation in human adipose tissue [72–75]. These mQTL studies support a strong effect of SNPs on methylation. In adipose tissue of 119 men, 101,911 SNP-CpG pairs in cis and 5342 SNP-CpG pairs in trans showed significant associations between genotype and DNA methylation. Of note, these mQTLs include reported obesity, lipid, and T2D loci, e.g. ADCY3, POMC, APOA5, CETP, FADS2, GCKR, SORT1, and LEPR. To test for causality, CIs were used to analyze whether SNPs mediate their effects on metabolic traits (e.g. body mass index (BMI), cholesterol, high-density lipoprotein (HDL), Hba1c, and homeostatic model assessment for Insulin Resistance (HOMA-IR)) via altered DNA methylation in human adipose tissue. This showed that, for example, SNPs annotated to CAMK1D and SLC22A16 mediate their effects on HOMA-IR and BMI, respectively, via differential methylation in adipose tissue.

4. Skeletal Muscle

4.1. T2D and DNA methylation in human skeletal muscle
The skeletal muscle is the tissue responsible for the majority of insulin stimulated glucose uptake and is essential for our ability to perform physical activity. Epigenetic changes have been reported in muscle biopsies and cultured myocytes from T2D subjects compared to non-diabetic controls (Table 1), as well as after exercise, diet interventions and with aging (see below) (Figure 3). A study in skeletal muscle from monozygotic twin pairs discordant for T2D analyzed DNA methylation in promoter regions as well as in repetitive LINE1, D4Z4, and NBL2 sequences [58]. Only one CpG site, in IL8, was found to be differentially methylated between the diabetic and non-diabetic twins. This small number may be explained by low power and small differences, which are commonly seen for complex diseases [76]. The overall methylation pattern associated with the interaction of twin pair and diabetes, indicating that differences between discordant twins are diabetes related. The largest variation in methylation between pairs of twins was found in LINE1 elements. Variation in methylation between twin pairs, expressed as standard deviation, correlated with that in BMI and plasma glucose after an OGTT. The authors concluded that variation in traits associated with T2D reflect the variation of LINE1 methylation in skeletal muscle. LINE1 elements make up approximately 17% of the genome [77], and it would be interesting to evaluate how changes in LINE1 methylation affect the muscle. Can it be used as a therapeutic target, and can changes over time predict future glucose intolerance and weight gain? A different study also analyzed DNA methylation of skeletal muscle of twin pairs discordant for T2D as well as in subjects with family history of the disease [78]. Here, multiple Hox genes showed lower methylation levels in subjects with T2D. A study by Barres et al. used methylated DNA immunoprecipitation (MeDIP) combined with arrays to study methylation of promoter regions in vastus lateralis muscle of age-matched individuals with T2D and controls [79]. The global methylation level did not differ between the groups, and the authors hypothesized that differences were more gene specific. 833 out of 25,500 promoter regions were differentially methylated with \( p < 0.05 \), which was not more than what can be expected by chance. Nevertheless, the authors technically validated some data and found higher methylation of PPARGC1A in muscle of T2D subjects, which correlated negatively with expression of the PPARGC1A mRNA. Methylation differences were mainly observed for non-CpG sites, cytosines followed by a nucleotide other than guanine. Non-CpG methylation has primarily been studied in embryonic stem cells and the brain, but was also observed in skeletal muscle [80]. Barres et al. then used human myoblasts, differentiated muscle fibers, to show that TNFα, palmitate, and oleate all increase methylation of the PPARGC1A promoter. Interestingly, silencing of DNMT3B prevented the palmitate induced methylation of PPARGC1A, as well as the reduced expression of PPARGC1A and other genes related to mitochondrial function. However, DNMT3B protein expression was not altered in myotubes upon palmitate treatment nor in myotubes from T2D individuals versus controls. Of course, enzymatic activity is not determined by protein levels alone. DNMT3B exists in several isoforms, of which some lack enzymatic activity, and may also be regulated by posttranslational modifications [81,82]. It would therefore be of interest to study DNMT3B activity in skeletal muscle and/or myocytes from individuals with T2D and test whether DNMT inhibitors have the same effect on PPARGC1A as siRNA mediated DNMT3B knockdown.

A more specific alternative is to genetically manipulate methylation of specific CpG sites. DNA methylation of nucleotide (nt) 780 of the PPARGC1A promoter has recently been shown to impact the 1 nucleosome position (the nucleosome closest upstream of the transcription start site (TSS), normally around nt 300 to 200) [83].
4.2. Interactions between genetics and DNA methylation in human skeletal muscle

Variation in both genetics and epigenetics has been linked to the expression and function of several key metabolic genes in the skeletal muscle [1]. One example is NDUFB6, which encodes a protein that is part of complex 1 in the respiratory chain and therefore essential for oxidative phosphorylation. NDUFB6 expression was reduced in skeletal muscle of T2D subjects and with age, and concomitant with increased promoter methylation [40,86]. Interestingly, a SNP (rs629566, A/G) in the NDUFB6 promoter introduces a CpG site. At old age carriers of the G/G genotype exhibited higher NDUFB6 promoter methylation and lower expression of the mRNA compared to carriers of the A/A and A/G genotypes [40]. Thus, genetic factors may predispose for epigenetic changes that will increase the risk of metabolic impairment in the skeletal muscle (Figure 3).

A genome-wide study found differences in DNA methylation between healthy sedentary men with or without a family history of T2D (FH⁻/FH⁺) [78]. Nitert et al. used MeDIP-Chip and they identified 60 genes with higher methylation and 5 genes with lower methylation in muscle of FH⁺ subjects. The methylation level of several genes was associated with SNPs nearby (minor allele frequency >5%), again pointing to a strong link between genotype and alterations in epigenetic marks. Genes showing both differential methylation and expression in FH⁺ versus FH⁻ were enriched for adipocytokine signaling, a signaling pathway that affects metabolism as well as insulin sensitivity in the muscle [78,87]. Moreover, 40% of the differentially methylated genes were also differentially methylated between MZ twins discordant for T2D. This further supports that the epigenetic differences observed by Nitert et al. play a role in the development of T2D. However, MZ twins are genetically identical and not all differences between FH⁻ and FH⁺ may therefore be explained by genetics; some may rather be due to a shared environment within families.

4.3. Associations between non-genetic factors and DNA methylation in human muscle

4.3.1. Age

Aging is associated with a decline in skeletal muscle mass and insulin sensitivity, which increases the risk of T2D [88]. DNA methylation in skeletal muscle seems to increase with age, mainly in gene bodies and not promoters [42]. In addition, gene specific studies in muscle have shown that promoter methylation of OXPHOS genes like NDUFB6 and COX7A1 increases with age, while their expression is reduced [40,41]. Also muscle PPARγ1 expression declines with age, and is affected by both genetic and environmental factors [89]. Thus, age-related methylation changes are likely to influence muscle metabolism and insulin sensitivity.

4.3.2. Exercise

Acute exercise increases glucose uptake [90,91] and chronic exercise improves mitochondrial function [92]. Several studies have examined the effect of exercise on the epigenome in skeletal muscle of healthy, obese, or individuals with T2D [78,93]. An acute bout of exercise in the fasting state decreased global methylation of CpG sites in the CCGG context in muscle biopsies [93]. Genes related to metabolism and/or exercise (PPARGC1A, TFAM, PDK4, MEF2A, CS, and PPARδ) showed decreased promoter methylation and increased gene expression, while there were no changes in the muscle specific genes MYOD and MYOG. The methylation changes observed by Barres et al. could also be induced ex vivo by contraction of mouse soleus muscle and by caffeine treatment of rat myotubes. Both contraction and caffeine raise intracellular calcium, which seems to play a role in promoter demethylation in response to exercise. Other studies have shown that activation of AMPK (an energy sensing kinase) and CaMK (a Ca²⁺/Calmodulin-dependent kinase) by exercise or caffeine leads to translocation of HDAC5 out of the nucleus and hyperacetylation of histone 3 [94,95]. HDAC5 inhibits MEF2 induced expression of e.g. PPARGC1A and GLUT4 [94,96,97]. HDAC5 knockdown or inhibition improved glucose uptake and metabolic flexibility, while prevention of the rise in Ca²⁺ levels or inhibition of Camk2 in mouse myotubes attenuated the effects on H3 hyperacetylation and Glut4 expression [94,98]. It is well known that histone modifications and DNA methylation affect each other, and future studies should investigate the relationship between H3 acetylation and DNA methylation of metabolic genes in skeletal muscle. Interestingly, exercise had similar effect as low methylation of nt —260 in the PPARGC1A promoter region. Exercise repositioned the —1 nucleosome and affected the expression level of PPARGC1A [83]. These exercise-induced alterations were only seen in individuals who
also responded to exercise in terms of improved metabolic profile. Thus, manipulation of −1 nucleosome positions and/or methylation of n1−260 in the PPARGC1A promoter may be an interesting target for the improvement of metabolism in low responders to exercise.

While studies of acute exercise may be advantageous to dissect molecular mechanisms, a single bout of exercise will neither prevent nor treat T2D. From a therapeutic point of view, it may be more relevant to study epigenetic changes induced by long-term exercise regimens. A six-month exercise intervention among sedentary healthy men changed the DNA methylation of 134 genes [78]. The vast majority of these genes, 115, showed decreased methylation. There was a negative correlation between methylation and gene expression for several genes, including OXPHOS genes, T2D candidate genes and genes involved in exercise (e.g. NDUFC2, THADA, MEF2A, and RUNX1). Also PPARGC1A methylation decreased after six months of exercise. 111 genes, including IL7, exhibited changes in both methylation and gene expression after the intervention. IL7 was hypomethylated in response to exercise, while mRNA expression and serum levels of IL7 increased. IL7 is a myokine and has been suggested to play a role in muscle development and repair, and may contribute to growth and maintenance of skeletal muscle mass [99].

In contrast to exercise, 5 days of bed rest changed the expression of OXPHOS genes, and PPARGC1A was downregulated while methylation of PPARGC1A increased [100]. This study indicates that physical inactivity has opposite effects on PPARGC1A promoter methylation when compared to exercise.

4.3.3. Diet

A HFD increases intracellular triglycerides, ceramides, and diacylglycerides, causing insulin resistance and impaired glucose metabolism in skeletal muscle [101–103]. PPARGC1A promoter methylation increased in myotubes treated with palmitate and oleate, as described above [79]. These fatty acids also altered expression and DNA methylation of PPARD and CPT1B, genes known to play a role in lipid oxidation [104,105]. Interestingly, the expression and methylation of these genes were lower in myocytes from obese insulin resistant women after incubation with lipids [104,105]. Lipid treatment differentially affected the methylation of two specific CpG sites in CPT1B in myocytes from non-obese and obese women. The methylation level of these sites seemed to regulate the occupancy of USF1 and USF2, which are known transcriptional repressors. Thus, changes in methylation of just a few CpG sites may improve response to lipid overload in individuals with low metabolic flexibility.

In vivo, five days of overfeeding altered the methylation of 7909 CpG sites measured by Illumina’s 27k Array [106]. These changes were not fully reversed after 6–8 weeks of “wash out,” suggesting that periods of excess food intake may build up methylation over time at certain loci. Additionally, PPARGC1A methylation in skeletal muscle biopsies was increased after five days of overfeeding [107]. Here, methylation levels were reversed by the control diet. Together, these studies demonstrate that lifestyle changes can have a strong impact on epigenetic modifications contributing to risk of disease (Figure 3).

4.3.4. Muscle memory

Skeletal muscle exhibits memory and can be metabolically programmed by environmental stimuli (reviewed by Sharples et al. [108]). Moreover, offspring of mothers exposed to nutrient restriction during early pregnancy have a higher risk for developing metabolic disease later in life [109]. Brons et al. showed that PPARGC1A promoter methylation is higher in muscle of individuals born with a low birth weight (LBW) compared to those with normal birth weight (NBW) [107]. This difference was abolished after five days of HFD as PPARGC1A promoter methylation increased in individuals with NBW but remained unchanged in LBW individuals [107]. Hence, PPARGC1A seems to be constitutively hypermethylated in individuals with LBW. This may reflect a lower grade of metabolic flexibility in response to different challenges in individuals with LBW and emphasize the role of epigenetics in muscle memory.

Skeletal muscle is a post-mitotic tissue but has the capacity to regenerate after injury in adults. The adult skeletal muscle stem cells, so-called satellite cells, are important for muscle growth and maintenance of a healthy skeletal muscle phenotype during life [110]. When satellite cells are isolated from muscle biopsies of individuals with T2D and differentiated in vitro, they retain a diabetic phenotype including impaired glucose uptake, lipid oxidation, and inflammation [111–113]. Epigenetics may contribute to this “memory” [114,115]. Indeed, a recent study demonstrates that satellite cells from obese subjects display an altered DNA methylation signature during in vitro differentiation into myotubes, despite being cultured in exactly the same conditions as those from non-obese subjects [115]. The same study showed that IL32 was differentially methylated and expressed during adult myogenesis and a factor that contributes to muscle growth and insulin sensitivity. Other myogenic regulatory factors are also known to be epigenetically regulated (reviewed by Dilworth and Blais [116]). Therefore, epigenetic changes in muscle stem cells induced by the environment may have a large impact on mature myofibers later in life. It is well known that muscle mass and strength decline with age, as do the myogenic potential of satellite cells [110]. Parabiosis studies in mice have suggested that age-related loss of regenerative muscle capacity is due to a changed in vivo environment rather than changes in the satellite cells [117,118]. However, research described here by Gaster et al., Broholm et al., Green et al. and Davegardh et al. clearly indicate that these cells can also be reprogrammed intrinsically.

5. LIVER

5.1. T2D and DNA methylation in human liver

The liver has a unique function in regulating blood glucose levels in both the fed and fasted states. After carbohydrate ingestion, the liver stores glycogen in response to insulin stimulation, whereas glucagon released during fasting increases glycogenolysis and gluconeogenesis in the liver to prevent hypoglycemia. In T2D, insulin action is impaired and glucagon action is enhanced, leading to increased hepatic glucose output and consequently to hyperglycemia [119]. Epigenetic modifications in human liver may contribute to the altered hepatic metabolism observed in T2D together with genetics and other risk factors (Figure 4). In fact, the liver is the main organ for one-carbon metabolism where the metabolic pathways of methyl donors are most active and where most methylation reactions occur [120]. Two recent EWAS showed that subjects with T2D display an altered DNA methylation pattern in liver compared to non-diabetic individuals [121,122] (Table 1). First, our group found that 35 subjects with T2D exhibited differential methylation of 251 CpG sites in the liver when compared to 60 non-diabetic subjects [122]. 94% of the significant sites showed lower methylation in the diabetics, which may be explained by the lower levels of folate observed in the same individuals as folate is one of the major dietary methyl donors. The differentially methylated sites include CpG sites in genes with already known functions in T2D, such as GNB10, ABC3, MOGAT1, and PRDM16. Moreover, 29 genes showed both differential DNA methylation and differential gene expression in the liver of diabetics, including the imprinted gene H19 and RIPK4. RIPK4 has been
associated with decreased insulin sensitivity in liver [123]. In accordance with Nilsson et al., Kirchner et al. showed that the majority of significant DNA methylation sites in obese T2D subjects were also hypomethylated [121]. This was also associated with increased mRNA and protein expression for genes regulating glucose and lipid metabolism, for example, within the ATF-motif regulatory site. Notably, Kirchner et al. found that hypomethylation of PRKCE was associated with increased expression of the PRKCE mRNA which might lead to the development of hepatic insulin resistance and hepatic steatosis. PRKCE may induce insulin resistance interfering directly with the activation of the insulin receptor kinase activity [124]. These two studies support a role for epigenetic regulation of hepatic gene expression in the pathogenesis of T2D. However, it is difficult to draw conclusions about causality and to elucidate whether epigenetic modifications in liver, as well as in other tissues, are the cause or the consequence of T2D. In this sense, longitudinal studies would provide a better approach for studying causality between DNA methylation in liver and T2D.

5.2. Interactions between genetics and DNA methylation in human liver

Hepatic gene expression has also been linked to genetic variants through the study of expression quantitative trait loci (eQTLs). For example, a few SNPs associated with metabolic disorders and lipid metabolism were also associated with hepatic expression of the fatty acid desaturase gene (FADS) in Caucasian and Asian populations [125,126]. As previously mentioned, epigenetic mechanisms also play an important role in regulating hepatic gene expression. Therefore, it is likely that genetic variants and DNA methylation interact with each other and together regulate the transcriptome in the human liver. There is only one study analyzing genetic and epigenetic interactions in human liver. This study identified effects of SNPs on both methylation (28,447 mQTLs) and gene expression levels (526 eQTLs) [127]. The latter suggests that unraveling the coordinated effects of genetic polymorphisms and DNA methylation is crucial for understanding the etiology of liver diseases.

5.3. Associations between non-genetic and genetic factors and DNA methylation in human liver

5.3.1. Obesity and aging associate with DNA methylation in human liver

It is well known that obesity increases the risk for T2D and this could take place through accelerated tissue aging. Horvath et al. showed that obesity is associated with increased DNA methylation age in human liver (3.3 years for each 10 BMI units), which, to a certain extent, may explain liver-related insulin resistance in T2D [128]. Furthermore, an EWAS further confirmed that age is associated with altered DNA methylation of 20,396 CpG sites in human liver, and some of these epigenetic changes also correlated with liver gene expression [44]. Among the most significant genes with associations between methylation and age in the liver were ELOVL2, KLF14, and FHL2, which were also top epigenetic markers of aging in blood, adipose tissue, and pancreatic islets [43,45]. Aging might affect DNA methylation through increasing gene expression of DNMT3A and DNMT3B in human liver [129].

5.3.2. NAFLD/NASH and DNA methylation in human liver

T2D can lead to liver complications such as non-alcoholic fatty liver disease (NAFLD) or non-alcoholic steatohepatitis (NASH), a chronic liver disease associated with insulin resistance. NAFLD/NASH has been associated with genome-wide alterations in DNA methylation in liver, and, interestingly, NAFLD/NASH-specific expression and methylation differences were observed for genes involved in insulin signaling [130,131]. Other studies have shown that regulation of mitochondrial biogenesis in liver through DNA methylation and hydroxymethylation might also be involved in the pathogenesis of NASH [132,133]. Importantly, Ahrens et al. revealed that weight-loss reversed NASH-associated methylation changes in liver from obese subjects who underwent bariatric surgery [131]. The PTPRE gene, which promotes insulin resistance in skeletal muscle [134], was hypermethylated and its mRNA downregulated after bariatric surgery [131]. Therefore, regulation of PTPRE may be involved in the restoring of hepatic insulin sensitivity after weight-loss.

5.3.3. Metformin and DNA methylation in human liver

Metformin is the most prescribed medication for T2D and one of its primary actions is in the liver where it is taken up through hepatic transporters. These transporters are essential for the pharmacological effect of metformin, and genetic as well as epigenetic factors seem to influence their hepatic expression, leading to either an impaired or improved metformin transport in the liver [135–137]. We recently showed that metformin decreases DNA methylation of metformin transporter genes in human liver, and these epigenetic marks were also associated with gene expression [138]. In addition, mQTLs in genes for important drug metabolizing enzymes may contribute to the variability in expression of these genes in the human liver [127,139]. Further research studying genetic variants and DNA methylation in liver could shed light on the causes of differences in metformin transport and response.
6. CONCLUSIONS AND FUTURE DIRECTIONS

Epigenetic alterations have been observed in tissues from individuals with T2D and those with high risk of developing the disease. Risk factors for T2D, such as obesity and aging, also affect the methylene in non-diabetic subjects, possibly triggering the development of insulin resistance, impaired insulin secretion, and T2D. Diet and exercise interventions may prevent disease development by inducing epigenetic changes followed by changes in gene expression and metabolism. The fact that risk SNPs may mediate their function on gene expression and metabolism via DNA methylation further supports the importance of epigenetic variation in T2D. Together, the studies reviewed in this summary support an important role of epigenetics in the pathogenesis of T2D.

It is important to consider that most human tissues consist of different cell types as well as cells from blood vessels and connective tissue. Alterations in the cell composition in diseased subjects may hence affect the epigenome. Thus, more efforts on studying the epigenome in sorted cells are needed. This issue should also be taken into account when manipulating the epigenome. Epigenetic inhibitors are already in clinical use; however, further studies are needed. This issue should also be taken into account when manipulating the epigenome. Epigenetic inhibitors are already in clinical use; however, further studies are needed.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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