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Epigenetics and Human Obesity

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ABSTRACT

Background: Recent technological advances in epigenome profiling have led to an increasing number of studies investigating the role of the epigenome in obesity. There is also evidence that environmental exposures during early life can induce persistent alterations in the epigenome, which may lead to an increased risk of obesity later in life.

Method: This paper provides a systematic review of studies investigating the association between obesity and either global, site specific, or genome-wide methylation of DNA. Studies on the impact of pre- and postnatal interventions on methylation and obesity are also reviewed. We discuss outstanding questions, and introduce EpiSCOPE, a multidisciplinary research program aimed at increasing the understanding of epigenetic changes in emergence of obesity.

Results: An electronic search for relevant articles, published between September 2008 and September 2013 was performed. From the 319 articles identified, 46 studies were included and reviewed. The studies provided no consistent evidence for a relationship between global methylation and obesity. The studies did identify multiple obesity-associated differentially methylated sites, mainly in blood cells. Extensive, but small, alterations in methylation at specific sites were observed in weight loss intervention studies, and several associations between methylation marks at birth and later life obesity were found.

Conclusions: Overall, significant progress has been made in the field of epigenetics and obesity and the first potential epigenetic markers for obesity that could be detected at birth have been identified. Eventually this may help in predicting an individual's obesity risk at a young age and opens possibilities for introducing targeted prevention strategies. It has also become clear that several epigenetic marks are modifiable, by changing the exposure *in utero*, but also by lifestyle changes in adult life, which implies that there is the potential for interventions to be introduced in postnatal life to modify unfavourable epigenomic profiles.

Keywords: obesity; epigenetics; DNA methylation; adipose tissue; developmental programming

INTRODUCTION

There is little contention that the rising incidence of obesity is a major public health issue world-wide [1]. Obesity is a major risk factor for co-morbidities, such as type 2 diabetes, cardiovascular disease, and certain forms of cancer [2]. Thus, the obesity epidemic threatens to reduce the length and quality of life of current and future generations, and it presents a significant challenge to future health care budgets. There is a strong need for safe and effective strategies for obesity prevention and treatment. A multitude of campaigns have been launched by governments and health agencies, but for the most part with limited effects on reducing obesity rates in the medium to longer term [3]. Part of the reason for this failure could be that these strategies are typically introduced after obesity is established, and it is becoming increasingly clear that at that stage obesity is difficult to reverse [4 5]. The focus of anti-obesity campaigns should therefore be on prevention in order to achieve maximum long-term health gains. To improve prevention and treatment strategies a better understanding of factors contributing to the development of obesity is essential.

Epigenetics and human disease

Over the last decade there has been increasing interest in the role of epigenetics in the development of complex conditions such as obesity. In contrast to genetic modifications, which lead to a change in the base sequence of DNA, epigenetic changes are typically reversible and refer to chemical modifications to DNA (or DNA-associated chromosomal proteins called histones) that occur in the absence of a change in the DNA sequence [6]. Epigenetic marks are heritable through mitotic cell division and can alter the way the transcription of genes is controlled within a cell. This occurs through a number of processes, the best described being the addition of methyl groups to DNA (methylation) and posttranslational modifications to histone proteins, such as acetylation and methylation.

Methylation of mammalian genomes occurs predominantly at cytosines adjacent to guanines ('CpG' sites). Epigenetic processes alter the accessibility of the transcriptional machinery to a particular gene, thereby determining whether or not the gene is active in a given cell at a given time. Importantly, whilst the DNA sequence of genes in an individual (the genome) is largely stable, the epigenome has the potential to be reversibly modified by exposure to a range of nutritional and environmental factors [6].

The importance of epigenetic processes in human disease was first identified in the field of cancer in the 1980s [7]. Since then, there have been a plethora of studies that have described epigenetic changes in cancerous tissues, and in the blood of cancer patients, and alterations in the methylation level of specific genes have been proposed as novel biomarkers in cancer screening [8].

More recently, the attention of the scientific community has turned to the potential role of epigenetic modifications in other disease states, including obesity. Initial studies were limited in sample size and number of CpG sites (CpGs) studied. With advances in technologies and the emergence of more affordable, high throughput methylation screening methods, there has been an increase in larger scale studies and the first epigenome wide association studies exploring the relationship between the environment, the epigenome and complex disease states. To date, DNA methylation, either at global, site-specific or genome-wide levels at single nucleotide resolution, is by far the most studied epigenetic mark. There have been few investigations of histone modifications in relation to obesity in humans, but some of the results to date do suggest an association between genome-wide histone modifications and the development of or susceptibility towards obesity [9].

There is accumulating evidence that the propensity toward adult obesity has early developmental origins and follows an intergenerational cycle [10 11 12 13 14 15]. Epidemiological studies have shown that exposure to a suboptimal nutritional environment during

development, as a result of either an excess or deficient maternal caloric or micronutrient intake, is associated with an increased risk of a range of chronic diseases, including obesity, type 2 diabetes and cardiovascular disease in later life [16 17 18 19 20]. These findings have led to the developmental origins of health and disease (DOHaD) hypothesis, which proposes that adult disease risk can be programmed by the perinatal environment [21].

One consistent theme relating to this hypothesis is that transient environmental influences experienced early in life can cause permanent effects that emerge as increased disease risk much later in life. The mechanisms underpinning this nutritional 'memory' response are not clear but may include changes in the developmental trajectories of tissues, reprogramming of stem cells, changes in tissue structure, and the reprogramming of neural, endocrine and metabolism regulatory circuits. Epigenetic programming may be mechanistically involved in these processes or provide a readout of their occurrence. Moreover, once established, early life nutritionally-induced epigenetic changes may lie dormant until their biological influence is triggered later in life.

Evidence to support a role of epigenetics in developmental programming of disease has been predominately derived from animal studies that have demonstrated the impact of a suboptimal intrauterine nutritional environment on the epigenome and phenotype of the offspring [22 23 24 25]. There are relatively few human studies in this area, but one of the most significant studies was conducted in children who were born to women exposed to severe undernutrition during pregnancy as a result of the Dutch Hunger Winter during World War II, which reported a reduced methylation of the imprinted gene *IGF2* in these individuals as adults [26 27]. This has particular relevance given that these individuals have also been shown to be at increased risk of obesity or glucose intolerance, depending on the timing of the exposure to famine [28 29 30].

In this review we provide a systematic overview of the most recent findings in the research area of epigenetics and obesity, specifically focused on human studies. Studies investigating the association between either global methylation, site specific methylation, or genome-wide methylation of DNA and obesity, are summarized and discussed. In addition, the impact of interventions on DNA methylation profiles and obesity in children are summarized. Moreover, it discusses outstanding questions and introduces EpiSCOPE, a multidisciplinary research program with the goal of increasing the understanding of epigenetic changes in emergence of obesity.

METHODS

The MEDLINE database was searched for relevant studies published between September 15, 2008 to September 15, 2013, using the search terms "obesity OR body mass index OR overweight OR body fat OR adiposity OR adipose tissue" AND "epigenetics OR methylation OR histone". The first search was restricted to primary studies in humans, and an additional search was performed for articles that were not labelled as a human or animal study. The titles and abstracts, and in several cases the full texts, were scanned to determine their relevance to the scope of this review. Studies to be included either described an association between epigenetic marks and obesity in humans or reported an effect of a defined intervention on epigenetic marks and obesity in humans.

Studies Identified by Search Strategy

From the 319 articles identified by the search strategy, 273 articles were excluded; 55 papers were reviews or commentaries, rather than original research articles, 62 studies were not conducted in humans, 71 did not assess any outcomes related to obesity, 18 did not assess an epigenetic outcome, 32 included only *in vitro* data and 35 were excluded for other reasons,

(e.g. methodology studies, or studies which included individuals with hereditary diseases or cancer). Thus, a total of 46 articles were included in this review.

RESULTS

Summary of included studies

Of the 46 studies included, 15 studies assessed relationships between measures of obesity and global DNA methylation, 13 studies assessed relationships with DNA methylation in specific candidate genes, five studies used genome-wide approaches to assess differences in methylation between obese/lean individuals or the association with obesity measures, eight studies assessed DNA methylation profiles in relation to weight loss interventions and nine studies assessed relationships of DNA methylation at early life with either parental health measures or later life health outcomes.

Global methylation and obesity

Global methylation refers to the overall level of methyl cytosine in the genome, expressed as percentage of total cytosine (%5meC). A lowered global methylation level has been associated with chromosomal instability and increased mutation events and is considered as a hallmark of cancer [31], but less is known about global methylation in other disease states.

Repetitive elements, such as ALU and Long Interspersed Nucleotide Element 1 (LINE1), comprise ~50% of the genome and the degree of methylation in these elements is often used as a surrogate to represent the overall methylation level of the genome. Because of the relatively low cost and high throughput, global methylation levels are reasonably easy to determine in large numbers of samples, which makes it ideal for screening purposes.

Details of the 15 studies that have examined global methylation in relation to obesity are summarised in Table 1. The majority of the studies used blood samples, but LINE1

methylation in DNA from muscle, placenta and colon has also been studied. All of the studies used BMI or changes in BMI to classify obesity, and two studies also used percentage body fat [32 33]. The majority of these investigations, including a large study combining four study populations, including up to 1254 individuals [34], did not find an association between obesity and global methylation[33,35–40]. Two studies that included only women[32 41], found reduced global methylation with increasing BMI, however in one of these studies this only occurred in the presence of low concentrations of the methyl donor folate [32]. In contrast, two other studies that included both men and women from two different populations (Samoa and China) reported a positive relationship between global methylation in Peripheral Blood Leukocytes (PBL) and BMI [42 43], and in a further study global methylation in placental tissues was higher in obese compared to lean women [40]. Only one study examined global histone methylation in obesity, showing substantially decreased levels of histone H3 lysine 4 dimethylation in adipocytes of overweight individuals compared with lean, with increased levels of lysine 4 trimethylation, observed in obese/diabetic individuals [9]. Thus, while some studies do report significant associations between global methylation and obesity-related measures, the direction of change is not consistent, and both global hypomethylation as well as global hypermethylation have been related to obesity-related measures [32 33 41 42 43 44 45].

There are a multitude of factors, including gender, ethnic background, age, exposure to chemicals, tobacco smoke, alcohol and diet [33 34 46 47 48 49], that are known to affect global methylation levels, which could influence a potential association between global methylation and obesity. In several studies corrections for at least a few of these confounding factors have been applied, but often not all confounding factors are known and taken into account.

Overall, the available global methylation studies in obesity do not provide consistent evidence for a relationship between global methylation and obesity. Compared to the situation in cancer, global methylation changes in obesity (if present) are likely to be more

subtle and thus difficult to detect considering the influence of multiple other factors on this measure. Consequently, more specific methylation analyses, either for specific loci of interest in obesity, or a genome-wide approach are likely to provide a better picture of the association between obesity and DNA methylation. Larger sample sizes (>1000) may also be required to produce more robust results. In addition, the studies that have been conducted to date point to the potential for sex differences in the relationship between BMI and global methylation, which also require further investigation and suggest that it is important to examine associations separately in males and females.

Gene-specific DNA methylation and obesity

The epigenetic environment of individual genes provides a critical component contributing to their regulation and level of expression. Because of the relative ease of analysis, gene-specific DNA methylation is the most extensively studied epigenetic mark in studies relating epigenetic changes to health outcomes, including obesity. Historically, elevated DNA methylation has been associated with repression of gene expression. However, with the advent of genome-wide methods of DNA methylation analysis, it is now recognized that the association of DNA methylation with gene expression is not as simple as previously thought, and appears to depend on where within the gene sequence the methylation occurs. In general, DNA methylation at gene promoters and enhancers is associated with gene silencing and higher methylation in the gene body with active gene expression, but even this is an oversimplification [50].

The majority of studies examining the relationship between site-specific DNA methylation and obesity are cross-sectional; i.e. both methylation levels and the phenotype are measured at the same time point. Hence it cannot be established whether the association between a

specific DNA methylation mark and obesity is a cause or a consequence of the obese phenotype.

Candidate gene studies

Multiple studies have used a hypothesis driven, candidate gene approach (summarised in Table 2) where methylation sites in, or near, known candidate genes for obesity susceptibility, have been the subject of investigation. In some cases the choice of genes has been based on prior analysis of gene expression differences in the same subjects. The candidate gene methylation studies have focussed on a range of genes implicated in obesity, appetite control and/or metabolism, insulin signalling, immunity, growth, circadian clock regulation and imprinted genes, and assessed their relationship with a variety of obesity markers. Collectively, these studies have identified lower methylation of tumour necrosis factor alpha (*TNFα)* in PBL [51], pyruvate dehydrogenase kinase 4 (*PDK4)* in muscle [52] and leptin (*LEP)* in whole blood (WB) [53] and increased methylation of proopiomelanocortin *(POMC)* in WB [54], PPARγ coactivator 1 alpha (*PGC1α*) [52] in muscle, and *CLOCK* and aryl hydrocarbon receptor nuclear translocator-like (*BMAL1)*[55] genes in PBL in obese compared to lean individuals. Associations between BMI, adiposity, and waist circumference, with methylation in *PDK4* in muscle[52], melanin-concentrating hormone receptor 1 (*MCHR1*) in WB [57], and the serotonin transporter (*SLC6A4*) gene [56], the androgen receptor (*AR*) [58], 11 b-hydroxysteroid dehydrogenase type 2 (*HSD2*) [59], period circadian clock 2 (*PER2*) [55] and glucocorticoid receptor (*GR*) [59] in PBL have also been reported. The most consistently observed epigenetic association has been that of methylation at the *IGF2/H19* imprinting region in blood cells with measures of adiposity [59] [60]. Collectively, these studies provide evidence that obesity is associated with altered epigenetic regulation of a number of metabolically important genes.

Genome-wide analyses and obesity

The recent development of genome-wide methods for quantifying site-specific DNA methylation has led to the initiation of studies that are not targeted to specific genes, but search for associations across a large number of genes and CpGs. Obesity-associated differentially methylated (DM) sites in peripheral blood cells were detected in four published genome-wide studies (Table 2) [61 62 63 64]. Extensive, but small, alterations in methylation at specific sites have been observed. In one case a signature of DM sites was used to predict obesity in a validation set [62], while in other studies specific DM CpGs were validated in a second cohort [63], or at a second time point [64].

Overall, obesity associated DM sites were enriched both in obesity candidate genes [62] and in genes with a wide diversity of other functions, such as immune response [63], cell differentiation [62] and regulation of transcription [61]. DM sites were also identified in or near genes with no known function related to obesity or adipose tissue functioning.

Intervention studies in adults

It has long been assumed that DNA methylation profiles would remain stable throughout adult life; however, this view is now changing. Interventions such as exercise, diets and weight loss surgery have been shown to modulate methylation profiles in different tissue types (Table 3) [52 65 66]. Interestingly, methylation profiles of obese individuals became more similar to those of lean individuals following weight loss surgery [52]. Although this was only demonstrated in a small study, it suggests that methylation profiles of obese individuals can be modified by reductions in body weight/fat mass. This conclusion may imply that some methylation marks are a consequence of the obese phenotype, rather than a programmed mark that predisposes people to become obese. These findings again highlight the importance

of studies in which methylation marks are measured early in life before disease manifests, to define which acquired marks become permanent, and thus potential early markers for disease risk, and which ones are semipermanent and modifiable in later life.

In a separate group of studies, comparison of the methylation profiles of people who successfully lost weight during interventions and those who did not, has been used in order to determine whether there may be biomarkers that predict individual responsiveness to weight-loss interventions (Table 3) [55 66 67 68 69]. Methylation differences between these individuals were identified in genes involved in weight control, insulin secretion, inflammation, and circadian rhythm. The association of such methylation differences with a propensity to lose weight may imply that methylation changes in those genes predisposes individuals to become and stay obese, even in situations of limited food intake. Adherence to the interventions was monitored in most studies through regular meetings with study dieticians, or attendance at group exercise or therapy sessions. However, it is notoriously difficult to accurately monitor compliance in nutritional intervention studies in humans, and it is therefore possible that failure to lose weight might also be a reflection of poor adherence of the participants to the intervention.

Prenatal and postnatal environment

The period of embryonic development has been recognised as a critical window in the establishment of the epigenome. There is compelling evidence that an adverse pre-natal and early postnatal environment can increase obesity risk in later life [27 70 71 72] and this has led to the search for nutritional interventions during pregnancy and lactation which have the potential to mitigate or overcome this adverse programming [12]. Diet and weight loss interventions in obese mothers may lead to a decreased risk of obesity in the offspring,

possibly mediated through changes in insulin signalling, fat storage, energy expenditure or appetite control pathways. Epigenetic mechanisms are likely to play a role in this altered risk profile and the findings of obesity associated methylation marks in genes involved in these processes also support this hypothesis. Human studies showing a direct relationship between specific prenatal (nutritional) exposures on methylation profiles of the offspring and subsequent risk of obesity in later life are scarce. However, there are a number of studies which have assessed differences in methylation of candidate genes in children in relation to maternal/paternal characteristics [44 73 74 75] or have explored relationships between epigenetic markers in the cord blood at delivery and obesity/metabolic outcomes in childhood (Table 4). One such study compared methylation profiles of siblings born before and after maternal weight loss surgery, and reported differences between the siblings in obesity characteristics and in methylation profiles for genes involved in the regulation of glucose homeostasis and immune function [76 77], some of which translated into alterations in gene expression and insulin sensitivity. Although this was a small study, its findings suggest that significant weight loss, and presumably improved metabolic health profiles, in the mother are associated with a distinct epigenome and lower weight and waist circumference in the children.

Additional studies have explored the association of DNA methylation at birth with adiposity in later life (Table 4) [78 79 80 81]. Methylation variation in the promoter of the retinoid X receptor alpha gene (*RXRα)* in umbilical cord tissue was found to explain up to 26% of the variation in childhood adiposity [79]. *RXRα* is a nuclear receptor with a known role in adipogenesis [82]; it forms a heterodimer with the transcription factor *PPARγ* to activate transcription of genes involved in adipocyte differentiation, glucose metabolism, inflammation and energy homeostasis. DNA methylation appears to be also important in the regulation of *PPARγ* [83], and variation in DNA methylation of *PGC1A*, interacting with PPARγ, has been associated with weight loss, obesity, and risk for T2DM [52 84 85]. Variation in

methylation within tumour-associated calcium signal transducer 2 (*TACST2*) at birth was also found to correlate with fat mass in later life, however, further analysis including Single Nuclear Polymorphism data of this gene showed that reverse causation or confounding was likely to account for the observed correlation [81]. *IGF2* is another example of a gene showing loci-specific variation in methylation at birth, and also at childhood, that is associated with growth characteristics and obesity in later life [60 78 59]. The epigenetic regulation of *IGF2* has been of particular interest given its role in control of fetal growth and development. Differences in the degree of methylation near *IGF2* has often been linked to exposure to a suboptimal environment *in utero* [27 26 59 86]. Contradictory findings, however, come from recent mice studies that showed no effect of maternal nutrition on *IGF2* DMRs (differentially methylated regions) [87].

The finding of an association between variation in matrix metallopeptidase 9 (*MMP9*) methylation levels at birth and childhood adiposity [80] is also of interest, given the critical role that metalloproteinases play in extra cellular matrix remodelling during adipose tissue formation, and coupled with the fact that altered MMP9 plasma levels and gene expression has previously been found in obese individuals [88]. Moreover, variation in methylation near *MMP9*, and another metalloproteinase called *PM20D1*, was associated with BMI in a genome-wide study, at two time points 11 years apart [64]. These findings show that these marks are most likely established at an early age and may be associated with adiposity at different stages in later life, which suggests that these methylation changes could be potentially useful to predict obesity risk from an early age.

DISCUSSION

Measurement of DNA methylation

The advent of genome-wide, array-based methods for determining site-specific DNA methylation is opening a new window for identifying phenotype differentially methylated sites or regions. An overview of different methods of DNA methylation analysis and discussion of their respective advantages and disadvantages, including those used in the reviewed studies, can be found in supplementary table 1. While a number of technologies have been used to date, the Illumina Infinium Methylation450 bead Chip is emerging as the most widely used platform. Early experience suggests that the technology provides reproducible quantitative data and its widespread use will facilitate cross-study comparisons. Since assessment is made of only a selected set of CpGs and regions (<5% of total number of CpGs), it will be important that other technologies that explore the genome more widely continue to be used.

Notably, both from candidate gene and genome-wide approaches, the methylation differences associated with obesity or interventions reported to date have generally been small. In array-based approaches, since the number of individuals is low compared to the high number of sites measured, often only a few DM sites remain significant after strict multiple testing corrections. The presence of co-ordinate variation at CpGs within a localised region can provide more confidence that differences seen are real and methods looking at variable methylated regions, as opposed to single sites, are increasingly being used in analysis of methylation data to overcome the problems associated with strict multiple testing corrections [89]. It has been proposed that particular regions of the genome show high levels of variance in methylation levels and that this might provide potential for gene expression levels to stably respond to environmental conditions [90]. This adaptability would confer a significant fitness advantage and has probably been subject to strong selection during the longer time scales of

evolution, i.e. the genetic blueprint has likely been altered by evolution and natural selection to enhance epigenetically mediated adaptability traits. In support of this a significant overlap in variably methylated regions and DM regions has been observed [62]. Given the modest levels of change in DNA methylation, it is expected that the effect size on phenotype of individual DM sites is generally likely to be small. However, it is noteworthy that methylation at the RXRA locus at birth could statistically account for 26% of variation in adiposity of children at 9 years[79]. Similar to genetic variations, it will probably not be one single DM site but more likely combinations of multiple (in)dependent DM sites, possibly in several genes, that can explain variations in phenotype and that will need to be used in combination to develop predictive signatures.

The vast majority of epigenetic studies published to date focused only on DNA methylation, thereby ignoring other epigenetic information, such as histone modifications and non-coding RNAs. Histone modifications can influence DNA methylation patterns and vice versa, and there is evidence that histone modifications play key roles in adipogenesis [91]. Thus, these are likely to have an important role in the development of obesity and should be taken into account in epigenetic studies. Integration of epigenome and transcriptome data will also be crucial to obtain a more complete picture of the interaction between epigenetic modifications and regulation of gene expression.

DNA methylation in different tissue types

DNA from peripheral blood cells is the most frequently used source of DNA for epigenetic studies. Since blood is easily accessible, it is often the only biological material that is routinely sampled in large scale studies. A potential issue with the use of blood is that it consists of a mixture of different cell types with different methylation profiles [92 93]. It has

been shown that although methylation of some CpGs is dependent on variation in blood cell types [94 58], global methylation and methylation at most sites appear to be unaffected [95 96 97]. However, if one wants to minimize potential effects of cellular heterogeneity on methylation profiles, a correction based on the numbers of each respective cell type in the sample may need to be applied [98 99].

Another important consideration when using blood in epigenetic studies is that the blood cell methylation profile in blood may not necessarily report the epigenetic state in other tissues. Since the hematopoietic system is established very early in development, it has been suggested that methylation changes induced around conception and in early embryo development may be reflected in all germ layers and thus detectable in blood and most tissue types [64]. Later in development, however, environmentally induced epigenetic changes might be more tissue specific and may not be detectable in blood. A recent study comparing methylomes across 30 human tissues and cell types showed that there is a large 'common methylation profile' across tissues and only a small fraction (~20%) of CpGs show dynamic regulation during development [100]. Efforts to provide a "Blue Print of Human Epigenomes", as undertaken by consortia like International Human Epigenome Consortium (IHEC), will be of considerable value to get a better insight in tissue specific epigenetic signatures and their role in disease development.

To increase our understanding of the role of epigenetics in obesity, adipose tissue is of high interest. Adipose tissue is not only the main tissue for energy storage, but also has important endocrine functions which are often disrupted in obesity. Adipose tissue contains functionally different cellular subtypes and different depots have distinct characteristics depending on their anatomical location. Metabolic disturbances are often linked to increased fat deposition in visceral depots, while storage in subcutaneous depots is considered less problematic from a

metabolic stand-point. Recently, there has been renewed interest in understanding the role of brown fat in humans, in particular in relation to human obesity, as a consequence of its fat burning properties [101]. Since DNA methylation is of major importance in defining cellular identity and differentiation of adipocytes, the study of DNA methylation profiles in different adipose tissue depots under different metabolic conditions could provide information about how epigenetic regulation of adipose tissue is involved in the development of obesity and associated co-morbidities, and how this could potentially be manipulated. Studies have already shown that DNA methylation in adipose tissue can change after exercise intervention and display differences between high and low responders to weight loss interventions [65 68 69], which indicates that epigenetic regulation in this tissue is likely to be of importance and should be further investigated.

For diagnostic purposes in a clinical setting, particularly when screening young children, epigenetic marks should be detectable in easily accessible samples, such as peripheral blood, or possibly buccal cells. Blood and buccal cells originate from different germ layers and the degree of similarity in methylation between both tissues varies across the sites that have been examined so far. An extensive comparison of genome-wide methylation profiles in both tissues has not yet been done and would be of significant interest to the field.

For screening at birth, DNA isolated from the umbilical cord might also be of interest, especially in light of the findings that variation in methylation in this tissue is highly associated with body fat mass in later life [79]. Cord blood and newborn blood spotted on filter paper (Guthrie cards) are other relatively accessible materials. Guthrie cards are routinely collected worldwide for screening of genetic diseases in newborns and a number of studies have demonstrated that DNA extracted from these spots can be successfully used for methylome profiling [102 103]. In countries where these samples are routinely stored for extended periods of time, they provide a unique resource for researchers to determine

whether specific methylation marks detected at older ages were already present in the individuals at birth.

Stability and inter-individual variation in DNA methylation

When considering methylation marks as biomarkers for disease risk, it is essential that they show substantial variation between individuals, and are relatively stable over time. For the design and interpretation of epigenetic studies, it is also important to know the actual scale and extent of inter-individual variation in DNA methylation.

Variability in DNA methylation across healthy individuals is a combination of genetic influence, environmental influence and stochastic events [104] [64]. A number of studies have investigated the stability and inter-individual variation in DNA methylation, mainly in blood, comparing changes in DNA methylation profiles over short time spans (days) to longer periods (years). The period from birth to childhood is considered a dynamic period for DNA methylation; Wang et al. showed that methylation status of ~5% of the measured 27,000 CpGs substantially changed from birth to two years of age [105] and in another study ~8% of the sites on the Illumina arrays showed age-associated methylation in childhood [106]. Multiple studies have demonstrated that some methylation marks show considerable variation over time while others are highly stable [64] [96] [107] [108]. It has been suggested that the stable epigenetic marks represent those determined by genetics, while the variable marks are more likely to be influenced by the environment [64]. A comparison of methylation profiles between populations of different ethnic backgrounds showed that about two-thirds of population-specific CpGs are associated with genetic background [104], while one-third might be influenced by other factors.

The stability and inter-individual variation in methylation is also dependent on its genomic location. Low levels of inter-individual variation have been found at commonly unmethylated

regions such as CpG islands, and higher variation is measured in regions adjacent to the CpG islands, such as the CpG shores [105 109 106 110]. DM regions also show enrichment of Single Nucleotide Polymorphisms (SNPs), which may help explain why several DMRs were found within or near obesity candidate genes. DNA methylation at sites that associate with SNPs might be a mechanism through which some SNPs affect gene function [111 112]. In this regard the presence of SNPs that are associated with cis-acting regional changes in DNA methylation status are of particular interest [113].

Study design and analysis

Robustly designed, well-conducted and adequately powered studies are crucial for the identification of early epigenetic biomarkers (whether causative or not) for obesity risk and in establishing the effects of the *in utero* environment.

Cross sectional studies

In most current studies, methylation levels and the phenotype are measured at the same time point, which makes it impossible to define whether specific DNA methylation marks are a cause or consequence of obesity. However, while they may not be contributing to the etiology of obesity they may still be useful for predicting obesity risk. The association between methylation and obesity may be indirect; there is most likely a third factor (nutritional or environmental) involved that independently affects both methylation and obesity.

Where no longitudinal data is available, statistical approaches have been developed in epidemiological studies to infer causality and these are beginning to be applied to epigenomic data. For example, Liu et al. inferred that methylation at specific sites mediated genetic risk for rheumatoid arthritis [98]. The Mendelian randomisation strategy, as proposed by Relton and Davey Smith [114], makes use of SNP data as a proxy for DNA methylation and the modifiable

exposure of interest, and may be a useful approach to gain a better understanding of the direction of causality. However, this approach also has its limitations, and suitable SNP data are not always available.

Longitudinal studies

While an effect of early nutritional exposures on later life disease risk in humans has been demonstrated, evidence showing involvement of epigenetic processes in linking early nutritional exposure to later obesity risk in humans is scarce. To successfully address this knowledge gap, large randomised controlled trials and prospective studies will be needed. It may not be ethical to conduct a randomized trial in all situations (e.g. restricting breastfeeding or randomly assigning children to be breast or formula fed). However, in the case of nutritional interventions applied during pregnancy, adequately powered randomized controlled trials are the only way to clearly dissect out the impact of the specific nutritional intervention from those of other environmental/demographic variables. In addition, in longitudinal studies, extensive data on parental phenotype and lifestyle, as well as parental blood or buccal cell samples, should ideally be collected before birth, and the phenotype and epigenome of the offspring should be followed prospectively from birth throughout their life course. Since DNA methylation profiles at birth have not been affected by postnatal environment or disease state in later life, such studies will provide better support for a link between early epigenetic marks and disease risk than cross-sectional studies. Another advantage of longitudinal studies is that genotype is constant and its direct effects on methylation profiles can be measured and removed. In addition, these studies may provide information on the dynamics of methylation changes during life time, this may help in identifying at what point during development an intervention would be most effective. Animal studies will also remain essential, particularly to give more mechanistic insight and

answer outstanding questions related to the underlying biology that are impossible to address in humans.

Human and animal data suggests that not only maternal health and diet might be of importance for the offspring; there are also indications that the paternal contribution to methylation of imprinted genes and phenotype should be taken into account[73].

Cohort studies

Cohorts including multiple generations, siblings, or ideally monozygotic twins discordant for obesity, would be valuable in unravelling the impact of genetics and environment on the epigenome and phenotype. These types of studies are expensive and time consuming, but some large scale initiatives such as ARIES, MuTHER, EpiTwin, PETS and KORA are underway [115 116 117 118 119].

EpiSCOPE (Epigenome Study Consortium for Obesity primed in the Perinatal Environment) is a recently initiated multi-institutional Australian research program that aims to add valuable new information to our understanding of the role of epigenetics in the aetiology of obesity. The research will centre around three areas; 1) The epigenome of human adipocytes; EpiSCOPE will develop high resolution epigenome and transcriptome maps of visceral and subcutaneous adipocytes of healthy, lean individuals for comparison with obese and obese/diabetic subjects. Cross-tissue comparisons will be performed to establish the translatability of epigenetic patterns in adipocytes to blood. 2) Early epigenetic marks for obesity and the effect of *in utero* fish oil exposure; this study is utilising blood samples collected both at birth and during childhood from children from the large randomized controlled DOMInO trial [120] that provides the opportunity to investigate both the relationship of epigenetic marks to childhood metabolic outcomes and the effect of *in utero* fish oil

exposure on the development of childhood obesity and to determine whether epigenetic changes are involved in mediating this. 3) The effect of periconceptional obesity, without the presence of obesity during pregnancy, on the epigenome of the offspring. A sheep model was chosen because of the similarities in the prenatal development of many organ systems, including adipose tissue, in sheep and humans. The model will be used to investigate the molecular mechanisms in specific organs involved in risk of obesity and that contribute to the transmission of a vulnerability to obesity in the offspring of obese mothers.

Using this three-pronged approach, EpiSCOPE aims to improve our understanding of the role of epigenetics in determining long-term metabolic outcomes and to provide an evidence base for the development of novel therapeutic and diagnostic targets to lower the burden and risk of a dominant chronic and to date intractable disease.

FUTURE DIRECTIONS

Overall, significant progress has been made in the field of epigenetics and obesity, but there is still much to be learned before we fully understand the role of the epigenome in development of complex diseases such as obesity. Epigenetics is a rapidly evolving area of research and the first steps are already being made in identifying potential biomarkers for obesity that could be detected at birth. Eventually this may help in predicting an individual's obesity risk at a young age, before the phenotype develops, and opens possibilities for introducing targeted strategies to prevent the condition. It is also now clear that several epigenetic marks are modifiable, not only by changing the exposure *in utero*, but also by lifestyle changes in adult life, which implies that there is the potential for interventions to be introduced in postnatal life to modify or rescue unfavourable epigenomic profiles.

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Table 1 Global DNA methylation and risk factors associated with obesity

	Obesity measure	Tissue type	Population	Association and reference*	Comment
%5meC					
	BMI	PBL	M and F, elderly (n=126)	↔ [35]	
LINE1					
	BMI	PBL	M and F, middle-aged (n=161)	↔ [33]	
	BMI	PBL	F, Premenopausal (n=470)	↓ [32]	Only in presence of low folate
	BMI	PBL	M and F, adults, Samoan (n=355)	↑ [42]	
	BMI	PBL	M and F, combination of 5 studies (n=1465)	↔ [34]	In separate studies also no effect
	BMI	PBL	M and F, middle-aged (n=228)	↔ [36]	
	BMI	PBL	M and F, adults (n=165)	↓ [37]	In multivariate linear regression
	BMI	Lymphocytes	F, obese, premenopausal (n=173)	↔ [38]	
	BMI	Placenta	F, pregnant (n=50)	↑ [40]	
	BMI	Cord blood	F, pregnant (n=50)	↔ [40]	
	BMI	Colon	M and F, middle aged, previous adenoma (n=388)	↔ [39]	
	Annual	PBL	M and F, school children (n=553)	↓ in M, ↔ in	
	Intra pair difference in BMI	Muscle	M and F, monozygotic twin pairs discordant for T2DM (n=11)	↑ [84]	Intra-pair differences in methylation associated with intra-pair differences in BMI
	BF %	PBL	M and F, middle-aged overweight (n=161)	↔ [33]	
	BF %	PBL	F, premenopausal (n=470)	↓ [32]	Only in presence of low folate
ALU					
	BMI	WB	F, postmenopausal (n=323)	↓ [41]	
	BMI	PBL	M and F, Chinese (n=286)	↑ [43]	Combined measure of methylation in ALU and SAT2 repetitive elements
	BMI	PBL	M and F, adults (n=1254)	↔ [34]	Combination of 4 studies, in separate study populations also no association
H3K4me2					
	BMI	Adipocytes	F, elderly (n=14 lean, n=19 overweight)	↓ [9]	Decrease of 40%
H3K4me3					
	BMI	Adipocytes	F, elderly (n=14 lean, n=19 overweight)	↔ [9]	No change in obese subjects, but increase in obese/diabetic subjects
H3K9me2					
	BMI	Adipocytes	F, elderly (n=14 lean, n=19 overweight)	↔ [9]	

Abbreviations: %5meC, Percentage 5-methylcytosine; BF%, Body Fat Percentage; H3K4me2, histone H3 lysine 4 dimethylation; H3K4me3, histone H3 lysine 4 trimethylation; H3K9me2, histone H3lysine 9 dimethylation; PBL, Peripheral blood leukocytes; T2DM, type 2 diabetes mellitus.

* ↑ Positive association with DNA methylation and health outcome. ↓ negative association with DNA methylation and health outcome. ↔ no association with DNA methylation and health outcome

Table 2 Specific gene methylation and obesity: Candidate gene and genome wide approaches

Study design and reference	Population	Tissue type	Methylation sites/ method	Main findings
Candidate gene approaches				
Comparison of subjects with low vs high central adiposity. Association with BF% and BMI [51]	Lean young women (n=40)	PBL	*TNFA* promoter, 20 CpGs/ Sequenom EpiTyper MassARRAY	Higher methylation levels of 2 CpGs in subjects with low compared to high central adiposity (7.1±1.8% vs 4.7±1.6 %, P<0.001). Higher methylation levels in these CpGs were also associated with lower BMI (r=-0.38, P=0.015) and BF% (r=-0.34, P=0.034)
Association with BMI [53]	Children 17 months (n=120)	WB	*LEP* , 10 CpGs in 7 CpG units / Sequenom EpiTyper MassARRAY	A 0.8% decrease in overall absolute *LEP* methylation level was associated with a 1.3 kg/m^2 increase in BMI (P=0.043)
Association with obesity characteristics [60]	Adolescents (n=315)	WB	*IGF2/H19* ICR 12 CpGs in 6 CpG units/ Sequenom EpiTyper MassARRAY	A 5.1% (males) and a 5.0% (females) increase in average *IGF/H19* methylation level was associated with a 2.5 mm (males) and a 2.0 mm (females) increase in abdominal skin fold thickness (P=0.04 to 0.001). Average methylation level was also associated with subcutaneous adiposity (P=0.023)
Comparison lean, overweight/obese and morbidly obese subjects. Association with obesity characteristics [55]	Lean (n=20), overweight/obese (n=20) and morbidly obese (n=20) adult women	PBL	*CLOCK* (39 CpGs, 2 regions), *BMAL1* (26 CpGs), *PER2* (27 CpGs)/ Sequenom EpiTyper MassARRAY	Lean individuals, compared to morbidly obese individuals, had different methylation levels in multiple CpGs in the *CLOCK* gene (CpG1: 2.4±0.6% vs 1.1±0.2%, CpG5-6: 17.4±0.9% vs 12.4±0.7%, CpG8:10.8±0.8% vs. 8.7±0.8%, CpG 11-14 (49.5±2.7% vs 59.5±4.0%)), and lower methylation levels in 6 CpGs in *BMAL1* . Associations between methylation levels of multiple CpGs in *CLOCK, BMAL1* , *PER2* and BMI, BF% and WC (r=-0.440 to 0.531, P=0.042 to <0.001)
Association with obesity characteristics [59]	Adults (n=34)	PBL	*HSD2, GR, IGF2* (DMR0, DMR2) and *H19* (ICR)/ Pyrosequencing	Positive association of methylation at several CpGs within *HSD2, GR* and *IGF2* and WC and BMI (partial r= 0.42 to 0.57, P= 0.007 to 0.04)
Association with BMI [37]	Adults (n=165)	PBL	*IL-6* promoter, 6 CpGs/ Pyrosequencing	No association found
Comparison lean and obese [54]	Lean (n=36) and obese (n=71) adolescents. Validation in 2nd cohort of children (n=154)	WB	*POMC* , 2 CpG islands in 5'-promoter region and around the intron 2 and exon 3 boundary/bisulfite sequencing	A lower overall methylation score in lean compared to obese adolescents (25% vs 40%, P<0.001) in a region adjacent to an intronic Alu element in the *POMC* locus. Highest difference between groups at a CpG at position +1 (methylation in 5% of lean children, but in 55% of obese children)
Association with obesity characteristics [56]	MZ middle-aged twin pairs (n=84)	PBL	*SLC6A4* promoter region, 20 CpGs/pyrosequencing	On average, a 1% increase in mean methylation level was associated with a 0.33 kg/m^2 increase in BMI (95% CI: 0.02-0.65; P=0.03) and 0.78cm increase in WC (95% CI: 0.05-1.50; P=0.03). Intra-pair differences in methylation level were correlated with intra-pair differences in BMI and WC at multiple CpGs.
Association with BMI for different alleles *MCHR1* [57]	Adults (n=39)	WB	*MCHR1* , 2 CpGs in SNPs and 15 additional CpGs/ Bisulfite specific PCR	Methylation status of the GT allele is negatively associated with BMI (r=-0.814, P=0.024)
Comparison MZ twins discordant for BMI [121]	MZ twins (n=16) adults, discordant for BMI	Saliva	20 CpGs in 9 growth-related imprinted regions/single nucleotide extension on bisulfite converted DNA (MS-SNuPE)	Mean methylation indexes were very similar among co-twins, no associations of intrapair methylation and BMI differences
Association with BMI [58]	Elderly men (n=170)	PBL	*AR* promoter, 8 CpGs in 5 CpG units/ Sequenom EpiTyper MassARRAY	Methylation status of 1 CpG unit in *AR* promoter was positively associated with BMI (r=0.24, P<0.05) and BF% (r=0.16, P<0.05) after adjustment for cell type variation in methylation
Association with BMI [52]	Obese (n=8) and matched lean (n=9) women	Muscle	*PGC1* α and *PDK4* promoter region/Bisulfite sequencing	Methylation levels of *PGC1* α were positively correlated (r=0.423, P=0.048), and methylation levels of PDK4 were negatively correlated (r=-0.707, P<0.001) with BMI
Genome wide approaches				
Association with BMI and WC [122]	Adults (n=183), families, 59% obese	WB	1505 CpGs/ llumina GoldenGate Methylation Cancer Panel I	Without multiple testing corrections 27 CpGs were nominally associated with WC and 12 CpGs with BMI, some in genes with role in obesity and diabetes. None significant after multiple testing corrections
Comparison lean and obese [62]	Lean (n=48) and obese (n=48) adolescents. Sample split into discovery and validation set	PBL	>480,000 CpGs /Illumina Infinium HumanMethylation450 BeadChip	Differential methylation in 23,305 CpGs between lean and obese, Top ranked differentially methylated site mean β value of 0.15 in lean, in obese 0.18. Differentially methylated sites identified in discovery set can predict presence obesity in validation set.
Comparison lean and obese [61]	Lean (n=24) and obese (n=23) preadolescent girls	WB	27,000 CpGs/ Illumina Infinium HumanMethylation27 BeadChip	Differential methylation in 20 sites between the lean and obese group, multiple sites in/near transcriptional regulator genes. Percentage change in obese relative to lean from -16.7 to 15.5 % (P=0.007 to 0.04)

Comparison lean and obese [63]	Lean (n=7) and obese (n=7) adolescent males Validation in 2nd cohort (n=92)	PBL	27,000 CpGs/ Infinium HumanMethylation27 BeadChip, validation of 6 CpGs by pyrosequencing	No significant differentially methylated sites after multiple testing corrections. Selection of highest 6 differentially methylated sites for validation. In validation step, methylation status of 1 in *UBASH3A* was 3.3% higher (P=0.002) and in the *TRIM3* was 1.21% (P <0.001) lower in the obese versus the lean group and significant after adjustment for age.
Association with BMI at 2 time points 11 yrs apart [64]	Adults (n=74)	Lymphocytes	4 million CpGs/ CHARM with custom designed NimbleGen HD2 microarray	Covariation with BMI for variable methylated regions in *MMP9* (β-coefficient b=11.6, P=0.007), *PM20D1* (b=7.6, P=0.003), *PRKG1* (b=11.8, P=0.013) and *RFC5* (b= -11.8, P=0.018), also at 2nd time point. Variable methylated regions in or near 9 other genes only correlated with BMI at 1st time point.

Abbreviations: BF%, Body Fat Percentage; CHARM, comprehensive high-throughput arrays for relative methylation; CpGs, cytosine-phosphate-guanine sites; DMR, differentially methylated regions; FDR, False Discovery Rate; ICR, Imprinting Control Region; MZ, monozygotic; PB, peripheral blood; PBL, peripheral blood leukocytes; WB, whole blood; WC, waist circumference

Table 3 Intervention studies

Study design and reference	Population	Tissue type	Methylation sites/method	Main findings
Comparison of methylation before and after 6 months exercise intervention [65]	Healthy, overweight men (n=23)	SAT	>450,000 CpGs/ Illumina Infinium HumanMethylation450 BeadChip	After exercise intervention changes in methylation (ranging from 0.2-10.9%) in 17,975 CpGs in 7,663 unique genes, including 18 obesity candidate genes.
Comparison of methylation before and 6 months after gastric bypass surgery [52]	Obese (n=8) and matched lean (n=9) women, obese men (n=6)	Muscle	In women *PGC1* α and *PDK4* promoter region and 14 other genes. In men genome-wide methylation analysis /Bisulfite sequencing, and methyl-CpG binding protein-based system for methylated DNA enrichment	After weight loss surgery promoter methylation of *PGC-1α* decreased (~2.2% to ~0.8%) and of *PDK4* increased (~0.3% to ~1.1%) in obese women, to levels comparable with lean women. Among the 14 metabolic genes analysed, promoter methylation of 11 genes was normalized to levels in lean individuals. In men, 409 differentially methylated regions after weight loss surgery.
Comparison of methylation between low and high responders to 10 wk diet and exercise weight loss intervention [67]	Overweight and obese adolescents (n=24). Validation in n=107	WB	>27,000 CpGs/ Illumina Infinium HumanMethylation27 BeadChip27k, validation using Sequenom EpiTyper MassARRAY	Differential methylation in 97 CpGs between the low and high responders (absolute methylation difference >5%, P <0.05). After validation, 5 DMRs in or near *AQP9, DUSP22, HIPK3, TNNT1* , and *TNNI3* showed methylation differences between the high and low responders and/or correlated with changes in weight
Association between baseline methylation and weight loss after 16-wks weight loss intervention [55]	Lean (n=20), overweight (n=20) and obese (n=20) women	PBL	CpGs *CLOCK, BMAL1, PER2* / Sequenom EpiTyper MassARRAY	Weight loss was associated with baseline methylation levels of *CLOCK* CpG 1 (r=0.377, P=0.01) and *PER2* CpGs 2-3 (r=0.318, P=0.016) and CpG 25 (r=0.318, P=0.016)
Comparison of methylation before and after weight loss due to bariatric surgery [123]	Severely obese (n=8)	SAT	305 bp region in *LEP* promoter, 31 CpGs/ clonal bisulfite sequencing	No change in methylation level
Comparison of baseline and after intervention methylation levels between high responders and low responders to 8 wk caloric restriction [69]	Obese women (n=27)	SAT	*LEP* and *TNFA* promoter methylation/ Methylation specific PCRs	At baseline, responders to the weight loss intervention showed lower promoter methylation levels of *LEP* than the non-responders (~18% vs ~33%, P=0.017). No change in methylation of *LEP* or *TNFA* after intervention.
Comparison of baseline and after intervention methylation levels between responders and low responders to 8 wk caloric restriction [66]	Overweight or obese men (n=6 high responders, n=6 non responders). Validation in the same subjects and n=13 extra	PBMC	>27,000 CpGs/ Illumina Infinium HumanMethylation27 BeadChip, validation using Sequenom EpiTyper MassARRAY	At baseline, 1034 differentially methylated CpGs between high and low responders. Validation of CpGs *ATP10A* (3-9% difference) and *CD44* (8% difference). Changes in methylation of 170 CpGs as result of intervention. Validation of increases in methylation CpGs in WT1 and ATP10A, change in methylation associated with change in fat mass and BMI.
Comparison of baseline and after intervention methylation levels between high and low responders to 6 months caloric restriction [68]	Overweight/obese premenopausal women, (n=7 high responders, n= 7, low responders)	SAT	14,923 CpGs/ Human CpG-island 8.1K array and 6800 additional CpG island loci, validation using Sequenom EpiTyper MassARRAY	At baseline, 35differentially methylated CpGs between high and low responders (fold change -1.32 to 1.44). After intervention 3 CpGs differentially methylated (fold change 1.18 to 1.38). Some differentially methylated genes involved in weight control, insulin secretion and in imprinted genomic regions

Abbreviations: CpGs, cytosine-phosphate-guanine sites; FDR, false discovery rate; PBL, peripheral blood leukocytes; PBMC, peripheral blood mononuclear cells; SAT subcutaneous adipose tissue; WB, whole blood; WC, waist circumference

Table 4 DNA methylation at early life; effect of interventions and association with later life obesity

Study design and reference	Population	Tissue type	Methylation sites	Main findings
Parental BMI and early life DNA methylation				
Association of methylation in children with maternal BMI [44]	School children (n=533)	PBL	Global (LINE1)/ pyrosequencing	Children in the lowest category of maternal BMI (<18.5 kg/m^2) had notably lower DNA methylation than those in the other three BMI categories (79.88% ±0.66 vs 80.27% ± 0.63, 80.27% ±0.67 and 80.29% ±0.71, P=
Association of methylation in cord blood with maternal BMI [74]	Mother and child pairs (n=319)	Cord blood	Global (LINE1)/ pyrosequencing	No association found between maternal prepregnancy BMI and LINE1 methylation
Association of methylation in placenta with maternal BMI [75]	Mother-child pairs (n=50)	Placenta	*IGF2* (DMR0, DMR2) and H19 (DMR) 19 CpGs/ pyrosequencing	No association found with maternal BMI
Association of methylation in newborns and obesity of father [73]	Newborns (n=79)	Cord blood	DMRs at *IGF2* and *H19*/ pyrosequencing	Methylation at the *IGF2* DMR was negatively associated with paternal obesity (b= -5.28, P = 0.003)
Parental intervention and DNA methylation				
Comparison of methylation in children born before and after maternal bariatric surgery [76 77]	Mothers (n=20), Siblings born before (n=25) and after (n=25) surgery, age 2-24 yrs	PBL	>480,000 CpGs,Infinium HumanMethylation450 BeadChip, validation using Sequenom EpiTyper MassARRAY	Children born before, compared to those born after surgery, had higher weight and WC and 14,466 differentially methylated CpGs in 5698 genes, involved in glucose regulation, vascular disease, immunity/inflammation. Validation of CADPS2 (Δ b=0.09), LAMC3 (Δ b=0.07) and SHANK2 (Δ b=0.06)
Early life DNA methylation and later life adiposity				
Association of methylation at birth with adiposity at age 10 [80]	Children (n=178)	Cord blood	29 genes, 1–3 sites per gene Selection based on gene expression comparison of lean and obese children/ Illumina GoldenGate Cancer Panel I array	Methylation in *CASP10, CDKN1C, EPHA1, HLA-DOB, NID2, MMP9, MPL* was associated with body composition at age 9 yrs. No association remained significant after multiple testing corrections.
Association of methylation at birth and childhood with body composition in childhood [81]	Cohort 1: children born preterm (n=91) Cohort 2: children, born term (n=131)	Cohort 1: WB at age 11yrs. Cohort 2: cord blood and WB at age 7 yrs	7 CpGs in *TACSTD2*/ pyrosequencing	In cohort 1, mean *TACSTD2* methylation was negatively correlated with fat mass at age 11 (r=-0.22, P=0.037). In cohort 2, *TACSTD2* methylation in cord blood was positively correlated (r=0.20, P=0.040) with fat mass at age 15, similar pattern at age 7 yrs, no association at age 9 yrs
Association of methylation at birth with adiposity at age 9 [79]	Children (n=78), Validation in 2nd child cohort (n=239)	Cord tissue	5 candidate genes (*RXRA eNOS, SOD1, IL8, PI3KCD*), 68 CpGs/ pyrosequencing	Sex-adjusted childhood fat mass was associated with methylation in *RXRA* (exponentiated b= 17% per SD change in methylation, 95% CI: 4–31, P = 0.009) and in *eNOS* (exponentiated b = 20%, 95% CI: 9–32, P = 0.001) at birth. *RXRA* methylation and sex explained 26% of the var iance in childhood fat mass. Validation of association between *RXRA* methylation and fat mass in 2nd cohort.
Association of methylation at birth with weight for age at 1 yrs[78]	Children (n=204)	Cord blood	*IGF2* DMR 3 CpGs, *H19* DMR 4 CpGs/ pyrosequencing	Methylation level of *H19* DMR at birth is 3.4% higher in overweight or obese children compared to normal weight children at age 1 yrs (P= 0.003)

Abbreviations: CpGs, cytosine-phosphate-guanine sites; DMR, differentially methylated region; PBL, peripheral blood leukocytes; WB, whole blood