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Predictive Epigenetic Biomarkers of Adiposity

by

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ABSTRACT

FACULTY OF MEDICINE
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Doctor of philosophy

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The early life environment, acting via epigenetic processes, can influence the risk of future obesity. Altered DNA methylation that is induced in early life that associates with future adiposity has the ability to predict individuals at an increased risk of becoming obese. There is little evidence about the effect of development and puberty during childhood on the stability of DNA methylation, and whether altered DNA methylation can be related to future phenotype. Therefore, the experiments in this thesis were completed in order to address these issues.

In order to investigate the effect of increasing age in childhood and puberty on the stability of DNA methylation, pyrosequencing was used to measure the methylation of specific CpG loci in genes that have been suggested to be important in the development of obesity. DNA was extracted from peripheral blood collected annually between 5 and 14 years from a subset of children (n=40, 20 boys, 20 girls) from the EarlyBird cohort. Peripheral blood is a heterogeneous mix of blood cell populations, which is a confounder when measuring DNA methylation. Therefore, any CpG loci where the methylation was significantly associated with the proportion of neutrophils and lymphocytes were excluded from further analyses.

Specific loci in the Sirtuin 1 (SIRT1) and Peroxisomal Proliferator Activated Receptor Gamma Coactivator 1 Alpha (PGC1α) promoters that exhibited temporal stability also predicted future adiposity, with methylation at 5-7 years in peripheral blood predicting percentage body fat up to 14 years, after adjusting for age, gender, physical activity and pubertal timing. The methylation of these loci in the children was not predicted by the methylation of these loci in the mother or the father in peripheral blood.

Electrophoretic mobility shift assays were used to exemplify the effect of differential methylation at one of the CpG loci that predicated future adiposity on transcription factor binding. Methylation of CpG -783 in the PGC1α promoter enhanced the binding of the pro-adipogenic HOXB9-PBX1 heterodimer, consistent with a positive relationship between methylation of this locus and adiposity. Loss of methylation resulted in PBX1 binding with an unidentified partner. Co-transfection of PBX1 with the unmethylated PGC1α promoter resulted in decreased gene expression, suggesting that PBX1 may negatively regulate PGC1α gene expression.

In order to determine if the methylation of these predictive markers in SIRT1 and PGC1α were also able to predict future adiposity when measured at birth, pyrosequencing was used to measure the methylation in fetal tissues. DNA was extracted from umbilical cord (n=440) and umbilical cord blood (n=300) from the Southampton Women's Survery (SWS) cohort. The methylation of SIRT1 and PGC1α at birth was associated with several measures of adiposity in childhood including fat mass, lean mass and skinfold thickness, suggesting that the methylation of these marks may be determined *in utero*. Furthermore, the methylation of three loci in the PGC1α promoter was increased in the subcutaneous adipose tissue of obese subjects (n=42) compared to lean subjects (n=23) from the BIOCLAIMS cohort, and associated with BMI, % fat and waist circumference.

The expression PGC1α was decreased in human liposarcoma cells supplemented with α-linolenic acid *in vitro*, however there were no changes in methylation of the seven CpG loci measured within the promoter. This suggests that fatty acid supplementation may not influence the methylation of these predictive epigenetic biomarkers *in vivo*.

Taken together, the findings of these studies suggest that the methylation of SIRT1 and PGC1α can predict future adiposity, and if measured at birth or during early childhood may have utility in identifying individuals who would benefit from targeted preventive mechanisms.

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Academic Thesis: Declaration Of Authorship

I, Rebecca Clarke-Harris, declare that this thesis entitled

Predictive Epigenetic Biomarkers of Adiposity

and the work presented in it are my own and has been generated by me as the result of my own original research.

I confirm that:

1. This work was done wholly or mainly while in candidature for a research degree at this University;

2. Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;

3. Where I have consulted the published work of others, this is always clearly attributed;

4. Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;

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6. Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;

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Signed: ……

Date: ……

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Now onto the next adventure…

Abbreviations

Abbreviation	Full Term
5-mC	5-Methyl Cytosine
10-H=DA	10-Hydroxy-2E-Decenoic Acid
10-HDA	10-Hydroxydecanoic Acid
ALA	α-Linolenic Acid
ALSPAC	The Avon Longitudinal Study of Parents and Children
ANOVA	Analysis of Variance
APHV	Age at Peak Height Velocity
ATP	Adenosine Triphosphate
BIOCLAIMS	Biomarkers of Robustness of Metabolic Homeostasis for Nutrigenomics-Derived Health Claims Made on Food
BMI	Body Mass Index
ChIP	Chromatin Immunoprecipitation
COV	Coefficient of Variance
CpG	Cytosine-Phosphate-Guanine
CT	Computed Tomography
DHA	Docosahexaenoic Acid
DNA	Deoxyribonucleic Acid
DXA	Dual-Energy X-Ray Absorptiometry
DNMT	DNA Methyltransferase
dNTPs	Deoxynucleotide Triphosphates
DOHaD	Developmental Origins of Health and Disease
EMSA	Electrophoretic Mobility Shift Assay
EPA	Eicosapentaenoic Acid
EZH2	Enhancer of Zeste Homolog
FTO	Fat Mass and Obesity Associated Gene
G6P	Glucose 6 Phosphate
GCK	Glucokinase
GLUT	Glucose Transporter Type
GR	Glucocorticoid Receptor
GWAS	Genome Wide Association Studies
HNF1α	Heptocyte Nuclear Factor 1 Alpha
HNF1β	Heptocyte Nuclear Factor 1 Beta
HNF3β	Heptocyte Nuclear Factor 3 Beta
HNF4α	Heptocyte Nuclear Factor 4 Alpha
HATs	Histone Acetylases
HDACs	Histone Deacteylases
HDMs	Histone Demethylases
HMTs	Histone Methyltransferases
HOMA-IR	Homeostasis Model Assessment of Insulin Resistance
HOXB9	Homeobox b9
IGF2	Insulin-like Growth Factor 2
IL10	Interleukin 10
INSR	Insulin Receptor
IRS1	Insulin Receptor Substrate 1
IRS2	Insulin Receptor Substrate 2
IUGR	Intrauterine Growth Restriction

K	Lysine
KCNJ11	Potassium Inwardly-rectifying Channel, Subfamily J11
LA	Linoleic Acid
lincRNA	Large Intergenic Non-Coding RNA
MeCP2	Methyl CpG Binding Protein 2
MeDIP	Methylated DNA Immunoprecipitation
miRNA	Micro RNA
mRNA	Messenger RNA
MODY	Maturity Onset Diabetes of the Young
MRI	Magnetic Resonance Imaging
MVPA	Moderate-to-Vigorous Physical Activity
N-3	Omega-3
N-6	Omega-6
NAD	Nicotinamide Adenine Dinucleotide
NEFA	Non-Esterified Fatty Acids
NTC	No Template Control
OA	Oleic Acid
PA	Palmitic Acid
PBX1	Pre B Cell Leukemia Homeobox 1
PCR	Polymerase Chain Reaction
PDX1	Pancreatic and Duodenal Homeobox 1
PGC1α	Peroxisomal Proliferator Activated Receptor Gamma Coactivator 1 Alpha
PI3K	Phosphatidylinositol 3 Kinase
piRNA	PIWI-Interacting RNA
POMC	Proopiomelanocortin
PPARα	Peroxisomal Proliferator Activated Receptor Alpha
PPARγ	Peroxisomal Proliferator Activated Receptor Gamma
PPRE	Peroxisomal Proliferator Response Element
PUFA	Polyunsaturated Fatty Acids
R	Arginine
RAR	Retinoic Acid Receptor
RXRα	Retinoid X Receptor alpha
RNA	Ribonucleic Acid
SAM	S-Adenosyl Methionine
SD	Standard Deviation
SEM	Standard Error of the Mean
siRNA	Small Interfering RNA
SIRT1	Sirtuin 1 (Silent Mating Type Information Regulation 2 Homolog 1)
SNP	Single Nucleotide Polymorphism
SnRNA	Small Nuclear RNA
SWS	Southampton Women's Survey
T2D	Type 2 Diabetes Mellitus
TSS	Transcription Start Site
WAT	White Adipose Tissue
WHO	The World Health Organisation

Chapter 1

Introduction

1.0 Introduction

The worldwide prevalence of obesity has rapidly increased over the past thirty years. In 2008, The World Health Organisation (WHO) stated that 10% of men and 14% of women were obese worldwide, classified by a body mass index (BMI) of more than 30 kg/m^2. Being overweight or obese increases the risk of developing many metabolic diseases including hypertension, insulin resistance and type 2 diabetes mellitus (T2D), and also increases mortality rates (1)(2)(3).

The rate of childhood obesity is increasing at an alarming rate globally. In 2007, Hossain et al. estimated that 155 million children worldwide were overweight or obese, and with these numbers increasing annually is now a worldwide health concern (4). Childhood obesity is associated with several short and long term health consequences including metabolic disturbances and learning difficulties. Parental obesity is known to increase the risk of childhood obesity, which is a major concern as this generates a constant cycle of obesity (5).

Whilst genetics is known to contribute to the development of obesity, there is also evidence to suggest that the environment throughout the life course may contribute to the onset of disease (3)(6). For example, the western lifestyle involves decreased physical activity and energy intake in excess of requirements, which has coincided with the rapid rise in obesity in adults and children (7). Further understanding of the aetiology of obesity is required in order to improve human health worldwide. Therefore, the overall aim of this project was to identify epigenetic biomarkers of adiposity, which may be beneficial for predicting individuals at an increased risk of developing obesity later in life.

1.1 Causes of Obesity

1.1.1 Adipose tissue

Obesity is broadly defined as an increase in adipose depots as a result of an imbalance of energy intake and energy expenditure (8). It is now well understood that the type, amount and distribution of body fat are important in the development of obesity related diseases. There are 2 types of adipose tissue; brown adipose tissue and white adipose tissue, which differ in their biological function. Brown adipose tissue contains a large amount of mitochondria and is important in thermogenesis (9). White adipose tissue is important in storing excess fat as triglycerides known as lipogenesis, and is an endocrine organ which releases hormones called adipokines that have effects on many process throughout the entire body (10). Altered adipokine levels such as adiponectin are associated with obesity in humans (11). White adipose tissue can be further subdivided into subcutaneous adipose tissue and visceral adipose tissue, which differ in their location and biological function. Visceral adipose tissue surrounds the inner organs, whereas

subcutaneous adipose tissue is located under the skin in the abdomen, lower body, and intra muscularly (9). Visceral adipose tissue undergoes lipolysis more readily than subcutaneous adipose tissue and has a greater impact on development of obesity related diseases such as insulin resistance (9).

Adipose tissue mass is determined by the formation of mature adipocytes via differentiation, known as hyperplasia, and by the accumulation of excess fat increasing adipocyte size, known as hypertrophy (12). The accumulation of excess fat in adipose tissue leads to inflammation as a result of increased recruitment of macrophages, which secrete inflammatory cytokines such as tumor necrosis factor α (TNFα) and interleukin 6 (IL6) (10). Eventually mature adipocytes become so enlarged that they are unable to store or oxidise excess lipids and excess fat is stored in non-adipose tissue such as muscle, liver and heart, known as lipotoxicity (8). Therefore, increasing the number of adipocytes, rather than the size might be beneficial in the prevention of lipotoxicity, inflammation and insulin resistance.

In humans, it has been suggested that the stage in development when increased preadipocyte proliferation occurs is important in determining the amount of adipose tissue that is present in adulthood (13). Preadipocyte proliferation is highest between 1 year of age and puberty, and increased proliferation during childhood can have an impact on long term health (14)(15). For example, a longitudinal study by Knittle et al. showed that obese adults had an earlier increase in adipocyte cell number and size during childhood, suggesting that childhood obesity increases the risk of obesity later in life (15).

1.1.2 Determining obesity in children and adults

There are several techniques that are widely used to evaluate obesity risk, which vary in complexity, ease of use, accuracy and reliability. Obesity is often defined by BMI, where adults with a BMI of more than 25kg/m^2 are classified as being overweight, whereas adults with a BMI of more than 30kg/m^2 and 35kg/m^2 are classified as obese and morbidly obese, respectively (7). Whilst the BMI cut off points that define overweight or obesity in adults are not affected by age or sex, using BMI to determine obesity in children has been criticised because in children BMI varies with both age and sex (16). BMI increases substantially with age in children, with a steep increase during the 1st year of life, followed by a slower increase up to 6 years, and a period of increase again during adolescence (17). Another drawback to using BMI to define childhood obesity is that puberty occurs earlier in females, which is a period in development where adiposity is known to increase (18). Therefore, BMI in children is compared to a reference-standard which takes into account age and sex, and is known as a BMI z-score (16). BMI z-scores

are often converted into BMI-for-age percentiles using growth charts, which can be used to track relative weight throughout childhood. There is also conflicting data for determining the age that should be used as a cut off point for defining if it is childhood or adulthood obesity that is present (18). The WHO 2007 growth reference is used internationally and utilises BMI-for-age as a reference of defining childhood obesity from 5-19 years in order to overcome some of these caveats (19).

BMI is the most commonly used technique in adults for determining if a subject is overweight or obese and correlates highly with obesity related diseases such as T2D (20). However, BMI is a measure of weight (kg) divided by the square of height (m) and does not directly measure adiposity. BMI is dependent on fat and lean mass and provides no information on the type or distribution of fat (21)(16). There is evidence to suggest that central or abdominal obesity may be more detrimental to human health, rather than fat depots elsewhere in the body (22). Therefore, waist circumference and waist to hip ratio, which both measure central abdominal obesity, may be more useful for identifying those individuals at greatest risk of developing obesity related diseases. However, waist circumference and waist to hip ratio do not distinguish between subcutaneous and visceral fat, and waist to hip ratio relies on two measurements making this a less accurate and unreliable technique (20).

Measuring skinfold thickness at sites such as the subscapular, triceps and thighs, is a technique that provides information on the size of specific subcutaneous fat depots (20). Measuring skinfold thickness is a quick and easy method for determining regional adiposity and is suitable for use in children and adults, but does not provide any information on lean mass or visceral fat (20). Bioelectrical impedance determines percentage fat mass based on the rate at which an electrical current travels through the body and the amount of resistance, or bioelectrical impedance. However, bioelectrical impedance is sensitive to hydration status and varies for different ethnicities (16). Dual-energy X-ray absorptiometry (DXA) scans are commonly used to determine regional and total measurements of fat mass, lean mass and bone mass. DXA scans are advantageous as they accurately measure adiposity in children, however there is conflicting evidence on whether DXA scans are able to distinguish between subcutaneous and visceral fat (23)(24)(25). DXA scans determine visceral fat by deducting the amount of subcutaneous fat from the total abdominal fat, however to measure true visceral fat a magnetic resonance imaging (MRI) scan or computed tomography (CT) scan is required (20)(23)(24)(25).

Because each method for determining adiposity has advantages and disadvantages, it is unlikely that a single technique is suitable to measure obesity risk in all populations, particularly as the distribution of fat depots is known to differ with age, sex and ethnicity (26). Therefore,

using a combination of these techniques may be the best way to identify those individuals at greatest risk of becoming obese and developing obesity related diseases.

1.1.3 Role of fatty acids in the development of obesity

Fatty acids are important in many cellular processes including the formation of new tissues during fetal development (27). Fatty acids can be saturated, which do not contain any double bonds in the hydrocarbon chain; monounsaturated, which contain a single double bond; or polyunsaturated, which contain two or more double bonds. Polyunsaturated fatty acids (PUFAs) are further subdivided into the omega-3 (n-3) or omega-6 (n-6) families (28). In humans, there are 2 essential PUFAs; α-linolenic acid (ALA; 18:3n-3) and linoleic acid (LA; 18:2n-6), which cannot be synthesised *de novo* and must be consumed within the diet (29). Therefore, the level of PUFAs within the developing fetus is dependent on the dietary intake of the mother, and also the efficiency of placental transport (29). ALA and LA are known as the parents of the n-3 and n-6 families respectively, which undergo a series of desaturation and elongation steps in order to produce longer chain PUFAs.

Over the past few decades there has been a reduction in the consumption of saturated fats in the western diet, which have been replaced with plant based PUFAs as they are considered to be the 'healthier' option (28). However, these alternatives have higher levels of LA which has altered the ratio of n-3 to n-6 PUFAs and has been suggested to have contributed to the development of obesity. For example, Massiera et al. have shown that the offspring of mice fed a high fat diet rich in LA during pregnancy and lactation had increased body weight and fat mass, and increased adipocyte size, compared to the offspring of dams fed a high fat diet enriched in equal amounts of LA and ALA, suggesting that LA may promote hypertrophy of adipocytes (30). These differences persisted into adulthood, which suggests that PUFA intake during early life can influence the risk of obesity in adulthood.

Several studies in animals and humans have shown that dietary supplementation with n-3 PUFAs decreases adiposity, which may be the result of increasing the n-3 to n-6 ratio. For example, Flachs et al. have shown that adult male mice fed a diet where 44% of dietary lipids were replaced with an n-3 concentrate had a 22% reduction in body weight and 24% reduction in white adipose tissue mass after only 5 weeks, with no reduction in food consumption, compared to mice fed a high fat diet (31). Another study in humans has shown that n-3 dietary supplementation for 2 months reduces total fat mass and subcutaneous adipocyte diameter in women with T2D, compared to controls receiving a placebo (32). Therefore, dietary supplementation with n-3 PUFAs may be beneficial for decreasing the risk of obesity.

1.1.4 Obesity and insulin resistance

Obesity is a risk factor associated with the development of insulin resistance, with weight loss know to improve insulin resistance (33). These diseases are often present together and are considered the two greatest risk factors in the development of the metabolic syndrome (1)(2).

Insulin is a hormone that is produced by the pancreatic β cells and is essential for regulating glucose homeostasis in many tissues including the liver, skeletal muscle and adipose tissue (34)(35). Glucose stimulated insulin secretion from the β cells occurs when plasma glucose concentration increases, for example in response to eating. Glucose enters the β cell via the glucose transporter GLUT2, and becomes phosphorylated by glucokinase and enters glycolysis generating pyruvate (36). This results in an increase in adenosine triphosphate (ATP) and causes the ATP-sensitive potassium channels at the cell membrane to close (36). Depolarisation of the β cell opens the calcium channels and this influx of calcium into the cell increases the rate of insulin exocytosis leading to the release of insulin (36). Once released, insulin binds to the extracellular alpha subunits of the heterotetrameric insulin receptor found on the surface of target cells, which transmits a signal across the plasma membrane and activates the intracellular tyrosine kinase domain of the β subunit (34). The insulin receptor undergoes a series of phosphorylation reactions, whereby one β subunit autophosphorylates specific tyrosine residues on the adjacent β subunit, leading to activation of the receptor (35). Once activated, the insulin receptor phosphorylates the tyrosine residues of numerous substrates, such as insulin receptor substrate 1 and 2 (IRS1 and 2), known as the phosphorylation cascade. Phosphorylation of IRS1 and IRS2 creates a recognition site for the regulatory subunit of phosphatidylinositol 3 kinase (PI3K), leading to its activation (35). PI3K activity is essential for insulin-stimulated glucose uptake into skeletal muscle and adipose tissue for storage, which occurs via the translocation of the glucose transporter, GLUT4, to the membrane (35).

Insulin resistance occurs when normal insulin concentrations are insufficient to maintain glucose homeostasis, resulting in the gradual development of hyperglycaemia. Insulin resistance and β cell dysfunction often occur together, although it is unclear whether insulin resistance causes β cell dysfunction, or whether β cell dysfunction causes insulin resistance (33). One hypothesis is that insulin resistance precedes β cell dysfunction as a result of decreased sensitivity of the insulin receptors, which results in increased insulin secretion in order to maintain normal glucose concentration, leading to an eventual decline in β cell function (37). Another hypothesis is that β cell dysfunction occurs as a result of increased apoptosis and decreased regeneration of the β-cells, leading to a decrease in β-cell mass, β-cell exhaustion and eventually insulin resistance (33).

When energy demands increase, for example during fasting and exercise, triglycerides stored in adipose tissue are hydrolysed by hormone-sensitive lipase releasing NEFAs and glycerol, known as lipolysis (9)(2). NEFAs are then either oxidised in the mitochondria to generate ATP, or re-esterified into triglycerides in the liver where they are repackaged with cholesterol into lipoproteins for use in many cellular processes (38). Therefore, the release of NEFAs from adipose tissue is essential for maintaining many metabolic processes , however, when circulating NEFAs levels become too high they contribute to the development of peripheral insulin resistance (27)(2).

Obese subjects often have increased levels of NEFAs which occur as a result of reduced NEFA clearing via the mitochondria or re-esterification in the liver, and also increased NEFA release from enlarged adipocytes under oxidative stress (39). In 1963, Randle proposed that NEFAs compete with glucose to be the major energy substrate in muscle and adipose tissue, which results in decreased glucose uptake (27). NEFAs are taken up into skeletal muscle and are transported to the mitochondria, where they undergo β oxidation to produce acetyl CoA that enters the tricarboxylic acid (TCA) cycle in order to release ATP (40). However, accumulation of acetyl CoA inhibits pyruvate dehydrogenase, which in turn leads to an excess of glucose-6-phosphate (G6P) and inhibition of hexokinase (40). This reduces glucose uptake into the skeletal muscle via the glucose transporter GLUT4, leading to increased plasma glucose concentration and increased insulin secretion from the pancreatic β cells (8)(41)(42). Excess NEFAs also increase hepatic gluconeogenesis, which increases circulating glucose concentrations further and increases the demand for insulin secretion from the pancreatic β cells (27). Increased NEFAs cause lipotoxicity, which is the accumulation of fat in non-adipose tissue, which occurs in the pancreas, resulting in reduced insulin secretion and the development of insulin resistance (8)(9). Normally insulin inhibits lipolysis in adipose tissue, therefore decreased insulin secretion results in further NEFA release and a more insulin resistant state (2)(35). Taken together, this suggests that the ability of adipose tissue to store excess lipids is essential to prevent peripheral insulin resistance.

1.1.5 Obesity and genome wide association studies

One possible mechanism for the development of obesity is the presence of a predisposing polymorphism, which led to the development of genome-wide association studies (GWASs). GWASs have allowed thousands of samples from different populations to be compared leading to the identification of more than 800 single nucleotide polymorphisms (SNPs) associated with over 100 diseases, including several SNPs specifically associated obesity (43)(3)(44)(45). For example, a common SNP within the fat mass and obesity associated (FTO) gene is associated with increased

BMI and fat mass in European populations (44). Adults who were homozygous for the risk allele were on average 3kg heavier and had a 1.67 fold increase in the chance of being obese, compared to homozygotes without the risk allele (44). Furthermore, the presence of the risk allele associated with BMI from age 7 years and persisted into adulthood, suggesting that this SNP may predispose an individual to childhood and adult obesity. Another SNP has been identified in the melanocortin-4 receptor (MC4R) gene that is associated with BMI and fat mass in children and adults (45).

SNPs that are associated with obesity have also been identified within two genes described as master regulators of metabolism; Sirtuin 1 (SIRT1) and peroxisomal proliferator activated receptor gamma coactivator 1 alpha (PGC1α). For example, the presence of a minor allele within the SIRT1 gene was associated lower BMI in a Dutch population, with a 13-18% decrease in risk of obesity compared to subjects with the major allele (46). Furthermore, when subjects were followed up 6.4 years later, those with the minor allele had a smaller weight gain over time, compared to subjects with the major allele, suggesting a potential role in the prevention of obesity (46). Another SNP within the SIRT1 gene is associated with increased waist circumference, waist to hip ratio, total abdominal fat and visceral fat in men only, with no association with subcutaneous fat (47). This suggests that SNPs can influence the risk of obesity in a sex and tissue specific manner. A common polymorphism in the PGC1α gene, Gly482Ser, is associated with an increased risk of obesity and T2D in several populations (48)(49)(50).

A major disadvantage of GWAS is once a polymorphism is detected it becomes associated with the gene that is nearest in proximity, which may not be the gene that is influenced by the SNP and may not be responsible for the development of a particular phenotype such as obesity. Whilst GWAS has identified several genetic variants associated with an increased risk of obesity, they cannot solely account for the rapid rise in obesity over recent years. It is more likely that obesity occurs as a result of a combined interaction between genetics and the environment. Environmental factors such as diet and physical activity are known to be important in the development of obesity, and there is evidence to suggest that the prenatal and postnatal environment can influence the risk of developing obesity later in life.

1.1.6 Developmental origins of health and disease (DOHaD) and fetal programming

The developmental origins of health and disease (DOHaD) hypothesis suggests that the prenatal and early life environment can influence the risk of disease later in life. Environmental factors including maternal diet, gestational weight gain and smoking status have all been shown to influence the risk of obesity in the offspring. This is thought to occur as a result of 'fetal

programming' whereby the prenatal environment can induce permanent changes to the physiology and metabolism in the offspring, predisposing them to an increased risk of obesity (6).

Barker et al. were first to show that a lower birth weight correlates with an increased risk of coronary heart disease, using the Hertfordshire birth cohort, which has resulted in further investigation into the relationship between the prenatal and early life environment and its role in disease susceptibility later in life (51)(52)(53). Barker et al. showed there was an increased rate of death from ischaemic heart disease in lower birth weight babies (≤5lb), with the weight at one year predicting death from ischaemic heart disease (51). This was investigated further and it was discovered that birth length and head circumference provided further information on thinness at birth which is also associated with disease risk (6). These studies provide a proxy measurement of the intrauterine environment and can be useful to determine if growth has been compromised as the result of suboptimal maternal diet, placental insufficiency and other environmental factors such as smoking and stress.

The influence of maternal nutrition in the developmental origins of disease has been shown in studies of the Dutch Hunger Winter Famine, where daily calorie intake was reduced from 1800Kcal/day to between 800-400Kcal/day during world war II (54). Because the length of the famine was known, it was possible to look at the effect of the dietary restriction at different time points during gestation to determine relationships between the timing of the exposure and the incidence of disease in adult life (54). Individuals exposed to the famine during mid-gestation had reduced birth weight with an increased susceptibility to mental health problems, whilst those exposed during late gestation also had lower birth weight and remained small throughout adult life but had an increased risk of hypertension and insulin resistance (55). Children who had been exposed to the famine during early gestation only were a normal birth weight; however they had an increased susceptibility to obesity, T2D and cardiovascular disease (55). This suggests that this effect may be caused by a 'mismatch' between the prenatal and postnatal environments. For example, those children exposed to the famine during early gestation may have predicted a sparse diet and may have altered their growth and metabolism in response to poor nutrition *in utero*. This is known as the 'thrifty phenotype' whereby the fetus can adapt to environmental cues in order to have a phenotype that is most suited to aid survival (56). However, because they were born into an environment with a plentiful diet, there was a mismatch which resulted in an increased risk of obesity in these children.

These findings in humans have been further investigated and replicated using animal models where specific aspects of nutrition can be controlled. These animal studies have shown that both maternal under and over nutrition can induce persistent metabolic changes in the

offspring, that are associated with an increased risk of obesity (57). For example, the offspring of pregnant rats fed a protein restricted diet during late pregnancy and throughout lactation had increased abdominal fat depots, when compared to controls (58). Samuelsson et al. have shown that the offspring of mice fed a high fat diet (16%) and high sugar diet (33%) before and during pregnancy and lactation were heavier and had increased abdominal obesity, when compared to the offspring of mice fed a control diet (59). These changes persisted into adulthood and the mice went on to develop cardiovascular and metabolic dysfunction and altered gene expression (59). These studies support the DOHaD hypothesis and suggest that the diet during the prenatal and early postnatal life can induce persistent metabolic changes that increase the risk of obesity in later life.

There is increasing evidence that other factors besides maternal diet may be important in the development of obesity in the offspring. For example, excessive gestational weight gain is associated with higher BMI z-scores and increased subscapular and tricep skinfold thickness in children at 3 years of age (60). Gestational diabetes is associated with an increased risk of childhood obesity between 5 and 7 years of age, irrespective of gestational weight gain, maternal age and birth weight (61). It is also well known that parental obesity increases the risk of childhood obesity, suggesting that there might be a cycle of obesity that needs to be addressed (5).

Whilst the prenatal environment is crucial to the development of the fetus, the early postnatal environment is also important as this can continue to increase disease risk. Developmental plasticity is a normal process whereby a single genome can potentially generate several phenotypes, which is thought to aid survival by producing a phenotype that is best suited to the environment (53) . It has been suggested that developmental plasticity does not stop at birth and that the environment continues to influence disease risk throughout the life course. There is some evidence to suggest that plasticity decreases with age, whereas exposure to environmental challenges usually increases throughout the life course, which may in part explain the increased incidence of diseases such as T2D with age (62).

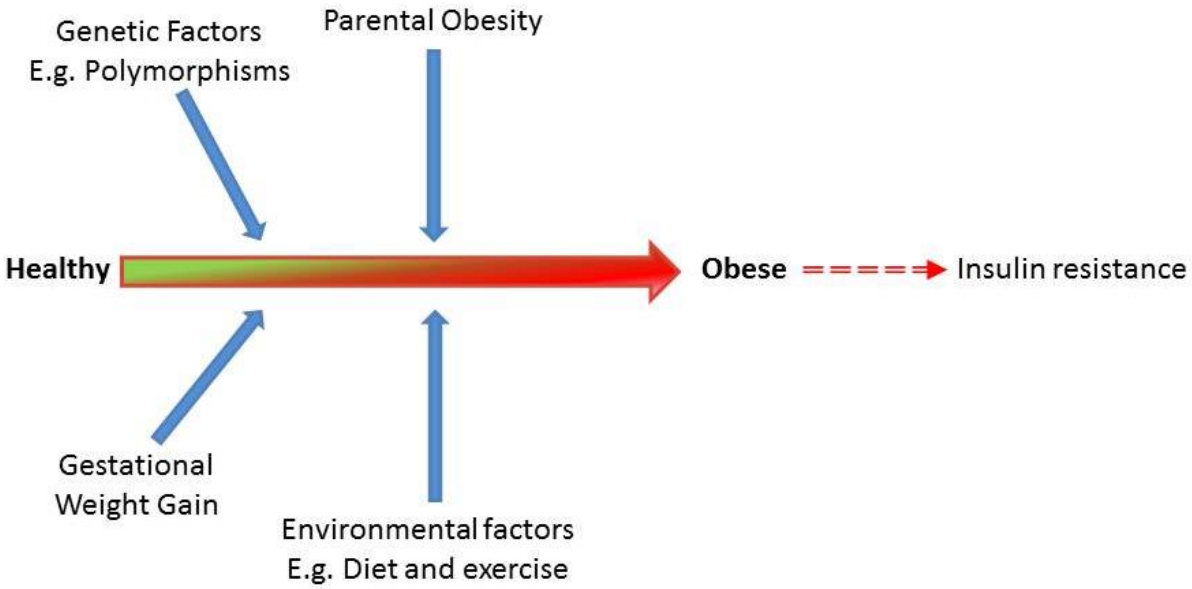


Figure 1.1- Factors involved in the development of obesity. This demonstrates how genetics and the early life environment can all influence the risk of obesity, and potentially insulin resistance, later in life.

1.1.7 Master regulators of metabolism

Sirtuin 1 (SIRT1) and peroxisomal proliferator-activated receptor gamma, coactivator 1 alpha (PGC1α) are often described as master regulators of metabolism. Both genes are expressed in a wide range of metabolic tissues where they play a key role in the regulation of many metabolic processes including adipogenesis, glucose homeostasis, muscle fibre-type switching and insulin secretion (10)(63)(64)(48)(65)(66).

SIRT1 is a NAD-dependent histone deacetylase which deacetylates a wide range of substrates including histones, transcription factors and corepressors (67)(68). SIRT1 is also known to deacetylate the coactivator PGC1α, which leads to its transcriptional activation (69)(70). PGC1α is then able to functionally interact with transcription factors such as peroxisomal proliferator activated receptor gamma (PPARγ), peroxisomal proliferator activated receptor alpha (PPARα) and hepatocyte nuclear factor 4 alpha **(**HNF4α**)** in order to respond to different stimuli in a tissue specific manner (69)(71).

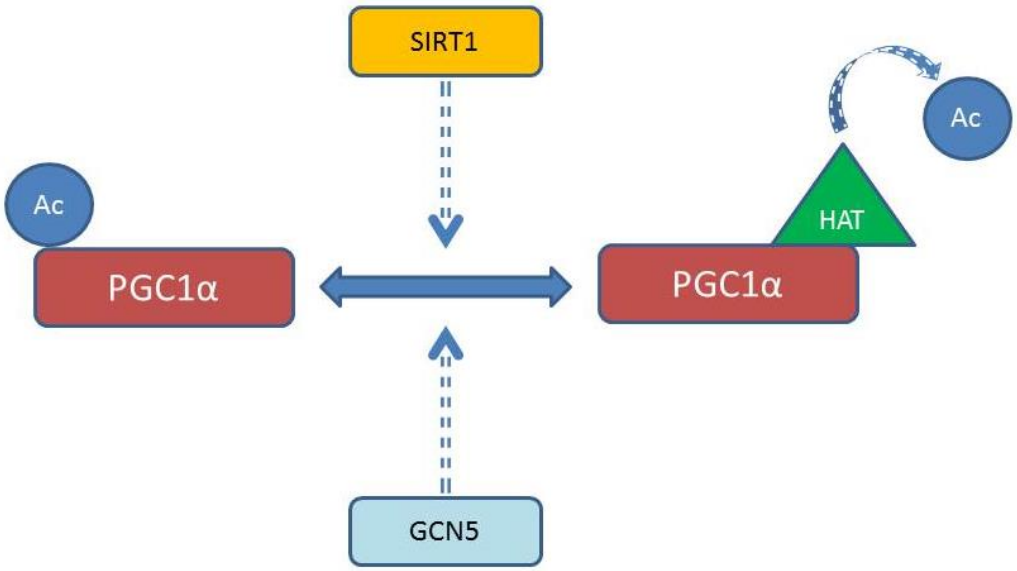


Figure 1.2- The regulation of PGC1α via SIRT1. SIRT1 deacetylates PGC1α which results in its activation. The acetyl group shown by 'Ac' is removed by a histone acetyl transferase (HAT). GCN5 leads to PGC1α acetylation and its deactivation, showing that this is a reversible process.

There is a substantial amount of evidence from *in vivo* and *in vitro* studies to suggest that nutrition, caloric restriction and exercise regulate SIRT1 and PGC1α expression in order to respond to changes in metabolic demand. Therefore, the environment during pre and postnatal life might be important in inducing persistent changes in gene expression.

Caloric restriction, defined as a 20-40% reduction in normal calorie consumption without malnutrition, is known to increase the expression of SIRT1, whereas overfeeding and high fat diets result in reduced SIRT1 expression (10)(72)(73)(74). In humans, caloric restriction has been shown to increase SIRT1 mRNA expression in abdominal subcutaneous adipose tissue, after as little as 6 days (73). It has been suggested that increased SIRT1 expression via caloric restriction may increase the life span, and SIRT1 is often described as the 'longevity gene' (10)(67).

SIRT1 and PGC1α play an important role in the metabolic response to fasting where hepatic expression is increased promoting gluconeogenesis and inhibiting glycolysis thereby increasing glucose concentration in the bloodstream (67)(68)(75)(70). During overfeeding, SIRT1 and PGC1α expression is decreased promoting glucose stimulated insulin secretion from the pancreatic β cells (46)(72)(68)(70).

SIRT1 is known to inhibit adipogenesis and animal models have shown that SIRT1 expression is decreased in adipose tissue in response to a high fat diet (74), whereas activation of SIRT1 by resveratrol protects against high fat diet induced obesity and insulin resistance (10)(46). SIRT1 is known to repress PPARγ, which increases fat mobilisation and lipolysis in adipocytes,

thereby reducing weight (63)(10). It has been shown that mice with SIRT1 knocked down specifically in adipose tissue, known as FKO mice, have increased subcutaneous and visceral fat when compared to wild type mice fed the same diet (76). The adipocytes from the FKO mice were enlarged and contained larger lipid droplets, when compared to adipocytes from the wild type mice (76). When wild type mice were fed a high fat diet for 12 weeks after weaning, they also had increased adiposity and mimicked the same response as the FKO mice, suggesting that a high fat diet can alter adipose tissue mass via the dysregulation of SIRT1 (76). In humans, SIRT1 expression has been shown to be inversely correlated with BMI and protein levels have been shown to be decreased in the subcutaneous adipose tissue of obese subjects, when compared to lean (72)(73). Taken together, these data suggest SIRT1 expression is decreased in adiposity.

PGC1α is known to play a key role in adipogenesis and energy homeostasis. Leone et al. have shown that PGC1α null mice have many metabolic abnormalities including increased adipose tissue mass, but with no difference in food intake when compared to control mice (77). Braun et al. have shown that pharmacological inhibition of PGC1α in preadipocytes *in vitro* results in increased fat storage during adipocyte differentiation, whereas pharmacological activation inhibited fat storage in preadipocytes (78). Activation of PGC1α in mature adipocytes *in vitro* resulted in increased fat mobilisation, suggesting that PGC1α negatively regulates fat storage in adipose tissue (78). Chen et al. have shown that PGC1α mRNA expression is decreased in the subcutaneous adipose tissue from obese subjects, compared to lean (79). Taken together, these data suggest that decreased PGC1α expression may be important in the development of obesity.

SIRT1 and PGC1α both play important roles in skeletal muscle and expression has been shown to be influenced by environmental factors. For example, SIRT1 mRNA expression increases in skeletal muscle following caloric restriction in mice and humans, which is associated with increased skeletal muscle glucose uptake (80)(81). PGC1α expression has been shown to be influenced by exercise. For example, Barrès et al. have shown that high intensity exercise is associated with an increase in gene expression after 3 hours, measured in skeletal muscle biopsies from healthy men (82).

Because SIRT1 and PGC1α are expressed in many tissues and involved in the regulation of many metabolic processes, environmental factors that influence their expression could potentially have huge effects on downstream processes. Therefore, these genes are targets for the potential prevention of obesity.

1.1.8 Childhood obesity and epidemiological studies

Childhood obesity is increasing worldwide and is a major public health concern as it is associated with long-term health issues including the metabolic syndrome and T2D (83). Epidemiological studies throughout childhood and adolescence are crucial to provide information on the aetiology of childhood obesity, in order to identify critical time points in development and risk factors associated with the development of disease.

The Avon Longitudinal Study of Parents and Children (ALSPAC) cohort is a longitudinal study investigating the health and development of 14,000 children in the UK (83). Data from the ALSPAC cohort shows that rapid growth during the first 2 years of life is associated with obesity at 5 years, determined by BMI and skinfold thickness (83). Data has also shown that parental obesity is a strong predictor of childhood obesity, which is increased further if both parents are obese (83). The Western Australian Pregnancy Cohort Study or the Raine cohort is a longitudinal study composed of 2868 subjects who were recruited when the mother was in the 16^{th} week of gestation, and were assessed at several time points between birth, during childhood, adolescence and into adulthood (84). Data from the Raine cohort has shown the time period when childhood obesity increases is between 3 and 8 years of age, suggesting this is a critical time in development when environmental factors can have an impact on the development of obesity (84). Data has also shown that 80% of overweight or obese children aged 6 years remain overweight or obese at 8 years, suggesting that these children may already be on a set trajectory determined during early life before the age of 6 years (84).

The Earlybird study is a longitudinal non-intervention study composed of 307 healthy children (137 girls and 170 boys) set up to answer several questions including 'is there an association between fat distribution and the development of insulin resistance?', 'are there sex differences?', 'does physical activity influence obesity and insulin resistance?' (85). Children were excluded if there was a family history of diabetes or any factors known to affect growth or body composition. Several measurements were taken annually between 5 and 16 years, including BMI and DXA scans in order to track potential changes that occur during childhood and puberty (85). The same parental measurements were also taken at the start of the study when the children were 5 years old, allowing for analysis of metabolic status a generation earlier (86). Parental obesity is a risk factor associated with childhood obesity. Data from the EarlyBird study has shown that at 5 years girls with overweight mothers had a higher BMI than boys with overweight mothers, and boys with overweight fathers had a higher BMI than girls with overweight fathers, suggesting there is a same-sex pairing effect (87). The daughters of obese mothers and sons of obese fathers gained excess weight between 5 and 8 years, whereas the daughters of normal

weight mothers and sons of normal weight fathers did not, suggesting these children may already be on a set trajectory (87). At 8 years, 41% of daughters of obese mothers were obese themselves, whereas 18% of sons of obese fathers were obese, suggesting that the effect is greatest in females during early childhood (87). It has also been shown that girls at 5 years have increased subcutaneous fat, BMI and waist circumference, when compared to boys, suggesting that females may be at greatest risk for childhood obesity, particularly if the mother of that child has a higher BMI (88). These results suggest that parental obesity may influence the risk of childhood obesity in a sex specific manner. The environment may be involved in the development of these sex specific associations as Y-linked traits are uncommon because the Y chromosome carries few genes (86). This study allows the temporal relationship between body mass and fat distribution be explored during childhood and puberty, and the effects of environmental factors such as physical activity to be determined during these potential critical periods in development. Therefore, this is a unique and novel cohort for identifying risk factors in childhood that may be associated with an increased risk of developing obesity and obesity related diseases in adulthood.

The Southampton Women's Survey (SWS) was established to measure the pre-pregnancy characteristics of 12, 579 women who were recruited during 1998-2002, between 20 and 34 years of age (89). The women were interviewed in order to gain information on body composition, diet, physical activity and lifestyle, including smoking status pre-conception (89). The women were monitored during pregnancy where ultrasound scans and blood samples were taken at various stages. Of these women, there were 2567 live births for which fetal tissues were collected, which included umbilical cord, umbilical cord blood and placental samples. The children had anthropometric measurements at birth, 6 months, and 1, 2 and 3 years of age, with a selection of children also having DXA scans at 4 and 6 years of age (89). Therefore, the SWS is unique because data was collected pre-conception, during pregnancy and after birth, allowing for examination of how the prenatal growth of a child interacts with their postnatal growth, and to determine risk factors that are associated with later health of the child. Findings from the SWS cohort have shown that several factors are associated with childhood adiposity. For example, breastfeeding is associated with lower fat mass at 4 years, compared to children who were never breastfed, suggesting diet during early postnatal life influences body composition in childhood (90). However, there was no association between breastfeeding and BMI of the children at age 4 years, showing the importance of using other measures of adiposity besides BMI for predicting high risk individuals. It has also been shown that children born to women with lower maternal vitamin D status during late pregnancy have increased fat mass at 4 and 6 years of age (91).

Data from the SWS cohort had also shown that maternal factors are associated with an increased risk of adiposity in the offspring. For example, children born to women who gained excessive weight during pregnancy and exceed the US Institute of Medicine weight-gain recommendations had greater fat mass at birth, 4 years and 6 years of age, when compared to those from women who gained adequate weight during pregnancy (21). There was also a positive association between pregnancy weight gain and birth weight, such that for each 5kg increase in weight during pregnancy there was a 76g increase in birth weight of the child, after adjustment for confounders including sex and gestational age (21). There was also a positive association between birth weight and fat mass and fat-free mass during childhood, suggesting that pregnancy weight gain predisposes the offspring to increased adiposity from birth which remains throughout early childhood (21). Interestingly, maternal education is positively associated with maternal prudent diet during pregnancy suggesting that educating women before they conceive may be beneficial for the prevention of childhood obesity (89). These findings suggest that children may be on a set trajectory from birth that determines their risk of adiposity in later childhood, which supports the fetal programming and DOHaD hypothesis.

Taken together, these data suggest that the prenatal and early postnatal life can influence the risk of becoming obese in later life. There is increasing evidence that epigenetic processes are involved in the mechanism by which the early life environment can increase susceptibility to obesity in childhood and adulthood.

1.2 Epigenetics

1.2.1 Epigenetics mechanisms

Epigenetics is typically defined as heritable changes in gene expression that are not encoded directly in the nucleotide sequence (92). Every cell contains identical genetic information; however the epigenome is regulated by mechanisms that determine tissue specific gene expression and cellular differentiation (93). Epigenetic regulation occurs via three main mechanisms; histone modification, non-coding RNAs and DNA methylation.

In eukaryotes, DNA is packaged into nucleosomes in which 146bp of DNA is wound around an octamer core of histone proteins made up from two copies of each histone (H2A, H2B, H3 and H4), which is referred to as chromatin (94). The N-terminal tails of these histone proteins can be modified altering the accessibility of the DNA resulting in in heterochromatin, which is condensed and transcriptionally silent, or euchromatin which is less condensed and transcriptionally active (95). Acetylation via histone acetylases (HATs) is involved in the formation of euchromatin whereby an acetyl group is added to specific lysine residues of the H3 and H4 tails

leading to a more open configuration and increased transcription because the positively charged lysine residue no longer interacts as tightly with the negatively charged DNA (95). For example acetylation of lysine 9 (K9) on H3 is a common feature of active promoters (96). Histone deacetylases (HDACs) remove acetyl groups, thereby causing a tighter interaction between the histone tails and the DNA, resulting in a less open configuration. Histone methylation occurs via histone methyltransferases (HMTs) which can lead to transcriptional activation or repression depending on the specific lysine (K) or arginine (R) residues that are modified (96). For example mono-, di- or tri-methylation of K9 on H3 or K20 on H4 is associated with a repressed chromatin state and gene silencing (92)(95), whereas the methylation of K4 on H3 and R3 on H4 is associated with an accessible chromatin state and gene activity (97). Methylation of the histone tails is reversible which occurs via histone demethylases (HDMs) which remove the methyl groups (95).

Non-coding RNAs include microRNA (miRNA), PIWI-interacting RNA (piRNA), small interfering RNA (siRNA), small nuclear RNA (snRNA) and large intergenic non-coding RNA (lincRNA) (94). There has been an increased interest in miRNAs over recent years, as increasing evidence suggests altered expression of miRNAs may be involved in the development of human disease, including obesity and T2D. For example, pancreatic miR-375 expression is known to be increased in T2D patients (98). MiRNAs are about 22 nucleotides in length which bind to the 3'-untranslated region of their target mRNAs leading to posttranscriptional silencing via chromatin condensation, or by inducing degradation of the mRNA molecule (99). Because a single miRNA can recognise and bind to multiple mRNAs they can influence many different metabolic pathways and therefore potentially can have a huge impact on downstream processes (100).

DNA methylation is closely linked with histone modifications, and is the most studied and understood form of epigenetic regulation. Therefore, DNA methylation will be the focus of epigenetic regulation from this point forward.

1.2.2 DNA methylation

DNA methyltransferases (DNMTs) catalyse the transfer of methyl groups from S-adenosylmethionine (SAM) to the 5^{th} carbon of the cytosine ring in CpG dinucleotides, producing 5-methylcytosine (5-mC) (92), as shown in figure 1.3. In humans, it is estimated that 70-80% of CpG dinucleotides are methylated (101). CpG dinucleotides are clustered into CpG islands which are defined as a 200bp sequence of DNA with a GC content of >55%, and are found in the 5'-promoters of about 60% of all human genes (92)(102). CpG islands occur every 100kb and are typically unmethylated or hypomethylated, which is associated with transcriptional activity and an active open chromatin configuration (103).

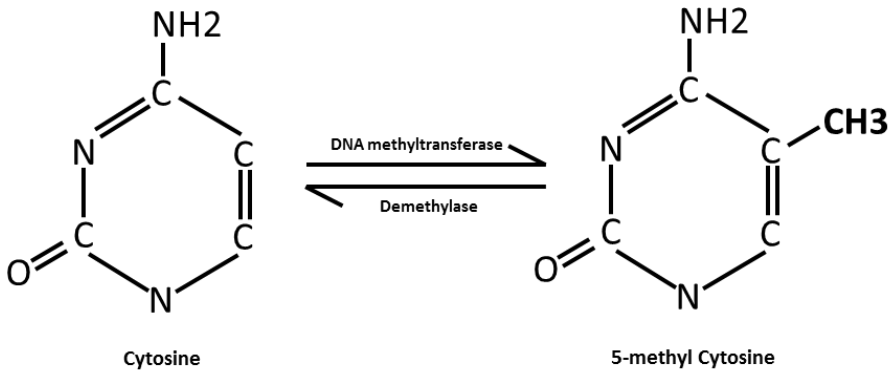


Figure 1.3- 5-methyl cytosine. DNA methyltransferase catalyses the transfer of a methyl group to the 5th carbon of the cytosine ring, to give 5-methyl cytosine. This is a reversible reaction and demethylases catalyse the removal of the methyl group to produce unmethylated cytosine.

DNA methylation within a promoter or within regulatory regions is usually associated with gene silencing where the methyl group physically blocks the binding of transcription factors to their recognition sequences, however, there is increasing evidence to suggest that methylation can enhance the binding of some transcription factors (104)(105)(106). Therefore, depending on whether these transcription factors are activators or repressors, methylation can activate or supress gene transcription. DNA methylation also represses gene expression via the recruitment of proteins that bind to methylated cytosines. Methyl cytosine binding protein 2 (MeCP2) is the most characterised methylcytosine binding protein, which contains a methyl binding domain which preferentially binds to 5-mC, and a transcriptional repression domain which interacts with the transcriptional machinery and SIN3A, a transcriptional regulatory protein (101)(103). Once bound, MeCP2 recruits HMTs and HDACs and this complex physically blocks the DNA preventing RNA polymerase and transcription factors from binding, shown in figure 1.4 (62). The MeCP2/HMT/HDAC complex causes further gene silencing via deacteylation and methylation of the histone tails, leading to a more closed compact chromatin structure that is less accessible to the transcription machinery (95).

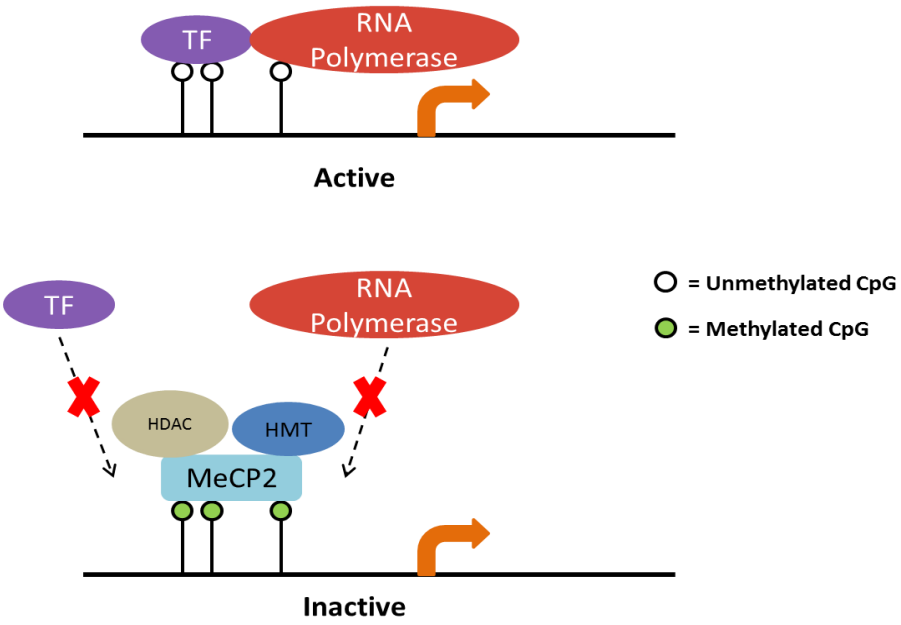


Figure 1.4- Epigenetic silencing of transcription. When CpG dinucleotides are unmethylated, shown by an empty circle, transcription factors (TF) and RNA polymerase are able to bind. When CpG dinucleotides are methylated, shown by a green circle, this prevents transcription factors (TF) and RNA polymerase from binding. Methylated CpGs also recruit MeCP2 recruits HMTs and HDACs and this complex physically blocks the DNA further preventing RNA polymerase and transcription factors from binding.

DNA methylation plays a key role in both normal and disease processes. DNA methylation is essential for normal embryonic development, and is important for the function of normal cells due to its role in the maintenance of chromatin structure and its role in regulating gene expression (107). The differential methylation of gene promoters and individual CpG loci is associated with subtle changes to gene expression and with tissue specific gene expression. However, DNA methylation is also responsible for the long term silencing of imprinted genes where either the maternal or paternal allele is switched off, and for the maintenance of X chromosome inactivation in females (62). Abnormal methylation can lead to the activation or inactivation of genes, often associated with the development of cancer, or within imprinted regions associated with diseases such as Angelman and Prader-Willi syndrome (107).

In humans, the epigenome undergoes genome wide demethylation during gametogenesis and between fertilisation and the 2-cell stage where methylation levels decrease from 54% in sperm and 48% in oocytes to 41% in the zygote, which decreases further to 32% by the 2-cell stage (108)(109). This is to ensure pluripotency in the zygote for cellular differentiation (109). Following implantation, *de novo* methylation occurs via DNMT3a and 3b, where CpG dense

regions tend to be hypomethylated and regions with lower CpG density are hypermethylated (62)(108). DNMT1 is a maintenance methyltransferase that recognises hemi-methylated DNA and catalyses the transfer of a methyl group from SAM to the cytosine of the unmethylated daughter strand, thereby maintaining methylation patterns throughout cell replication (101). Dnmt3a and $3b^{-/-}$ or $Dnmt1^{-/-}$ knockout studies in mice have shown a loss of imprinting and embryonic lethality when these enzymes are absent, highlighting their importance for normal development (110)(111).

1.2.3 Epigenetics and the early life environment

There is considerable evidence to suggest that the environment, particularly during prenatal and postnatal life, can alter the epigenome and epigenetic mechanisms, leading to long term changes in gene expression and phenotype (93). It has been estimated that the rate of epimutations is one to two orders of magnitude greater than that of somatic DNA mutations, and with more than 10^7 CpGs throughout the genome, altered DNA methylation may be involved in the development of many diseases.

Evidence suggests there are 3 critical periods in development during prenatal and early postnatal life where the epigenome is most susceptible to environmental factors. These are preconceptional exposures which influence the maternal and paternal gametes, prenatal exposure of the fetus *in utero*, and finally postnatal exposures during early life (112). However, some evidence suggests that plasticity does not stop at birth and the environment continues to have an impact on long-term epigenetic regulation (53). Therefore, the environment can potentially influence the epigenome throughout the life course and altered DNA methylation may induce permanent changes in specific genes that increase the risk of non-communicable diseases later in life.

1.2.4 The potential of epigenetic biomarkers in determining obesity risk

A biomarker is broadly defined as ‘a characteristic that is objectively measured and evaluated as an indicator of normal biological processes or pharmacologic responses to a therapeutic intervention’ (100). Therefore, if altered DNA methylation is associated with a particular phenotype, it has the ability to act as a biomarker of disease and could be used to identify those individuals at an increased risk of disease who may benefit from targeted intervention.

Evidence from animal studies suggest that adverse environmental conditions can lead to persistent changes in DNA methylation of specific CpG loci that can influence the susceptibility to

obesity in later life. For example, the Agouti mouse model has shown that supplementation of the maternal diet with methyl donors and cofactors (folic acid, vitamin B12, betaine and choline) induces hypermethylation of the IAP promoter/enhancer and decreased expression of the agouti gene in the offspring. This results in the pseudoagouti phenotype, which protects against obesity and hyperinsulinemia (54)(113). Therefore, the maternal diet can influence DNA methylation and gene expression in the offspring, which persists into adulthood. Plagemann et al. have shown that neonatal overfeeding in rats increases the methylation of the proopiomelanocortin (POMC) promoter in the hypothalamus which was associated with rapid weight gain and obesity (114). This suggests that the diet during early life can influence genes that are involved in appetite control.

In humans, it has been shown that increased methylation of a specific CpG in the RXRα gene in umbilical cord was strongly associated with increased adiposity in childhood at 6 and 9 years, after adjusting for maternal age, smoking, BMI and birth weight, such that methylation at birth explained more than 25% of the variation in adiposity in childhood (115). Relton et al. have shown that the methylation of specific CpG loci in CDKN1C (cyclin-dependent kinase inhibitor 1C) and EPHA1 (ephrin type-A receptor), in umbilical cord blood was associated with BMI and fat mass at 9 years of age, such that for a 1% increase in methylation, BMI increased by 2.08% and 0.80%, and fat mass increased by 5.16% and 1.84%, respectively (116). These data suggest that measuring the methylation of these potential epigenetic biomarkers in fetal tissue at birth may beneficial for identifying those individuals at an increased risk of later childhood obesity.

Maternal diet has been shown to influence the methylation of specific loci in genes involved in growth and metabolism. For example, Heijmans et al. demonstrated that 4 CpG loci in the insulin-like growth factor 2 (IGF2) gene were hypomethylated in peripheral blood from individuals exposed to the Dutch Hunger Winter Famine during early gestation, compared to unexposed same sex siblings (117). Whilst these methylation differences were small (less than 4%) and may not effect gene expression, it has been shown that these individuals have an increased susceptibility to obesity, T2D and cardiovascular disease, suggesting that altered methylation of these loci may be useful for determining disease risk (55).

There is evidence to suggest that maternal BMI and gestational weight gain can influence the methylation of specific loci in the fetus, which could influence the risk of obesity in later life. For example, increased gestational weight gain during early pregnancy associates with increased methylation of a specific CpG in MMP7 (matrix metallopeptidase 7), a gene involved in embryonic development and extracellular matrix remodelling that occurs during adipose tissue formation, in

umbilical cord blood (118). Therefore, maternal factors may be involved in determining the methylation levels of these loci in the offspring.

Previous studies have shown altered methylation of PGC1α in several tissue types is associated with obesity. For example, Chen et al. have shown that the mean methylation of 5 CpG loci in the PGC1α promoter were hypermethylated in abdominal subcutaneous adipose tissue from obese subjects, when compared to lean subjects (79). Ling et al. have shown that the methylation of 4 CpG loci in the PGC1α promoter are hypermethylated in the pancreatic islet cells from obese, T2D patients, when compared to normal weight, healthy controls, which associated with a decrease in mRNA expression (49).

Taken together, these data suggest that altered DNA methylation has the ability to be a biomarker of obesity. However, it is unknown if altered methylation in one tissue type may reflect the methylation of another tissue. It is also unknown if the association between altered DNA methylation and phenotype would be present in different tissues types.

1.2.5 Proxy tissues and tissue specific methylation

It is advantageous to measure potential epigenetic biomarkers in peripheral blood as it is a method of sampling large populations, and allows samples to be taken repeatedly throughout a longitudinal study (119). However, peripheral blood is a heterogeneous mix of blood cell populations, which has been identified as a potential confounder for measuring DNA methylation because different white blood cell types have specific DNA methylation patterns (120). The major cell types found within peripheral blood in children and adults are neutrophils (~52% children, ~54% adults), lymphocytes (~36% children, ~33% adults), monocytes (~8.5% children, ~9.5% adults), eosinophils (~3% in both) and basophils (~0.5% in both) (121)(120). Therefore, inter-individual variation may occur whereby 2 individuals differ in their cellular heterogeneity but may have the same % methylation (119). Intra-individual variation may also occur, which is particularly important in longitudinal studies because changes in DNA methylation over time may be explained by changes to the relative proportions of different cell types (119). However, these disadvantages are easily avoided by taking a full blood count in order to determine the relative proportions of each white blood cell, which can be corrected for in later statistical analyses.

Data from previous studies suggests that cellular heterogeneity does not affect DNA methylation equally across the epigenome and may be gene or even locus-specific. For example Talens et al. have shown that the variation in methylation of several imprinted (e.g. IGF2) and non-imprinted genes (e.g. FTO) was not explained by the proportion of neutrophils ($p>0.05$), whereas 50% of the variation in methylation in interleukin 10 (IL10) was explained by the

proportion of neutrophils (119). Adalsteinsson et al. measured the methylation of 4 CpG islands within hematopoietically expressed homeobox (HHEX), potassium inwardly-rectifying channel (KCNJ11), potassium voltage-gated channel KQT-like subfamily member 1 (KCNQ1) and peptidase M20 domain containing 1 (PM20D1) in peripheral blood, and showed that cellular heterogeneity determined by the proportion of neutrophils, lymphocytes, monocytes, eosinophils and basophils explained 40% of the variation in methylation measured in the HHEX CpG island ($p<0.0001$), whereas cellular heterogeneity did not explain the variation in methylation of the remaining CpG islands (120).

Epidemiological studies measure DNA methylation in fetal tissues in order to determine associations with future phenotype as they are easily accessible tissues (115)(116)(122). However, like peripheral blood, these tissues are a heterogeneous mix of cellular populations. For example, umbilical cord and umbilical cord blood contain mesenchymal stem cells which are multipotent and have the ability to differentiate into osteogenic, chondrogenic and adipogenic lineages (123)(124). Umbilical cord blood also contains nucleated red blood cells and hematopoietic stem cells (125).

Whilst it would be beneficial to discover an epigenetic biomarker of disease risk that could be measured in a proxy tissue such as peripheral blood and fetal tissues, the methylation of these loci may not represent the methylation of harder to access tissues that are involved in the development of the disease, such as the liver or pancreas. It is also unknown if the methylation of these loci would still be associated with a particular phenotype in these harder to access tissues. Evidence from human studies has suggested that the methylation of some loci differs across different tissue types, whereas other studies have shown methylation is consistent across different tissues. For example, Armstrong et al. have shown that the methylation of 7 gene promoters and global methylation varied across the placenta, umbilical cord blood and saliva at 3 and 6 months of age (126). Ollikainen et al. have shown that the methylation of the imprinted IGF2/H19 locus varies between several fetal tissues, including umbilical cord blood mononuclear cells and granulocytes, human umbilical vein endothelial cells, buccal epithelial cells and placental tissue (127). These data suggest that methylation is tissue specific and each tissue may have its own epigenetic signature. This could be important for tissue specific gene expression and the function of each tissue. Therefore, methylation in proxy tissue may not reflect the methylation in a different tissue, and the association between methylation and altered phenotype may not be present across different tissue types.

This idea has been challenged by other studies that have shown conflicting findings where methylation of specific loci is the same across different tissue types from different germ line

layers. For example, Talens et al. have shown that the methylation of several non-imprinted genes is highly correlated between peripheral blood and buccal cells, which are from the mesoderm and ectoderm respectively (119). Ma et al. have shown that methylation levels are conserved across multiple tissues from different germline layers, including peripheral blood leukocytes, artery and liver, such that surrogate tissue methylation was able to predict the methylation of specific target tissues (128). These data suggest that the methylation of specific loci can be measured in different tissues from different germline layers, which may be the result of an environmental challenge *in utero* which affected the germline layers equally. Therefore, the association between methylation and altered phenotype may be present across different tissue types.

Measuring an epigenetic biomarker in a proxy tissue is advantageous as it increases the number of individuals in whom such associations can be tested. If a potential epigenetic biomarker of disease is not present in different tissue types or the target tissue, they may still have utility for determining disease risk. Epigenetic biomarkers may not be stable throughout the life course and may change with disease progression.

1.2.6 Stability of DNA methylation

In humans, methylation patterns are set up following genome-wide demethylation and remethylation to ensure pluripotency for cellular differentiation, and these patterns are maintained during cell division via DNMT1 (129)(101). Evidence from animal studies suggests that once methylation is established in early development, it is then stably maintained throughout the life course and is not influenced by future environmental challenges. For example, Weaver et al. have shown that increased pup licking and grooming and arched-back nursing decreased the methylation of several CpG loci in the glucocorticoid receptor (GR) in the hippocampus (130). These differences in methylation were maintained throughout the life course between postnatal day 1 and day 90. This suggests that the environment can induce persistent changes in the epigenetic regulation of genes. Maternal dietary supplementation with methyl donors and cofactors induces hypermethylation of the IAP promoter/enhancer and decreased expression of the agouti gene, which associated with the pseudoagouti phenotype, which was maintained into adulthood (54)(113).

Evidence from the Dutch Hunger Winter famine suggests that maternal nutrition can alter DNA methylation and influence obesity risk in the offspring, which is maintained throughout the life course. For example, Heijmans et al. have demonstrated that individuals exposed to the famine during early gestation had decreased methylation of the imprinted IGF2 gene, 6 decades after the exposure, when compared to unexposed same sex siblings (117).

However, this idea of stable DNA methylation was challenged by the recent discovery of the ten-eleven translocation (TET) family of enzymes that catalyse the removal of the methyl group (131). There is now increasing evidence to suggest that plasticity does not stop at birth and the environment continues to have an impact on long-term epigenetic regulation (132)(53)(133). For example, Fraga et al. have shown that younger monozygotic twins who had spent their lives together had methylation patterns much more identical than older monozygotic twins who had spent time in different environments (133). These differences in methylation seen in the older twins, particularly those twins who had spent time apart, was present in several different cell types and tissues from different germline layers including lymphocytes, epithelial cells, skeletal muscle and intra-abdominal fat (133). These data demonstrate that methylation levels change with aging, known as 'epigenetic drift', and show the importance of the environment throughout the life course.

Environmental factors such as pollution and exercise have been shown to induce changes in DNA methylation of specific loci and this methylation change is reversed once the environmental factor has been removed, suggesting the environment can influence the epigenome throughout the life course (134)(135)(82). For example, Barrès et al. have shown that high intensity exercise induces hypomethylation of the PGC1α promoter immediately after exercise, followed by a significant increase in gene expression after 3 hours, measured in skeletal muscle biopsies from healthy men (82).

Of the studies that have looked at the stability of DNA methylation, there is little evidence on the stability during early childhood and puberty, which have been proposed to be periods in development where insulin resistance and hormone levels fluctuate and therefore may be a critical time in development where epigenetic mechanisms are influenced (95)(12). Of the evidence that does exist, there are conflicting findings (132)(136). For example, Wong et al. have shown that the methylation of several CpG loci in the promoter of 3 genes involved in neurological function is unstable between 5 and 10 years of age, when measured in buccal cells from 46 monozygotic and 45 dizygotic twins (136). Methylation levels varied between twin pairs suggesting that methylation differences between genetically identical or similar children is present as early as 5 years and is due to environmental factors (136). However, there is also evidence to suggest the methylation of some loci may be stable during early childhood when Wang et al. showed the methylation of 95.5% of CpGs from a 27K array was stable from birth up to 2 years of life, when measured in umbilical cord blood and peripheral blood (132). Of the 4.5% of CpGs that showed categorical methylation changes from the hypo, intermediate or hypermethylation groups, only 0.5% were significantly different during the first 2 years of life

(132). Another study by Heijmans et al. analysed the methylation of the maternally imprinted IGF2/H19 locus in 196 adolescent twins (mean age 16.7 years) and 176 middle aged twins (mean age 44.8 years). Data from the study suggest that environmental and stochastic factors did not have an effect upon the methylation of the IGF2/H19 locus in the middle-aged twins (137). Taken together these data suggest that for some CpG loci methylation is not altered during early life or throughout the life course.

Therefore, DNA methylation appears to be more dynamically regulated than previously thought and the environment potentially can influence the epigenome throughout the life course, not just during critical periods in development. For complex diseases such as T2D, there is an increased incidence of disease with age and the severity of disease increases with time, and therefore, it is possible that altered DNA methylation may contribute to the development of disease (138). Whist it is not essential that a potential epigenetic biomarker is stable throughout the life course; it would be beneficial if these biomarkers could predict future risk of obesity when measured at any age throughout the life course. However, epigenetic biomarkers that are associated with a particular phenotype at a single time point would still be useful for identifying individuals at an increased risk of disease.

More evidence is required on how environmental, hormonal and stochastic factors can influence potential epigenetic biomarkers and how DNA methylation may potentially change with disease progression.

1.3 Aims and hypotheses

Whilst genetic factors have been shown to be associated with an increased risk of obesity, there is now increasing evidence to suggest that the environment may be important in the epigenetic dysregulation of genes that are involved in the development of obesity. A candidate gene approach was used to measure the methylation of specific CpG loci in genes that have been shown to be causally involved in the development of obesity, insulin resistance and T2D.

A major question still remains about whether DNA methylation is stable throughout childhood and puberty or not, with recent studies reporting conflicting findings suggesting that the epigenome is not influenced equally by the environment throughout the life course (107)(132). Therefore, the methylation of specific CpG loci in 5 genes was measured annually during childhood and puberty.

There is some evidence in humans to suggest that altered DNA methylation associates with future adiposity (115). Therefore, more evidence is required to determine if altered DNA methylation associates with altered phenotype, and has the potential to be a suitable biomarker

of obesity risk. This would provide a method for identifying individuals at an increased risk of disease, which could allow for intervention and prevention thereby improving human health.

The general hypothesis is that DNA methylation in key metabolic genes is induced during early life and stably maintained, and contribute to an increased risk of obesity in later life. The following specific hypotheses were tested:

1) The methylation of specific CpG loci in genes involved in β cell function, glucose homeostasis, adipogenesis and energy metabolism, is stable throughout childhood and puberty.
2) DNA methylation is a predictive biomarker of future adiposity and/or insulin resistance.
3) Differential methylation of CpG -783 in the PGC1α promoter alters transcription factor binding.
4) The methylation of specific CpG loci in SIRT1 and PGC1α in children is predicted by the methylation of these loci in the parents, measured in peripheral blood.
5) The methylation of specific CpG loci in SIRT1 and PGC1α measured in fetal tissues associates with adiposity in childhood.
6) The methylation and mRNA expression of SIRT1 and PGC1α differs in the abdominal subcutaneous adipose tissue of lean and obese people.
7) Fatty acids can alter the expression and methylation of SIRT1 and PGC1α *in vitro*.

Chapter 2

Materials and Methods

2.1 Materials

Table 2.1- Reagents used in experiments

Chemical/Reagent	Details
10-Hydroxy-2E-decanoic acid	Sigma Aldrich, #379700
10-Hydroxy-2E-decenoic acid	Cambridge Bioscience, #CAY10976
10X Tris-Borate-EDTA Buffer (TBE)	Fisher Scientific, # T/P050/15
4-(2-Hydroxyethyl)-1-Piperazineethanesulfonic Acid (HEPES)	Sigma Aldrich, #H4034
40% Acrylamide Solution	Fisher Scientific, # BPE1402-1
α Linolenic Acid	Sigma Aldrich, #L2376
Agar	Fisher Scientific, #BP1423
Agarose	Melford, #MB1200
Ammonium Persulfate (APS)	Fisher Scientific, #BPE179-25
Ampicillin	Sigma Aldrich, #A9393
Anti-HOXB9 antibody	Abcam, #ab133701
Anti-PBX1 antibody [1D9]	Abcam, #ab119069
BCA Protein Assay Kit	Fisher Scientific, #23227
BlueJuice Gel Loading Buffer 10X	Invitrogen, #10816015
Chemiluminescent Nucleic Acid Detection Module	Fisher Scientific, #PN89880
Chloramphenicol	Sigma Aldrich, #C1919
Chloroform	Fisher Scientific, #10102190
CpGenome Universal Methylated DNA	Millipore, #S7821
CpGenome Universal Unmethylated DNA	Millipore, #S7822
Deoxynucleotide Triphosphates (dNTPs)	Promega, #U1420
Dithiothreitol (DTT)	Melford, #MB1015
DNase and RNase free water, DEPC treated	Fisher Scientific, #BPE561-1
DNase I Amplification Grade Kit	Sigma Aldrich, #AMPD1
Docosahexaenoic Acid	Sigma Aldrich, #D8768
Dulbecco's Modified Eagle Medium (DMEM)	PAA, #E15-843
Eicosapentaenoic Acid	Sigma Aldrich, #E6627
EpiTect Control Unmethlyated DNA	Qiagen, #59665
Ethanol 99%+	Fisher Scientific, #E/0600/05
Ethylenediaminotetraacetic Acid Disodium Salt (EDTA)	Sigma Aldrich, #03690
EZ DNA methylation gold kit (2x96)	Cambridge Bioscience, #D5007
FastDigest EcoRI	Thermo Fisher, #FD0274
FastDigest HindIII	Thermo Scientific, #FD0504
FastDigest NcoI	Thermo Fisher, #FD0574
FastDigest NotI	Thermo Fisher, #FD0596
FastDigest SacI	Thermo Scientific, #FD1133
FastDigest SalI	Thermo Fisher, #FD0644
FastDigest XhoI	Thermo Fisher, #FD0695
Fetal Bovine Serum	PAA, #A15-151
FuGENE HD Transfection Reagent	Active Motif, #32043
GelRed Nucleic Acid Stain 10,000X	Cambridge Bioscience, #BT41003
GeNorm Kit	Primer Design, #ge-SY-6

Glycerol	Sigma Aldrich, #G6279
HotStarTaq DNA Polymerase	Qiagen, #203205
HOXB9 cDNA Clone	Source BioScience, # IRAUp969H0951D
Human Genomic DNA	Promega, #G3041
Human Genomic DNA from Blood	Roche, #11691112001
Human Liposarcoma SW-872 Cell Line	Cell Line Service, #SW 872
Hyaluronidase	Sigma Aldrich, #H3506
Igepal CA-630	Sigma Aldrich, #I8896
Isopropanol	Fisher Scientific, #11398461
JM109 Competent cells	Promega, #L2001
Leupeptin Hemisulfate Salt	Sigma Aldrich, #L5793
Lightshift EMSA Kit	Fisher Scientific, #PN20148X
Linoleic Acid	Sigma Aldrich, #L8134
Luciferase Assay System	Promega, #E1500
Luria Bertani Broth (LB)	Sigma Aldrich, #L3022
M-MLV Reverse Transcriptase Kit	Promega, #M1701
O'generuler 1Kb	Fisher Scientific, #FQ-SM1163
Oleic Acid	Sigma Aldrich, # O7501
Opti-MEM Medium	Invitrogen, #31985062
PBX1 cDNA Clone	Source BioScience, # IRQLp5017E064D
pcDNA3+ Plasmid	Invitrogen, #V790-20
Penicillin-Streptomycin	PAA, #P11-010
PGL3-Basic Plasmid	Promega, #E1751
Phenol	Life Technologies, #15509-037
Phenylmethanesulfonyl Fluoride (PMSF)	Sigma Aldrich, #P7626
Phosphate Buffered Saline (PBS) Tablets	Fisher Scientific, #12821680
Poly(deoxyinosinic-deoxycytidylic) Acid Sodium Salt (Poly DIDC)	Sigma Aldrich, #P4929
Potassium Chloride (KCl)	Sigma Aldrich, #P9541
PPARGC1A pLightSwitch Promoter	SwitchGear Genomics, #S722424
Primers	Biomers
pRL-CMV Plasmid	Promega, #E2261
pRL-SV40 Plasmid	Promega, #E2231
Proteinase K	Qiagen, #19131
PureYeild Plasmid Midiprep System	Promega, #A2492
PyroMark Annealing Buffer	Qiagen, #979009
PyroMark Binding Buffer	Qiagen, #979006
PyroMark Gold Q96 Reagents	Qiagen, #972804
PyroMark Wash Buffer	Qiagen, #979008
QIAamp DNA Blood Mini Kit (50)	Qiagen, #51104
QIAamp Genomic DNA Mini Kit	Qiagen, #51304
QIAprep Spin Miniprep Kit	Qiagen, #27104
QIAzol Lysis Reagent	Qiagen, #79306
QuantiTect Primer Assay PGC1α	Qiagen, #QT00095578
QuantiTect Primer Assay PPIA	Qiagen, #QT01866137
QuantiTect Primer Assay SIRT1	Qiagen, #QT01886675

QuantiTect SYBR Green PCR Mastermix	Qiagen, #204143
Quick Ligation Kit	New England Biolabs, #M2200S
Random Nanomers	Sigma Aldrich, #R7647
RNase A	Qiagen, #19101
RNeasy Lipid Tissue Mini Kit	Qiagen, # 74804
Sodium Acetate Solution	Sigma Aldrich, #S8388
Sodium Chloride (NaCl)	Sigma Aldrich, #S3014
Sodium Dodecyl Sulfate	Sigma Aldrich, #L6026
Sodium Hydroxide(NaOH)	Sigma Aldrich, #S5881
Streptavidin Sepharose Beads	Fisher Scientific, #GZ17511301
Tetramethylethylenediamine (TEMED)	Sigma Aldrich, #T7024
Tri Reagent	Sigma Aldrich, # T9424
Tris Base	Sigma Aldrich, #T6791
Tris-acetate-EDTA (TAE) solution 50X	Fisher Scientific , #BPE1332-1
Trizma Base	Sigma Aldrich, #T1503
Trypsin EDTA	PAA, #L11-004
Zymoclean Gel DNA Recovery Kit	Cambridge Bioscience, #D4001

2.2 Methods

2.2.1 The EarlyBird cohort

The EarlyBird study is a longitudinal, non-intervention study composed of 307 healthy children (137 girls and 170 boys) set up to study the natural evolution of insulin resistance and obesity throughout childhood, between 5 and 16 years (88). Children were recruited from the 1995 Plymouth Birth Cohort and were stratified into quartiles based on their socio-economic status with an equal selection made from each quartile. Children were excluded if there was a family history of diabetes or any factors known to affect growth or body composition.

At the start of the study the children were randomly allocated a number and all data were anonymously stored on a database. The children were reviewed annually at Derriford Hospital, Plymouth, where several measurements were taken. These measurements were chosen in order to look at factors known to be involved in insulin resistance, the behaviour of insulin resistance, insulin secretary capacity, metabolic disease and cardiovascular disease (88). Table 2.2 provides details of each test and the EarlyBird study protocol can be found online at www.EarlyBirddiabetes.org/downloads/pdf/study_protocol.pdf.

Table 2.2- Details of measurements taken annually for the EarlyBird Cohort

Factor being measured	How the measurement was achieved
Anthropometry	Skin fold thickness at five sites, height and weight allowing BMI to be determined. BMI SD scores were calculated from the British 1990 standards (139).
Body composition	Regional fat mass, % body fat, fat-free mass water and bone mass measured using dual energy X-ray absorptiometry (DXA) and bio impedance.
Physical activity	Daytime activity at school and home, along with weekend activity measured over a seven day period using an accelerometer annually, measuring the length and intensity of movements.
Resting energy expenditure	Resting metabolic rate measured in the lying position by indirect calorimetry over 15 minutes.

Food types	Food questionnaires completed by both the child and parent and then compared.
Insulin resistance	Fasting insulin and glucose concentrations from a venous blood sample were used to determine HOMA-IR.
Insulin secretary capacity	Fasting insulin and glucose concentrations from a venous blood sample were used to determine HOMA-ISC.
Blood pressure	Measured by a semi-automated sphygmanometer.
Cholesterol and triglycerides	Measured in a fasted venous blood sample.
Serum IGF1 (Insulin-like growth factor 1)	Measured to determine pubertal timing.
Heart rate	ECG to measure beat to beat variation.

At baseline, when the children were 4.9 years old (mean age), several parental measurements were taken including height, weight, BMI, total cholesterol and a peripheral blood sample, allowing analysis of metabolic status a generation earlier and mother/offspring and father/offspring comparisons to be made (86).

40 subjects (20 boys and 20 girls) were selected from the EarlyBird cohort and stratified according to insulin resistance at 5 and 14 years, measured by HOMA-IR. Subjects were chosen to represent the range of insulin resistance across all subjects throughout childhood in the EarlyBird cohort. 10 subjects (5 boys and 5 girls) formed one of the following 4 groups:

1) low insulin resistance at 5 and 14 years
2) high insulin resistance at 5 and 14 years
3) low insulin resistance at 5 years and high insulin resistance at 14 years
4) high insulin resistance at 5 years and low insulin resistance at 14 years

The reason 10 subjects were selected per group to give a total of 40 subjects was due to feasibility of sample preparation and analysis. No power calculation was performed. No analysis was performed on the 4 groups and all samples were analysed together. Peripheral blood samples were available for these 40 subjects at most ages between 5 and 14 years, giving a total of 298 samples.

Table 2.3- Subject characteristics at 5 and 14 years for subjects selected from the EarlyBird cohort

	Boys		Girls	
	5 years	**14 years**	**5 years**	**14 years**
Age (Years)	5.0 ± 0.4	14.0 ± 0.4	5.0 ± 0.3	13.9 ± 0.3
Adiposity (% fat)		22.1 ± 10.2		31.0 ± 8.2
BMI SDS (Standard deviation score)	0.17 ± 0.85	0.68 ± 1.04	0.40 ± 0.59	0.75 ± 0.97
Moderate-vigorous physical activity (minutes per day)	51.2 ± 24.9	58.0 ± 36.1	51.7 ± 15.6	43.0 ± 20.0
Insulin resistance (HOMA-IR)	0.49 ± 0.52	1.21 ± 1.10	0.75 ± 0.66	0.98 ± 1.57
Age at peak height velocity (APHV)	13.3 ± 0.58		11.9 ± 0.97	
Proportion of neutrophils	0.49 ± 0.08	0.51 ± 0.08	0.46 ± 0.11	0.50 ± 0.10
Proportion of lymphocytes	0.38 ± 0.07	0.35 ± 0.09	0.42 ± 0.10	0.37 ± 0.08

Subject characteristics at 5 and 14 years are summarised in table 2.3, for boys and girls separately. Values are mean ± SD except for insulin resistance (median ± interquartile range). BMI standard deviation scores were standardised for age and gender.

2.2.2 Isolation of genomic DNA from peripheral blood from the EarlyBird cohort

Peripheral blood was collected annually into EDTA tubes following an overnight fast, and stored at -80°C. Genomic DNA was extracted from 400µl of peripheral blood using the QIAamp DNA blood mini kit (Qiagen) according to the manufacturer's instructions, with 100% sample recovery efficiency. Samples were eluted in 300µl of DNase and RNase free water.

The quality and quantity of the DNA samples were determined using the Nanodrop 1000. All samples had a 260/280 ratio between 1.6 and 2.0. All samples had between 1.27µg and 17.3µg total DNA. A selection of samples were visualised on a 0.8% agarose gel to determine the quality, shown in figure 2.1. Any samples with a concentration less than 40ng/µl were concentrated for an appropriate length of time to achieve 40ng/µl to enable 1µg of DNA to be bisulphite converted. All samples were stored at -20°C.

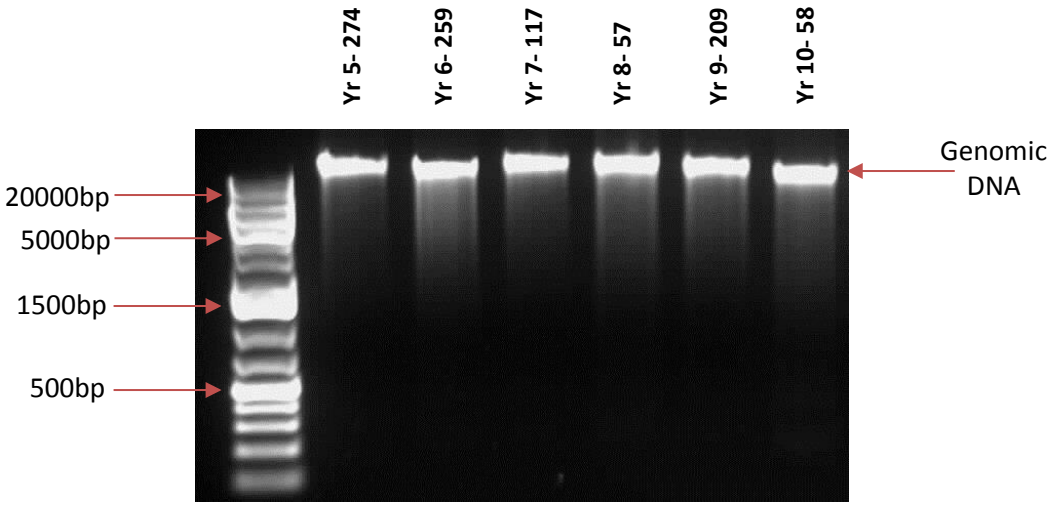


Figure 2.1- Genomic DNA from peripheral blood from the EarlyBird cohort. These example samples were selected to represent the range of concentrations and 260/280 ratios of all samples. Lanes are labelled with the age in years and subject number, which was randomly assigned to each child at the start of the EarlyBird study.

2.2.3 Bisulphite conversion

Genomic DNA was bisulphite treated (1µg) using the EZ DNA methylation gold kit (Cambridge Bioscience) according to the manufacturer's instructions. Each sample was eluted in 30µl of DNase and RNase free water. Each plate of EarlyBird samples also contained controls allowing for intra and inter-plate validation. Controls included unmethylated DNA (Millipore and Qiagen), methylated DNA (Millipore), human genomic DNA (Promega), subject 209 aged 6 years (in triplicate), subject 209 aged 14 years and a no template control (NTC) where the DNA was replaced with 1µl of water.

2.2.4 Polymerase chain reaction (PCR)

2.2.4.1 Primer validation

Details of how genes and specific CpG loci were selected for optimisation and validation can be found in section 3.1. Briefly, genes were selected based on previous literature to suggest they are epigenetically regulated, work within the same pathway, and/or are involved in the development of obesity, insulin resistance and T2D. The methylation of each CpG loci is shown in figure 3.2.

Bisulphite converted human genomic DNA (Promega) from peripheral blood was used to optimise and validate primers. The chemically modified DNA was used as a template for primers designed to amplify key regulatory regions of specific genes. Primers were rehydrated using

DNase and RNase free water (Fisher) to a concentration of 100µM upon arrival and stored at -20°C. Primers were validated using the standard PCR setup details listed below.

Table 2.4- Conditions used to validate primers

Description	Volume µl
10X Qiagen Buffer	5.00
dNTPs (10mM)	1.00
Forward Primer (10µM)	1.00
Reverse Primer (10µM)	1.00
Qiagen HotStar Taq (5u/µl)	0.25
Water	40.75
DNA (50ng)	1.00
TOTAL	**50.00**

95°C 15 minutes

{ 95°C 30 seconds
  45-60°C 30seconds
  72°C 1 minute } 45 cycles

72°C 10 minutes

4°C∞

Gene selection criteria included those that had CpGs with a varied level of DNA methylation encompassing a transition between regions of high and low methylation and had methylation levels above the bisulphite conversion control threshold (10%). Genes involved in the same pathway (67)(71), or contained CpG loci where the methylation has been shown to be important in gene function or to be associated with adiposity and/or T2D (82)(115)(49)(79), were chosen for amplification of the EarlyBird bisulphite DNA. Genes that were selected are shown in table 2.5.

Table 2.5- PCR primer sequences of assays that were selected for amplification

Assay Name	Forward Primer (5'-3')	Reverse Primer (3'-5')	Biotin tag?	Annealing Temperature °C	Product size (bp)
SIRT1 CpG -880 to -865	GGGTTTTTAGTTAAGATTAGATATGGAGTT	AAAAAAAAATAACTTAACCTCCATACAC	Reverse	47.8	279
SIRT1 CpG -760 to -668	AGATGTGTATGGAGGTTAAGTTAT	TTTTTTCCCCTCTCTCCCATCA	Reverse	55.4	247
PGC1α CpG -841 to -783	AAAGGTATTTTAAGGTAGTTAGGGAGGAAA	CCCCATCCTTTCAACTTCCTTCTA	Forward	53.4	168
PGC1α CpG -652 to -617	TGTGGTTTGTTTTTTTTATATGGAGTAAAG	TAAACAAACTCCTCCACCCAAAAT	Reverse	53.4	110
PGC1α CpG -521 to -515	GAATTTTGGGTGGAGGAGT	ATCTCCAAATAAACTCAAACTCAATT	Reverse	45.5	202
HNF4α CpG -121 to -79	TTTTGGGTGATTAGAAGAATTAATAAGA	ATCTTCCCCCAACCCCACTTA	Reverse	46.4	230
HNF4α Enhancer	TGAGTAAAGTTTTTTTTTTGGAGAGTAG	CCCTCCCTATCCTTAACCTAATT	Forward	51.4	236
RXRα CpG -2346 to -2335	TTTTAGGGATAAGGAGTGAGATGG	CCAACCAAAAAACTCAAACAACTT	Reverse	59.8	274
GCK CpG -322 to -282	GGGGTTAGGGATGTGAGATG	CTCCCACACTCCTATTCTAAT	Reverse	51.4	121
GCK CpG -192 to -172	TGTTTTAGGATTTGAATAGGTGGTAAAG	CCATCCTATCATTAAAACTACCATACAT	Reverse	53.4	149
GCK CpG +30 to +37	ATATTTGGTTGGAGTAGGAAATGT	TATATCTCTCACATCCTAACCTACTTC	Forward	51.4	73

2.2.4.2 PCR using EarlyBird samples

EarlyBird bisulphite converted DNA was used in a PCR using each assay and the optimised conditions in table 2.5. A no template control (NTC) was used on each plate where the DNA was replaced with 1µl of water. To confirm the product size, 10µl of each control and a random selection of 7 samples from each of the four plates were run on a 1.5% agarose gel, shown in figure 2.2.

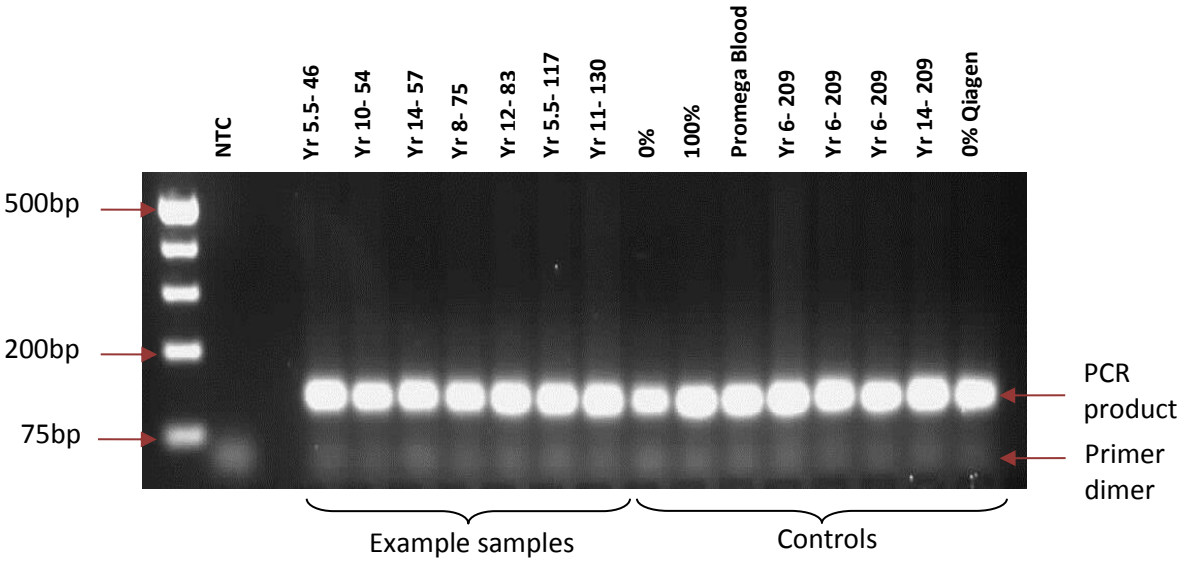


Figure 2.2- PGC1α CpG -652 to -617 Plate 1 PCR. For each PCR using EarlyBird bisulphite converted DNA, a selection of 7 samples and all controls were run on a 1.5% agarose gel. Lanes are labelled with the age in years and subject number, and each control is labelled. The PCR product was 110bp and a small amount of primer dimer was present as a by-product of the reaction.

2.2.4.3 Determining the genotype of two single nucleotide polymorphisms in EarlyBird subjects

CpG -2335 in RXRα and CpG -282 in GCK are known to contain a SNP within the CpG dinucleotide, where the cytosine residue becomes a thymine, as reported by Ensembl (http://www.ensembl.org). In order to determine the genotype of the 40 EarlyBird subjects, genomic DNA at age 14 years was diluted to a concentration of 12.5ng/µl for each subject. Primers were rehydrated using DNase and RNase free water (Fisher) to a concentration of 100µM upon arrival and stored at -20°C. Primers were validated using the conditions shown in table 2.6 and a temperature gradient of 45-65°C.

A total of 25ng of DNA of each sample was used in the PCR using the conditions listed in table 2.7. An NTC was used on the plate where the DNA was replaced with 2µl of water. To confirm the product size 10µl of each sample and the NTC were run on a 1.5% agarose gel.

Table 2.6- Conditions used to validate SNP primers

Description	Volume µl
10X Qiagen Buffer	5.00
dNTPs (10mM)	1.00
Forward Primer (10µM)	1.00
Reverse Primer (10µM)	1.00
Qiagen HotStar Taq (5u/µl)	0.25
Water	39.75
DNA (25ng total)	2.00
TOTAL	**50.00**

95°C 15 minutes

{ 95°C 30 seconds
45-60°C 30seconds
72°C 1 minute } 45 cycles

72°C 10 minutes

4°C∞

Table 2.7- SNP PCR primer sequences

Assay Name	Forward Primer (5'-3')	Reverse Primer (3'-5')	Biotin tag?	Annealing Temperature °C	Product size (bp)
RXRα SNP CpG -2335	TTTCAGGGACAAGGAGTGAG	TTTGGGATCACTGGTTCTTG	Reverse	57	274
GCK SNP CpG -282	ATAGGGGGGTTAGGGATGTG	TCCTGGGGCAGTGTCTCA	Reverse	60	148

2.2.5 Pyrosequencing

The methylation of individual CpG loci was determined by pyrosequencing. PCR products (10µl) were immobilised on sepharose streptavidin beads (Fisher Scientific), washed, denatured and released into annealing buffer containing the appropriate sequencing primer at a concentration of 10µM (table 2.8) (140). Pyrosequencing was carried out using PyroMark Gold Q96 Reagents (Qiagen) on a PyroMark Q96 MD Pyrosequencer (Qiagen). The percentage methylation was calculated using PyroMark CpG software 1.0.11 (Qiagen) and the genotype was determined using PyroMark MD 1.0 (Qiagen).

Table 2.8- Sequencing primers for pyrosequencing

Assay Name	Sequencing Primer	Direction	Sample Pass rate
SIRT1 CpG -880 to -865	ATTATTTAAGATATGAGAAAAATTA	5' to 3'	91%
SIRT1 CpG -760 to -722	TGTTTTTATATTGATTTAATAAATT	5' to 3'	86%
SIRT1 CpG -689 to -668	GGGGTTAGAAAGTAGAT	5' to 3'	90%
PGC1α CpG -841 to -783	ACTCCCAAAAAACAAATATTAATAA	3' to 5'	95%
PGC1α CpG -652 to -617	GGAGTAAAGAAAATTGTAGTAAT	5' to 3'	90%
PGC1α CpG -521 to -515	AAAAAAAAAAAAAAAAAAAAAAGT	5' to 3'	97%
HNF4α CpG -121 to -79	GTGATTAGAAGAATTAATAAGATA	5' to 3'	97%
HNF4α Enhancer	AAAACCTCCAAAATTATACA	3' to 5'	0%
RXRα CpG -2346 to -2335	GTTGTTTTTTTTTTTGTAG	5' to 3'	82%
GCK CpG -322 to -282	GGGGTTTGTTTTTGAG	5' to 3'	89%
GCK CpG -192 to -172	GGTAAAGGTTTAATAGGTTAG	5' to 3'	89%
GCK CpG +30 to +37	CCTACTTCCCTAAAACT	3' to 5'	93%
RXRα SNP CpG -2335	GCTCCTTCTCTCTGCA	5' to 3'	100%
GCK SNP CpG -282	CCTCTGCTTCCAGACT	5' to 3'	100%

Some subjects had missing DNA samples at either age 5, 6 or 7 years, therefore mean methylation values between 5-7 years were used for the statistical analysis. Each subject had a sample available at either age 8 or 9 years, but not both ages; therefore the data was combined into '8-9 years'. For all other years a sample was available for each subject up to 14 years, with the occasional sample missing, however no subject is missing more than one sample between 10 and 14 years.

The controls for all HNF4α Enhancer plates failed. Therefore, the data for this assay could not be used.

2.2.6 Co-efficient of variance (COV)

Within assay precision, or co-efficient of variance (COV), was determined for each CpG using bisulphite treated human genomic DNA. Each PCR was repeated 10 times for each assay using the conditions shown in table 2.5. An NTC was used on each plate where the DNA was replaced with 1µl of water. To confirm the product size 10µl of each sample and the NTC were run on a 1.5% agarose gel.

The methylation of each CpG loci was determined by pyrosequencing, as described in section 2.2.5. The 10 repeated PCR products were used on 3 separate pyrosequencing plates to determine the % variation in methylation for each CpG within and between plates, using the below formula:

Co-efficient of variance (%) = (Standard deviation/Mean) x 100

Table 2.9- The coefficient of variance for each assay using human peripheral blood

	CpG						
SIRT1	**-880**	**-865**	**-760**	**-742**	**-722**	**-689**	**-668**
Plate 1 Mean methylation	67	51	19	92	47	66	45
Plate 1 SD	1	1	1	4	2	2	2
Plate 1 COV %	**1**	**2**	**6**	**5**	**4**	**3**	**4**
Plate 2 Mean methylation	64	52	18	88	48	66	47
Plate 2 SD	1	1	1	3	1	2	3
Plate 2 COV %	**2**	**1**	**7**	**4**	**3**	**3**	**6**
Plate 3 Mean methylation	68	54	20	86	47	67	47
Plate 3 SD	1	1	2	2	2	2	3
Plate 3 COV %	**1**	**2**	**8**	**2**	**5**	**3**	**6**
All plates Mean methylation	66	52	19	87	46	67	47
All plates SD	2	2	2	4	3	2	3
All plates COV %	**4**	**4**	**9**	**5**	**7**	**3**	**6**

	CpG						
PGC1α	**-841**	**-816**	**-783**	**-652**	**-617**	**-521**	**-515**
Plate 1 Mean methylation	67	55	46	34	39	23	53
Plate 1 SD	0	1	1	1	1	0	1
Plate 1 COV %	**0**	**2**	**2**	**3**	**3**	**2**	**3**
Plate 2 Mean methylation	70	54	49	35	39	23	55
Plate 2 SD	1	1	1	1	1	1	1
Plate 2 COV %	**2**	**2**	**2**	**3**	**3**	**3**	**3**
Plate 3 Mean methylation	70	54	49	37	39	23	56
Plate 3 SD	1	1	1	1	1	1	2
Plate 3 COV %	**2**	**2**	**2**	**2**	**3**	**3**	**3**
All plates Mean methylation	70	54	48	35	39	23	55
All plates SD	1	1	2	2	1	1	2
All plates COV %	**2**	**2**	**3**	**4**	**3**	**3**	**4**

	CpG				
HNF4α	**-121**	**-117**	**-103**	**-94**	**-79**
Plate 1 Mean methylation	96	96	99	100	81
Plate 1 SD	1	1	1	0	1
Plate 1 COV %	**2**	**1**	**1**	**0**	**2**
Plate 2 Mean methylation	100	95	99	100	84
Plate 2 SD	0	1	1	0	2
Plate 2 COV %	**0**	**1**	**1**	**0**	**3**
Plate 3 Mean methylation	100	95	100	100	83
Plate 3 SD	1	1	0	0	2
Plate 3 COV %	**1**	**1**	**0**	**0**	**3**
All plates Mean methylation	99	95	99	100	83
All plates SD	2	1	1	0	3
All plates COV %	**2**	**1**	**1**	**0**	**3**

	CpG	
RXRα	**-2346**	**-2335**
Plate 1 Mean methylation	91	83
Plate 1 SD	1	2
Plate 1 COV %	**2**	**2**
Plate 2 Mean methylation	92	84
Plate 2 SD	2	1
Plate 2 COV %	**2**	**2**
Plate 3 Mean methylation	90	85
Plate 3 SD	1	2
Plate 3 COV %	**1**	**2**
All plates Mean methylation	91	84
All plates SD	2	2
All plates COV %	**2**	**2**

	CpG						
GCK	**-322**	**-282**	**-192**	**-172**	**+30**	**+34**	**+37**
Plate 1 Mean methylation	77	73	92	76	95	91	84
Plate 1 SD	1	2	0	1	0	1	1
Plate 1 COV %	**1**	**3**	**0**	**1**	**0**	**1**	**2**
Plate 2 Mean methylation	75	76	92	76	95	92	85
Plate 2 SD	1	1	0	1	0	1	1
Plate 2 COV %	**2**	**1**	**0**	**1**	**0**	**1**	**1**
Plate 3 Mean methylation	78	74	92	78	95	91	85
Plate 3 SD	1	1	0	1	0	1	1
Plate 3 COV %	**2**	**1**	**0**	**1**	**0**	**1**	**1**
All plates Mean methylation	77	75	92	77	95	91	85
All plates SD	2	2	0	1	0	1	1
All plates COV %	**2**	**3**	**0**	**2**	**0**	**1**	**1**

Table 2.9 shows the methylation of each CpG loci, which are numbered according to their distance from the transcription start site. The CpG loci in HNF4α, RXRα and GCK show the least intra and inter plate variation in % methylation (all <3.10%). The CpG loci measured in PGC1α show some intra (0.31-3.38%) and inter (2.01-4.36) plate variation in % methylation, however the variation is <5% for all CpGs. The CpG loci measured in SIRT1 show the most intra (0.91-7.95%) and inter (3.022-8.58%) plate variation in % methylation, with CpG -760 showing the most variation of all (8.58%). Therefore, methylation differences less than these COV values are explained by analytical thresholds.

2.2.7 Statistical analysis and modelling methylation data in the EarlyBird cohort

Statistical analyses were performed by Dr. Joanne Hosking (Plymouth University Peninsula School of Medicine and Dentistry), using IBM SPSS statistics version 19.0. An unpaired t-test with a bonferroni correction was used to determine any significant differences between gender and % methylation of each CpG loci. Pearson's correlation was used to determine the association between % methylation of each CpG at 5-7 years and % methylation at all subsequent ages. A longitudinal tracking co-efficient was determined for each CpG, where methylation at 5-7 years was regressed on all other measurements between 8-9 and 14 years simultaneously.

Pearson's correlation coefficient was used to determine if there were any significant associations between the proportions of the major circulating leukocyte populations; neutrophils and lymphocytes, and the methylation of individual CpG loci measured at each time point. Leukocyte proportions were measured by a leukocyte differential count on each peripheral blood sample, completed by Derriford Hospital, Plymouth, throughout the EarlyBird study.

In order to take into account the correlations between repeated measurements on the same children, mixed effects modelling was used to determine the association between methylation at 5-7 years and adiposity (DEXA % fat) and insulin resistance (HOMA-IR) between 8-9 and 14 years. Age was included in these models as a categorical variable, and covariates in the models included gender, age at peak height velocity (APHV) and minutes per week spent in moderate-to-vigorous physical activity (MVPA). All relevant 2-way interactions were tested.

2.2.8 Liposarcoma SW-872 cell line

Human liposarcoma cells, SW-872, (Cell Line Service) were thawed on arrival and seeded at $1.5x10^4$cells/cm^2. Cells were cultured in DMEM media (4500 mg/L glucose and L-glutamine) supplemented with 10% FBS and 1% PenStrep, and maintained at 37°C, in an atmosphere containing 5% CO_2. Cells were sub-cultured when 80% confluent, using 0.25% trypsin EDTA.

2.2.9 Nuclear extracts

Nuclear extracts were prepared from human liposarcoma cell pellets (SW-872). Cell pellets ($4x10^6$ cells) were resuspended in 800µl of ice-cold lysis buffer (10mM HEPES, pH 7.9, 10mM KCl, 0.1mM EDTA, 1mM DTT, 0.5mM PMSF, 0.025mM leupeptin) and incubated on ice for 10 min (141). Next, 50µl of 10% Igepal ca-360 was added, vortexed for 10 seconds and spun at 13,000rpm for 30 seconds, 4°C (Eppendorf, Centrifuge 5415 R). The supernatant was discarded and the pellet containing nuclei was re-suspended in 50µl of ice-cold nuclear extraction buffer (20mM HEPES, pH 7.9, 0.4M NaCl, 1mM EDTA, 1mM DTT, 0.5mM PMSF) (141). This was then incubated on ice for an hour and nuclear debris pelleted at 4°C for 5 minutes at 13,000rpm (Eppendorf, Centrifuge 5415 R). The protein concentration of nuclear extracts was determined with the BCA protein assay kit (Pierce) according to the manufacturer's instructions. The nuclear extracts were alliquoted (50µl) and stored at -80°C until use.

2.2.10 Analysis of transcription factor binding by electrophoretic mobility shift assay

2.2.10.1 Annealing oligonucleotides

Complementary single stranded oligonucleotides (100µM) were mixed together in a 1:1 molar ratio and diluted to the required concentration in 1ml of Tris buffer containing salt (10mM Tris, 1mM EDTA, 50mM NaCl)(pH 8.0) (http://www.piercenet.com/files/TR0045-Anneal-oligos.pdf). Oligonucleotides were annealed by heating to 95°C for 5 minutes and slowly cooling to room temperature. Biotin-labelled double-stranded oligonucleotide probes corresponding to a

region of the PGC1α promoter from -797 to -764 with respect to the transcription start site containing either an unmethylated or methylated cytosine base at -783 were diluted to 1pmol. Unlabelled double-stranded specific and non-specific competitors, and oligonucleotides containing transcription factor binding consensus sequences, were diluted to 1pmol and 5pmol. Double-stranded oligonucleotides were aliquoted (50µL) and stored at -20°C.

Table 2.10- EMSA oligonucleotide sequences

	Forward	Reverse
Unmethylated PGC1α CpG -783 Probe	GAGCAGAGCAGCAG**C**GACTGTATTTACTAACACT	AGTGTTAGTAAATACAGT**C**GCTGCTGCTCTGCTC
Methylated PGC1α CpG -783 Probe	GAGCAGAGCAGCAG^{me}**C**GACTGTATTTACTAACACT	AGTGTTAGTAAATACAGT^{me}**C**GCTGCTGCTCTGCTC
Non-Specific Competitor	CCCCAGCCTCCCGGCGGGGTCACCC	GGGTGACCCCGCCGGGAGGCTGGGG
HNF1α Consensus Sequence (142)	GTTAATNATTAAC	GTTAATNATTAAC
HNF3β Consensus Core Sequence (142)	T[A/G]TT[G/T]AC	GT[A/C]AA[C/T]A
HNF3β Consensus Sequence (142)	CATT[A/G]TT[G/T]ACTT	AAGT[A/C]AA[C/T]AATG
HOXB9 Consensus Sequence (143)	TT[A/T]ACGAC	GTCGT[A/T]AA
PBX1 Consensus Sequence (144)	TTGATTGAT	ATCAATCAA
HOXB9-PBX1 Dimer Consensus Sequence (143)	ATGATTTACGAC	GTCGTAAATCAT

2.2.10.2 Electrophoretic mobility shift assay (EMSA)

Electrophoretic mobility shift assays were performed using the LightShift Chemiluminescent EMSA Kit (Fisher Scientific). All binding reactions were carried out in the presence of the non-specific DNA competitor poly (dI-dC) (1µg) and final concentrations of 0.5x binding buffer, 2.5% glycerol, 0.05% NP-40, 5mM MgClg (145). For reactions carried out in the presence of nuclear extract from SW-872 cells, a total of 5µg was utilised. Where applicable, competition was performed in the presence of 25-500-fold excess of either unlabelled specific oligonucleotides (unmethylated or methylated), unlabelled non-specific oligonucleotides, or unlabelled transcription factor binding consensus sequences (table 2.10). Where applicable a total of 1µg of the anti-HOXB9 (monoclonal, AbCam) or anti-PBX1 (polyclonal, AbCam) antibody was added to the reaction in replacement of specific competitor or transcription factor binding consensus sequences. All binding reactions were made up to a final volume of 18µl with DNase and RNase free water and incubated on ice for 30 minutes prior to the addition of biotin-labelled double-stranded PGC1α probe (10fmol), either unmethylated or methylated, followed by a further 20 minute incubation on ice (145).

Complexes were resolved on a pre-run 5% non-denaturing polyacrylamide gel in 0.5X Tris-borate-EDTA for 50 minutes at 100V, 4°C, followed by semi dry transfer (200mA, 1hr) to a positive nylon membrane and UV cross-linking (145). After incubation in blocking buffer for 15 min at room temperature, the membrane was incubated with streptavidin-HRP conjugate for 15 minutes. The membrane was then washed and visualised with a chemiluminescent substrate according to the manufacturer's instructions (Fisher Scientific).

2.2.11 Cloning the PGC1α promoter

2.2.11.1 Excising the PGC1α promoter from the SwitchGear Genomics PGC1α plasmid

The PGC1α promoter fragment was excised from the SwitchGear Genomics plasmid by digesting 2µg of plasmid with 4µl SacI, 4µl HindIII and 8µl of Fast Digest buffer (Thermo Fisher), made to a total volume of 80µl with DNase and RNase free water. The PGL3-basic plasmid (1µg) was digested with 2µl SacI, 2µl HindIII and 4µl of Fast Digest buffer, made to a total volume of 40µl with DNase and RNase free water. Both reactions were incubated at 37°C for 1 hour before running on a 1% agarose gel. The gel was visualised under low UV and the appropriate sized bands were excised; PGC1α promoter 951bp, PGL3-basic backbone 4776bp. The DNA was purified using the ZymoClean Gel DNA Recovery kit according to the manufacturer's instructions and eluted in 6µl of DNase and RNase free water.

2.2.11.2 Ligation of the PGC1α promoter into PGL3-basic plasmid

A 3-fold molar excess of PGC1α promoter insert was added to 50ng of cut PGL3-basic plasmid using the equation below, and then made to a total volume of 10µl with DNase and RNase free water.

Calculating ng of insert:

= [ng vector x size of insert (in Kb)]/ size of vector

= [50ng x 0.951kb]/4.776kb
= 9.95ng (1:1 ratio)
= 9.95ng x 3 (3:1 ratio)
= **29.86ng of PGC1α promoter insert**

A negative control, where the PGC1α promoter insert was replaced with water, was added to 50ng of cut PGL3-basic plasmid. 10µl of 2x quick ligation buffer was added to the positive and negative reactions, before adding 1µl of Quick T4 DNA ligase (New England Biolabs), and mixed well, before briefly centrifuging and incubating at room temperature for 5 minutes. The reaction was chilled on ice until required for the transformation.

2.2.11.3 Agar plates and luria broth

Agar (7.5g) and LB (10g) were added to 500mL of water, autoclaved and left to cool to 50°C before adding the required concentration of antibiotic and mixing; Ampicillin at 100µg/ml (PGC1α-PGL3-basic plasmid, pcDNA3+ plasmid) and ampicillin at 50µg/ml (PBX1 cDNA clone plasmid). Agar plates were made using under sterile technique by pouring 20ml into a petri dish and left to set for 30 minutes. Plates were stored at 4°C upside down until they were required, to avoid condensation forming.

2.2.11.4 Transformation

JM109 competent cells (Promega) were thawed on ice and 50µl added to a pre-chilled eppendorf, before adding 5µl of the positive and negative ligation reactions. A negative control where the DNA was replaced with 5µl of water, and a positive control where 50ng of intact SwitchGear Genomics PGC1α plasmid were each added to 50µl of JM109 competent cells. The eppendorfs were gently mixed by flicking, and then incubated on ice for 20 minutes before heating to 42°C for exactly 45 seconds. The eppendorfs were added to ice immediately for 2 minutes before adding 450µl of LB

(no antibiotics) using sterile technique and incubated at 37°C for 1 hour with shaking (225rpm). The cells were pelleted at 10,000rpm for 5 minutes (Eppendorf, Centrifuge 5415 R) and the supernatant removed, except 100µl in which to resuspend the pellet. The transformation mixture was plated onto pre-warmed LB agar plates containing 100µg/ml ampicillin in 3 volumes to enable single colonies to be picked (10µl, 30µl and 60µl). Plates were incubated upside down for 16 hours at 37°C. After the incubation, plates were stored at 4°C until colonies were picked. The negative plates did not contain colonies confirming both the ligation and transformation were specific.

2.2.11.5 Inoculation

Using a sterile tip, a single colony was picked from the positive ligation plate containing the PGC1α promoter in the PGL3-basic plasmid, and added to 10ml of LB media containing 100µg/ml ampicillin. Several colonies were picked from each plate (10µl, 30µl and 60µl). The bacterial culture was incubated for 16 hours at 37°C with shaking (225rpm). A negative control containing just LB media without a colony was incubated also. After 16 hours, 1ml of each culture was removed and stored at 4°C for further use.

2.2.11.6 Minipreperation of PGC1α-PGL3-basic plasmid DNA

Bacterial cultures were pelleted by centrifugation at 13,000rpm for 10 minutes and the supernatant discarded (Eppendorf, Centrifuge 5415 R). Tubes were left upside down for 2 minutes in order to remove excess media. Pellets were resuspended in cell resuspension solution before adding cell lysis solution and gently mixing by inversion 5 times. The cell lysis reaction was terminated after 3 minutes by adding neutralisation solution and mixing thoroughly by inversion 5 times. Unwanted proteins and genomic DNA were pelleted by centrifugation at 13,000rpm, 10 minutes (Eppendorf, Centrifuge 5415 R). The lysate containing the plasmid was added to a binding column and spun by centrifugation, followed by a wash step with endotoxin removal solution then an ethanol wash solution. Plasmid DNA was eluted in 50µl of DNase and RNase free water according to the Qiagen kit recommendations. The DNA concentration and purity of the plasmid was determined using the Nanodrop.

2.2.11.7 Confirming the presence of PGC1α-PGL3-basic plasmid DNA

Purified plasmid DNA (250ng) prepared in section 2.2.11.6 was digested with 1µl NcoI, 1µl Fast Digest buffer (Thermo Fisher) and made to a total volume of 10µl with water. NcoI is a double cutter within the PGC1α-PGL3-basic construct; however NcoI does not cut the backbone of the original PGC1α SwitchGear Genomics plasmid and only cuts the PGL3-basic plasmid once, and therefore confirms the promoter fragment is present within the PGL3-basic plasmid. The reaction was incubated at 37°C for 1 hour before running on a 1% agarose gel to confirm the presence of 2 bands, shown in figure 2.3.

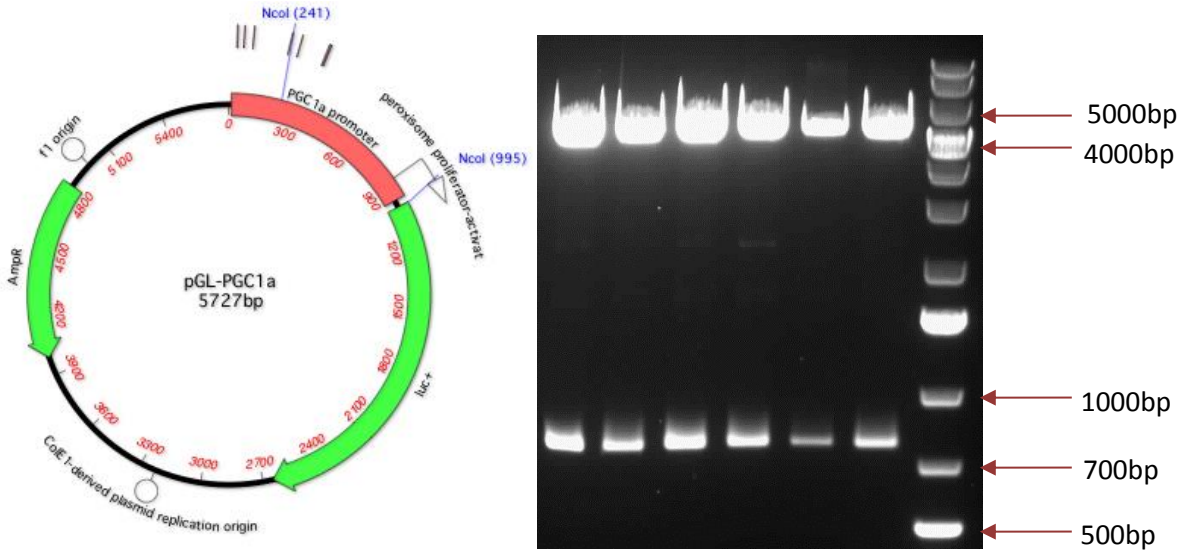


Figure 2.3- Digesting the PGC1α-PGL3-basic construct with NcoI. This schematic diagram represents the PGC1α-PGL3-basic construct, detailing where the two NcoI recognition sequences are. Each restriction enzyme digest was run on a 1% agarose gel. Two bands of 754bp and 4973bp confirmed the PGC1α promoter had correctly ligated into the PGL3-basic plasmid.

All samples were confirmed to contain the PGC1α promoter within the PGL3-basic plasmid. Therefore, the 1ml of each culture that was stored at 4°C was added to 50ml of LB media containing 100µg/ml ampicillin and incubated for 16 hours at 37°C with shaking (225rpm), in order to purify larger quantities of plasmid DNA. A negative control containing just LB media without a colony was incubated also.

2.2.11.8 Midipreperation of PGC1α-PGL3-basic plasmid DNA

Bacterial cultures were pelleted by centrifugation, and pellets were resuspended, lysed and neutralised as previously described in section 2.2.11.6. Unwanted proteins and genomic DNA was removed by centrifugation through a clearing column at 1,500g for 5 minutes. The lysate containing the plasmid was added to a binding column and spun by centrifugation, followed by a wash step with endotoxin removal solution then an ethanol wash solution. Samples were eluted in 600µl of DNase and RNase free water according to the Promega kit recommendations. The DNA concentration and purity of the plasmid was determined using the Nanodrop. All purified plasmid DNA was digested with NcoI (described in section 2.2.11.7) to confirm the presence of the PGC1α promoter fragment within the PGL3-basic plasmid. Samples were also confirmed to be correct by Sanger sequencing.

2.2.11.9 Creating glycerol stocks for long-term storage

Glycerol (99%) was diluted to 30% using ultrapure water, and 350µl aliquots were autoclaved. Liquid culture (350µl), removed before bacterial cultures were pelleted in section 2.2.11.8, was added to a glycerol aliquot to give a final concentration of 15%, and vortexed for 30 seconds to ensure through mixing before snap freezing. Glycerol stocks were stored at -80°C for future use.

2.2.11.10 Generating plasmid DNA from stab cultures

Upon arrival, the PBX1 cDNA clone and pcDNA3+ stab cultures were scraped with a sterile loop and streaked onto an agar plate containing the appropriate antibiotic (section 2.2.11.3). Plates were incubated upside down for 16 hours at 37°C. After the incubation, plates were stored at 4°C upside down until single colonies were picked from each plate and added to 10ml of LB media containing the appropriate antibiotic. The bacterial culture was incubated for 16 hours at 37°C with shaking (225rpm). A negative control containing just LB media without a colony was incubated also. After 16 hours, 1ml of each culture was removed and stored at 4°C for further use.

Plasmid DNA was prepared by minipreparation, as described in section 2.2.11.6, and 250ng of PBX1 was digested with NotI and SalI (1µl of each), 1µl Fast Digest buffer (Thermo Fisher) and made to a total volume of 10µl with water. The reaction was incubated at 37°C for 1 hour before running on a 1% agarose gel to confirm the presence of 2 bands at 1830bp and 4375bp for PBX1. The restriction enzyme digest confirmed the plasmid DNA was correct. Therefore, the 1mL of bacterial culture stored at 4°C was added to 50ml of LB media containing the appropriate antibiotic and incubated for 16 hours at 37°C with shaking (225rpm), in order to purify larger quantities of plasmid DNA by

midipreperation, as described in section 2.2.11.8. A glycerol stock was generated for each plasmid for long term storage at -80°C, as described in section 2.2.11.9.

2.2.12 Transfection

2.2.12.1 Preparing plasmid DNA for tissue culture

Plasmid DNA was sterilised by adding 1/10th of the volume of sodium acetate (pH 5.2), 2 volumes of ice cold 100% ethanol and incubating at -20°C overnight. Plasmid DNA was pelleted by centrifugation at 13,000rpm for 15 minutes and the supernatant removed (Eppendorf, Centrifuge 5415 R). The DNA was washed in 1ml of 70% ethanol and spun at 13,000 for 5 minutes. The supernatant was carefully removed and the pellet was air dried for 5 minutes, before resuspending in sterile water to a concentration of 100ng/µl (SV40 control plasmid (Promega), PGC1α-PGL3-basic and PGL3-basic) or 1000ng/µl (PBX1 cDNA clone (Source BioScience) and pcDNA3+ (Invitrogen)).

2.2.12.2 Transfection of PGC1α-PGL3-basic plasmid DNA

Human liposarcoma cells, SW-872, (Cell Line Service) were seeded at 5 x 10^3 in a 96 well plate. Cells were plated in triplicate for each separate transfection, along with 9 wells in a separate plate to allow cell density to be determined each day. Cells were grown overnight in 100µl of media, as described in section 2.2.8, at 37°C, in an atmosphere containing 5% CO_2.

After 16 hours, cells were 50% confluent and were transfected with plasmid DNA using FuGENE HD Transfection Reagent (Active Motif) according to the manufacturer's instructions. FuGENE HD and opti-MEM were left to reach room temperature before use, and FuGENE HD vortexed prior to use. The appropriate volume of opti-MEM was added to each eppendorf (to make the total volume to 5µl), and 0.6µl of FuGENE HD added directly to the media without touching the sides of the tube, before incubating at room temperature for 5 minutes. The appropriate firefly reporter plasmid DNA (PGC1α-PGL3-basic, SV40 or PGL3-basic) (98.75ng) and Renilla CMV control plasmid (1.25ng), were added and mixed, achieving a 6:1 FuGENE HD to DNA ratio, before incubating at room temperature for 30 minutes. Transfection mix (5µl) was added to the appropriate wells of a 96 well plate containing 100µl of media. The media was gently mixed by pipetting up and down, before incubating for 48 hours at 37°C, in an atmosphere containing 5% CO_2.

2.2.12.3 Co-transfection

Cells were plated, as described in section 2.2.12.2. After 16 hours, cells were 50% confluent and were co-transfected using FuGENE HD Transfection Reagent, as described previously. Cells were co-transfected with PGC1α-PGL3-basic DNA (98.75ng), Renilla CMV control plasmid (1.25ng), PBX1 plasmid DNA (1000, 750, 500, 250, 0ng) and pcDNA3+; an empty pCMV promoter plasmid to serve as a positive control (0, 250, 500, 750, 1000ng). In each combination, PBX1 plasmid and pcDNA plasmid totalled 1000ng DNA, to ensure the total amount of DNA was consistent between different conditions. After incubating the DNA with FuGENE HD and opti-MEM for 30 minutes at room temperature, 5µl of transfection mix was added to the appropriate wells of a 96 well plate containing 100µl of media. The media was gently mixed by pipetting up and down, before incubating for 48 hours at 37°C, in an atmosphere containing 5% CO_2.

2.2.12.4 Preparing cells for the Dual-Luciferase assay

48 hours after transfection, media was removed and the cultured cells were rinsed with 100µl of PBS. All traces of liquid were removed before adding 20µl of 1x Passive Lysis Buffer (Promega) to each well and mixed by pipetting up and down. Cells underwent 3 cycles of freeze thawing before incubating at room temperature on a rocking platform for 15 minutes. The lysed cells were transferred to a white 96 well plate in order to read luciferase activity on the Thermo Varioskan Flash Luminometer.

The luminometer was primed with 1500µl of Luciferase Assay Reagent II and 1500µl Stop and Glo reagent (Promega). 100µl of Luciferase Assay Reagent II was added to each well, followed by 100µl of Stop and Glo reagent. The level of firefly luciferase activity was normalised to renilla luciferase activity.

2.2.13 Comparing parental EarlyBird DNA methylation with DNA methylation of the child

2.2.13.1 Measuring parental EarlyBird DNA methylation

Peripheral blood from the parents of the EarlyBird children was collected into EDTA tubes following an overnight fast at the start of the study, when the children were aged 5 years. Genomic DNA was extracted by Exeter University, using a high salt method. The quality and quantity of the DNA was determined using the Nanodrop 1000. All samples had a 260/280 ratio between 1.5 and 2.2. All samples had between 1.16µg and 15.66µg total DNA. Samples were stored at -20°C.

Genomic DNA (1µg) was bisulphite treated, as described in section 2.2.3. Control samples included unmethylated DNA (Millipore and Qiagen), methylated DNA (Millipore), human genomic DNA in triplicate (Promega), and a no template control (NTC) where the DNA was replaced with 1µl of water. Bisulphite converted DNA was used in a PCR using each assay and the optimised conditions in table 2.5. An NTC was used for each assay where the DNA was replaced with 1µl of water. To confirm the product size, 10µl of each control and a random selection of 6 samples were run on a 1.5% agarose gel, shown in figure 2.4.

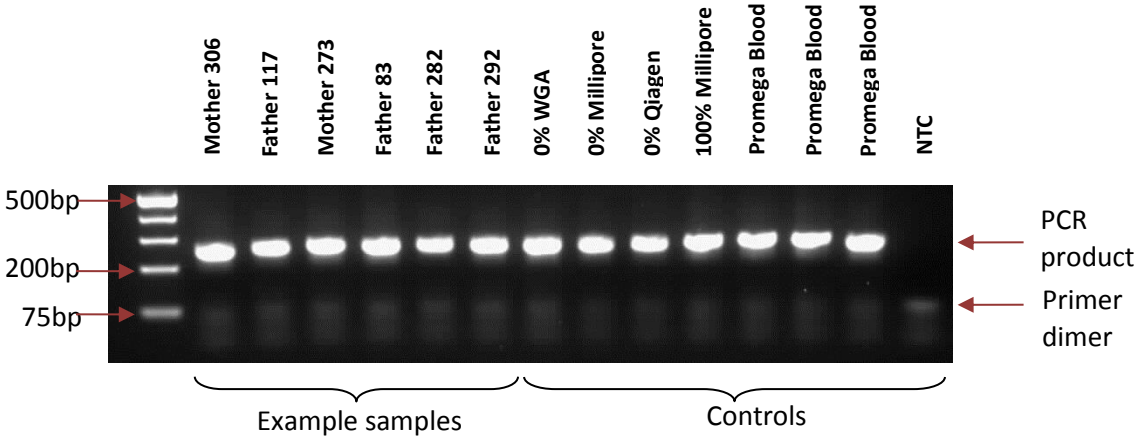


Figure 2.4- SIRT1 CpG -760 to -668 parental PCR. For each PCR using parental EarlyBird converted DNA, a selection of 6 samples and all controls were run on a 1.5% agarose gel. Lanes are labelled with the corresponding EarlyBird subject number and each control is labelled. The PCR product was 247bp and a small amount of primer dimer was present as a by-product of the reaction.

The methylation of individual CpG loci was determined by pyrosequencing using 10µl of PCR product, as described in section 2.2.5. The pass rates for each assay are shown below in table 2.11.

Table 2.11- Pass rates for parental EarlyBird pyrosequencing assays

Assay Name	Sample Pass rate
SIRT1 CpG -880 to -865	90%
SIRT1 CpG -760 to -722	90%
SIRT1 CpG -689 to -668	90%
PGC1α CpG -841 to -783	94%
PGC1α CpG -652 to -617	97%
PGC1α CpG -521 to -515	93%
HNF4α CpG -121 to -79	92%
RXRα CpG -2346 to -2335	92%
GCK CpG -322 to -282	93%
GCK CpG -192 to -172	90%
GCK CpG +30 to +37	93%

2.2.13.2 Statistical analysis of parental and child EarlyBird methylation

Statistical analyses were performed using IBM SPSS Statistics version 21.0. The methylation of each child at 5-7 years was compared to the methylation of the corresponding mother and father separately, using a linear regression, for each CpG loci. Where appropriate, the data was transformed when the methylation was not normally distributed. The methylation of the parent was the independent variable and the methylation of the child was the dependent variable. The methylation at 5-7 years was used because the blood was taken from the parents at the start of the study when the children were aged 5 years. The statistical analysis was also repeated comparing the methylation of each child at 14 years with the methylation of the corresponding mother and father separately, using a linear regression. This was because the methylation at 14 years was a single methylation value rather than a mean. The statistical analysis was repeated using the methylation at 5-7 years and 14 years for just the girls and comparing to the methylation of the corresponding mother and father separately, using a linear regression. This was then repeated using the methylation for just the boys and the methylation of the corresponding mother and father, to determine if there were any sex specific associations.

2.2.14 Measuring DNA methylation in the Southampton Women's Survery (SWS) cohort

2.2.14.1 Isolation of genomic DNA from umbilical cord

DNA was isolated from umbilical cords which were collected as part of the Southampton Women's Survey (SWS). Umbilical cords were snap frozen upon collection and stored at -80°C long term. Umbilical cord was crushed using a pestle and mortar, in the presence of liquid nitrogen, and tissue was not allowed to thaw during crushing. Hyaluronidase solution (500µl) (0.0125mg/ml) was added to 500mg of powdered umbilical cord and gently mixed by flicking. Samples were briefly spun to ensure the tissue was fully immersed in the hyaluronidase solution, before incubation at 37°C for 30 minutes with shaking (150rpm).

Genomic DNA was extracted using a high salt method, with 100% sample recovery efficiency. TNES solution (500µl) (50mM Tris, 400mM NaCl, 100mM EDTA and 0.5% SDS) was added to each sample and gently tapped to ensure mixing before homogenisation (30 seconds per sample) using a dispomix homogenizer (Miltenyi Biotec). Each sample was briefly spun before the addition of 5µl of proteinase K (20mg/ml) and incubation at 55°C for 16 hours. Then, 500µl of NaCl (2.6M) was added before vigorous shaking for 15 seconds, followed by centrifugation at 12,000rpm for 20 minutes at room temperature (Eppendorf, Centrifuge 5415 R). The supernatant was removed and the DNA was precipitated by adding 900µl of ice cold 100% ethanol, which was split equally between two clean eppendorfs. One eppendorf was stored at -20°C for future use; the other was processed by transferring the precipitated DNA to a fresh eppendorf containing 500µl of DNase and RNase free water with 5µl RNase A (10mg/ml), ensuring excess ethanol was not transferred. Samples were incubated at 37°C for 30 minutes before 500µl of phenol/chloroform (50:50) was added to each sample and inverted 5 times to ensure thorough mixing. Samples were centrifuged at 12,000rpm for 5 minutes at room temperature and the top aqueous layer (~500µl) was transferred to a clean eppendorf, without disturbing the middle protein layer (Eppendorf, Centrifuge 5415 R). The phenol/chloroform wash step was repeated and the top aqueous layer (~450µl) was transferred to a clean eppendorf. Next, 45µl of NaAc (3M, pH 5.2) and 900µl of ice cold 100% ethanol was added to each sample and mixed well. The precipitated DNA was transferred to a clean eppendorf and was resuspended in 500µl of DNase and RNase free water.

The quality and quantity of the DNA was determined using the Nanodrop 1000. All samples had a 260/280 ratio between 1.5 and 1.8. All samples had between 2.04µg and 177.10µg total DNA. 500ng of each sample was run on a 0.8% agarose gel, shown in figure 2.5. All samples were stored at -20°C.

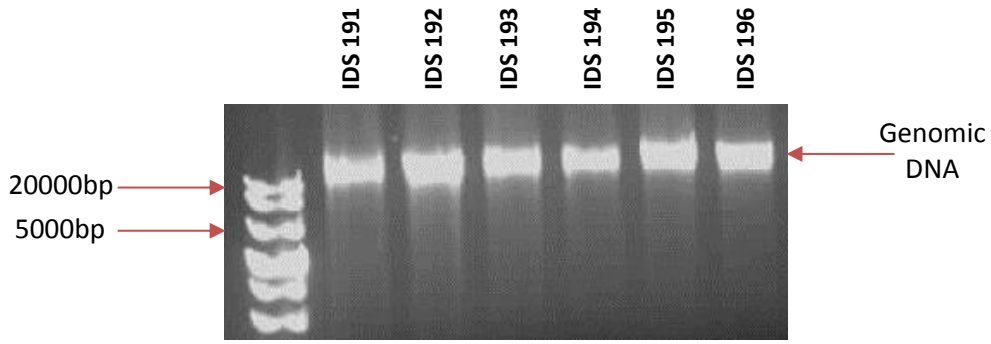


Figure 2.5- Genomic DNA from umbilical cord from the SWS cohort. These example samples were selected to demonstrate the quality of DNA isolated using the high salt method. These samples represent the range of concentrations and 260/280 ratios of all samples. Lanes are labelled with the IDS number, which was randomly assigned when extracting the DNA.

2.2.14.2 Isolation of genomic DNA from umbilical cord blood

Genomic DNA was extracted from 500µl umbilical cord blood using the high salt method described in section 2.2.14.1, with 100% sample recovery efficiency. The precipitated DNA was resuspended in 500µl of DNase and RNase free water and the quality and quantity of the DNA was determined using the Nanodrop 1000. All samples had a 260/280 ratio between 1.5 and 2.1. All samples had between 1.10µg and 35.10µg total DNA. 500ng of each sample was run on a 0.8% agarose gel. All samples were stored at -20°C.

2.2.14.3 Measuring DNA methylation in the SWS cohort

The number of samples selected for each tissue was determined by the amount of remaining DNA in the SWS cohort. Samples with more than 30µg of umbilical cord and umbilical cord blood were used for measuring DNA methylation. Genomic DNA (1µg) from 440 SWS umbilical cords and 299 SWS umbilical cord bloods was bisulphite treated, as described in section 2.2.3. Control samples included unmethylated DNA (Qiagen), methylated DNA (Millipore), human genomic DNA in triplicate (Promega and Roche), and a no template control (NTC) where the DNA was replaced with 1µl of water. Bisulphite converted umbilical cord DNA was used in a PCR using the optimised conditions listed in table 2.5 for SIRT1 CpG -880 to -865, PGC1α CpG -841 to -783 and PGC1α CpG -521 to -515. Bisulphite converted umbilical cord blood DNA was used in a PCR using the optimised conditions listed in table 2.5 for each assay for SIRT1 and PGC1α. An NTC was used for each assay where the DNA

was replaced with 1µl of water. To confirm the product size, 10µl of each control and a random selection of 4 samples were run on a 1.5% agarose gel, shown in figure 2.6.

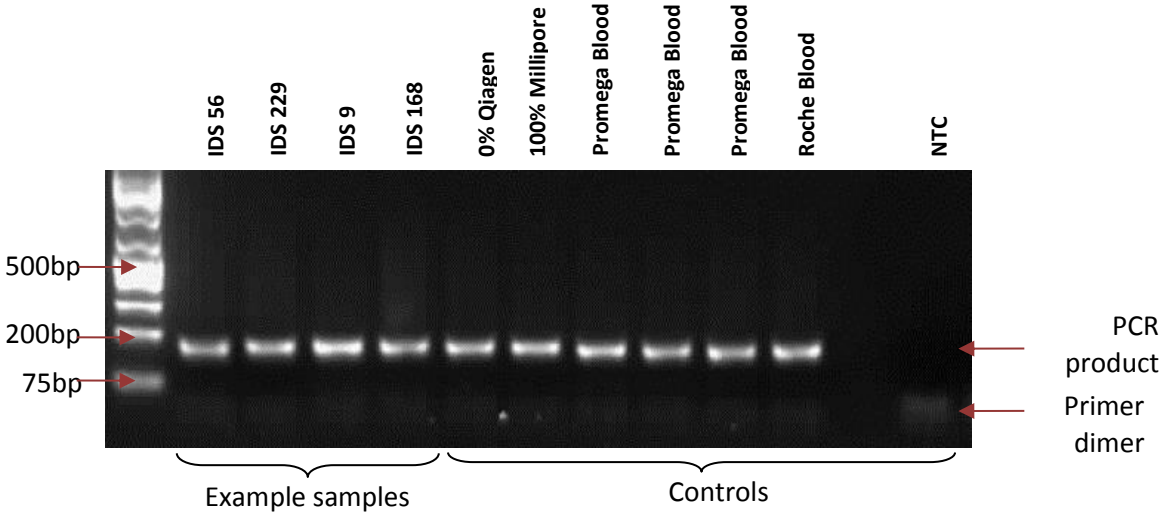


Figure 2.6- PGC1α CpG -841 to -783 SWS Umbilical Cord Blood PCR. For each PCR using SWS umbilical cord and cord blood converted DNA, a selection of 4 samples and all controls were run on a 1.5% agarose gel. Lanes are labelled with the corresponding IDS subject number and each control is labelled. The PCR product was 168bp and a small amount of primer dimer was present as a by-product of the reaction.

The methylation of individual CpG loci for SIRT1 and PGC1α was determined by pyrosequencing using 10µl of PCR product, as described in section 2.2.5. The pass rates for each assay are shown below in table 2.12.

Table 2.12- Pass rates for SWS umbilical cord and cord blood pyrosequencing assays

Assay Name	Sample Pass Rate Umbilical Cord	Sample Pass Rate Umbilical Cord Blood
SIRT1 CpG -880 to -865	77%	96%
SIRT1 CpG -760 to -722	Not measured	83%
SIRT1 CpG -689 to -668	Not measured	95%
PGC1α CpG -841 to -783	95%	91%
PGC1α CpG -652 to -617	Not measured	97%
PGC1α CpG -521 to -515	82%	91%

2.2.14.4 Statistical analysis in the SWS cohort

Statistical analyses were performed using IBM SPSS Statistics version 21.0. Where appropriate the data was transformed when the methylation or phenotypic outcomes were not normally distributed. The methylation of each CpG loci was the independent variable and the phenotypic outcome was the dependent variable, and the statistical model accounted for age and gender of the child. The association between the methylation of each CpG loci and several phenotypic outcomes at several different ages including total fat mass, % fat mass, total lean mass, % lean mass, total bone mineral content, % bone mineral content, total prentice bone mineral density and skinfold thickness, was determined using a linear regression.

2.2.15 Measuring DNA methylation and expression in subcutaneous adipose tissue from the BIOCLAIMS cohort

2.2.15.1 Isolation of genomic DNA from abdominal subcutaneous adipose tissue

Abdominal subcutaneous adipose tissue was collected as part of the **Bio**markers of robustness of metabolic homeostasis for nutrigenomics-derived health **claims** made on food (BIOCLAIMS) cohort. Subjects were classified as lean or obese according to their BMI. Samples were available for 23 lean subjects and 42 obese subjects. Subjects were not on any medications. Subject characteristics are summarized in table 2.13, for males and females separately. Values shown are mean ± SD.

Table 2.13- Subject characteristics for subjects in the BIOCLAIMS cohort

	Lean		Obese	
	Males	**Females**	**Males**	**Females**
Number	4	19	11	31
Age (years)	34.8 ± 19.2	33.2 ± 15.6	40.7 ± 14.4	44.3 ± 10.6
Height (m)	1.8 ± 0.0	1.6 ± 0.0	1.8 ± 0.1	1.6 ± 0.1
Weight (kg)	76.0 ± 4.3	57.4 ± 5.4	114.4 ± 12.5	93.8 ± 12.5
BMI (kg/m^2)	22.9 ± 1.0	21.7 ± 1.8	34.5 ± 4.5	34.9 ± 2.8
Waist (cm)	86.5 ± 11.1	73.7 ± 5.2	117.0 ± 13.2	104.3 ± 9.5
Hip (cm)	96.9 ± 3.4	91.7 ± 6.1	117.4 ± 10.7	118.1 ± 8.8
Waist to hip ratio	0.9 ± 0.1	0.8 ± 0.1	1.0 ± 0.0	0.9 ± 0.1
Total fat mass (kg)	13.2 ± 5.4	15.5 ± 3.6	35.1 ± 9.3	41.9 ± 8.3
% fat mass	17.2 ± 6.8	26.8 ± 4.5	30.9 ± 6.0	44.4 ± 3.7
Lean mass (kg)	62.8 ± 5.9	41.9 ± 3.6	77.1 ± 6.2	51.9 ± 5.2

Genomic DNA was extracted from 50mg of adipose tissue using the QIAamp DNA mini kit (Qiagen), according to the manufacturer's instructions, with 100% sample recovery efficiency. Samples were eluted in 100µl of DNase and RNase free water twice as this recovered the largest amount of DNA with a suitable concentration for downstream processing. Both elutions for each sample were combined to give a total volume of 200µl.

The quality and quantity of the DNA was determined using the Nanodrop 1000. All samples had a 260/280 ratio between 1.5 and 2.1. All samples had between 1.6µg and 13.3µg total DNA. 500ng of each sample was run on a 0.8% agarose gel, shown in figure 2.7. Any samples with a concentration less than 25ng/µl were concentrated for an appropriate length of time to achieve 25ng/µl to enable 0.5µg of DNA to be bisulphite converted. All samples were stored at -20°C.

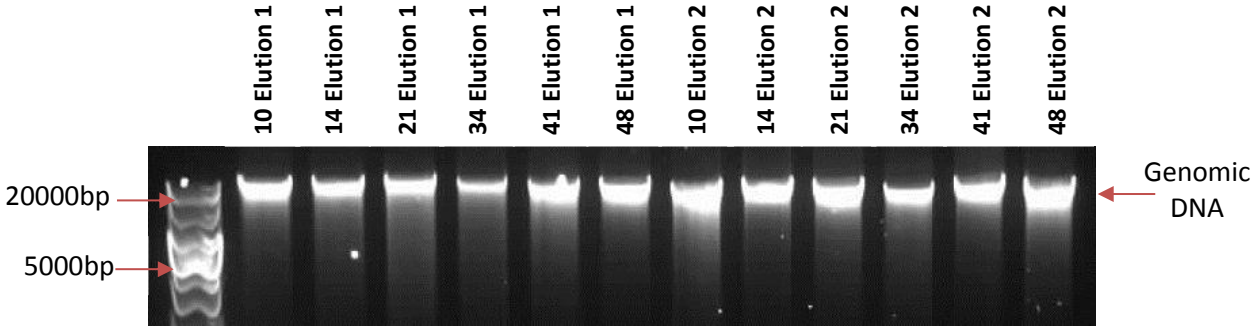


Figure 2.7- Genomic DNA from adipose tissue from the BIOCLAIMS cohort. These example samples were selected to represent the range of concentrations and 260/280 ratios of all subjects, for each elution. Lanes are labelled with the subject number, which was randomly assigned at the start of the BIOCLAIMS study.

2.2.15.2 Measuring DNA methylation in the BIOCLAIMS cohort

Genomic DNA (500ng) was bisulphite treated, as described in section 2.2.3. Control samples included unmethylated DNA (Qiagen), methylated DNA (Millipore), human genomic DNA (Promega and Roche), and a no template control (NTC) where the DNA was replaced with 1µl of water. Bisulphite converted DNA was used in a PCR using the optimised conditions listed in table 2.5 for SIRT1 CpG -880 to -865 and each assay for PGC1α. An NTC was used for each assay where the DNA was replaced with 1µl of water. To confirm the product size, 10µl of each control and a random selection of 6 samples were run on a 1.5% agarose gel. The methylation of individual CpG loci for SIRT1 and PGC1α was determined by pyrosequencing using 10µl of PCR product, as described in section 2.2.5. The pass rates for each assay are shown below in table 2.14.

Table 2.14- Pass rates for BIOCLAIMS subcutaneous adipose tissue pyrosequencing assays

Assay Name	Sample Pass rate
SIRT1 CpG -880 to -865	98%
PGC1α CpG -841 to -783	98%
PGC1α CpG -652 to -617	95%
PGC1α CpG -521 to -515	100%

2.2.15.3 Isolation of total RNA from subcutaneous adipose tissue

Human subcutaneous adipose tissue (75mg) was homogenised in 1ml of QIAzol Lysis Reagent (Qiagen), before incubation at room temperature for 3 minutes. Total RNA was extracted using the RNeasy Lipid Tissue Mini Kit, according to manufacturer's instructions, with 100% sample recovery efficiency. Samples were eluted in 30µl of DNase and RNase free water and the quality and quantity determined using the Nanodrop 1000 and the Agilent Technologies Bioanylser. All samples had a 260/280 ratio between 1.7 and 2.2 and between 0.55µg and 4.82µg total RNA. All samples had an RNA Integrity Number (RIN) of more than 5.40.

2.2.15.4 cDNA synthesis

RNA from each subcutaneous adipose sample was converted to cDNA, plus 10 extra samples for controls. RNA (0.5µg) was incubated with 1µl dNTP mix (10mM) and 1µl random nanomers (5µM) for 10 minutes at 70°C. Samples were placed on ice before adding 1µl M-MLV reverse transcriptase (200 units/µl), 4µl 10x M-MLV reverse transcriptase buffer and 5µl DNase and RNase free water, to make a total volume of 20µl. Samples were incubated at room temperature for 10 minutes, before incubation at 37°C for 50 minutes, followed by 90°C for 10 minutes to denature the enzyme. cDNA was diluted with DNase and RNase free water to a total volume of 160µl (6.25ng/µl). cDNA from the 10 extra samples was combined and mixed thoroughly. The pooled cDNA (200µl) was mixed with 600µl of DNase and RNase free water, giving a final concentration of 12.50ng/µl. This sample was then serially diluted by adding 400µl of the sample to 400µl of DNase and RNase free water, and repeating 6 times. All samples were stored at -20°C.

2.2.15.5 Reference gene selection for RT-PCR

Optimal reference genes for accurate normalisation for RT-PCR were determined using the geNorm Reference Gene Selection Kit (Primerdesign). Six reference genes were tested which included B2M, CYC1, GAPDH, PPIA, TOP1 and YWHAZ, and were ranked according to their expression stability. To determine the optimal reference gene for subcutaneous adipose tissue, 6 samples from lean subjects and 6 samples from obese subjects were randomly selected and cDNA was synthesised, described in section 2.2.15.4.

A total of 25ng of cDNA (4µl) of each sample was mixed with 5µl QuantiTect SYBR Green PCR mastermix, 0.5µl of specific PrimerDesign primer mix and 0.5µl of DNase and RNase free water, giving a total volume of 10µl for each reaction. Each cDNA sample was measured in duplicate on a 384 well plate using the LightCycler 480 real time PCR machine (Roche Diagnostics Limited, UK). Each assay had 2 negative controls where the cDNA was replaced with 4µl of DNase and RNase free water. Cycle conditions were 95°C for 15 minutes, 45 cycles of 95°C for 15 seconds, 60°C for 30 seconds and 72°C for 30 seconds, followed by a melt curve of 95°C for 15 seconds, 60°C for 1 minute and a gradual increase to 95°C.

2.2.15.6 Real time PCR using cDNA from subcutaneous adipose tissue

A total of 25ng of cDNA (4µl) was mixed with 5µl QuantiTect SYBR Green PCR mastermix and 1µl of QuantiTect primer assays (Qiagen), giving a total volume of 10µl for each reaction. Each cDNA sample was measured in duplicate on a 384 well plate using the LightCycler 480 real time PCR machine (Roche Diagnostics Limited, UK). Each assay had 2 negative controls where the cDNA was replaced with 4µl of DNase and RNase free water. A standard curve was used for each assay using 7 serially diluted samples described in section 2.2.15.4, which was measured in duplicate. Cycle conditions were 95°C for 15 minutes, 45 cycles of 95°C for 15 seconds, 55°C for 30 seconds and 72°C for 30 seconds, followed by a melt curve of 95°C for 15 seconds, 60°C for 1 minute and a gradual increase to 95°C. Data for SIRT1 and PGC1α were normalised to cyclophillin (PPIA) as this was stably expressed in adipose tissue.

2.2.15.7 Statistical analysis in the BIOCLAIMS cohort

All statistical analyses were performed using IBM SPSS Statistics version 21.0. A Mann Whitney U test was used to determine any significant differences between the methylation of each CpG loci in lean and obese subjects. An unpaired t-test was used to determine any significant differences between normalised mRNA expression of SIRT1 and PGC1α in lean and obese subjects.

Subjects were split into case and control for lean and obese respectively, according to BMI, % fat, waist measurement and waist to hip ratio. A binary logistic regression was used to determine if there was a statistically significant difference between the case and control groups for each CpG loci and mRNA expression measured for SIRT1 and PGC1α.

2.2.16 Supplementing human liposarcoma cells with fatty acids

2.2.16.1 Culturing human liposarcoma cells supplemented with fatty acids

A stock concentration of each fatty acid was made to 200mM in ethanol and stored at room temperature. Fatty acids included n-3 polyunsaturated fatty acids (PUFAs)(ALA, EPA and DHA), n-6 PUFA (LA), monounsaturated fatty acids (10-H=DA and OA) and a saturated fatty acid (10-HDA) and Human liposarcoma cells, SW-872, were seeded at 1.5 x 10^5 in 6 well plates. Cells were grown overnight in 4ml of media, as described in section 2.2.8, at 37°C, in an atmosphere containing 5% CO_2. After 16 hours, cells were 50% confluent and all traces of media were removed.

Each fatty was diluted to 2mM in media and filter sterilised using a 2µM filter and syringe. Control media was also filter sterilised, which contained the equivalent amount of ethanol as each fatty acid media solution at 2mM. Media was added to each well of the 6 well plates, followed by the appropriate fatty acid to make a final concentration of 100µM, 50µM and 10µM in a total volume of 4ml. The control plate had the same volume of the 2mM media solution for the 100µM plate, to ensure the maximum amount of ethanol (0.1%) was added to determine if ethanol had any effects on cell growth. Cells were grown in fatty acid supplemented media for 72 hours before harvesting.

2.2.16.2 Isolation of total RNA from liposarcoma cells

All traces of fatty acid supplemented media was removed and cells were washed in 2ml of PBS. Cells in each well were scraped into 1ml of Tri-reagent and left to incubate at room temperature for 5 minutes, before transferring to a 1.5ml eppendorf and adding 200µl of chloroform. Samples were shaken vigorously for 15 seconds and left at room temperature for 3 minutes, before centrifugation at 12,000rpm for 15 minutes at 4°C (Eppendorf, Centrifuge 5415 R). The colourless

upper aqueous layer was transferred to a fresh eppendorf and the RNA was precipitated by adding 500µl of isopropanol. Samples were incubated at room temperature for 10 minutes and centrifuged at 12,000rpm for 20 minutes at 4°C (Eppendorf, Centrifuge 5415 R). The supernatant was removed and the RNA was washed by addition of 1ml of 75% ethanol, vortexing and centrifugation at 8,000rpm for 5 minutes at 4°C (Eppendorf, Centrifuge 5415 R). All traces of ethanol were removed and the pellet was left to air dry at room temperature for 10 minutes. RNA was redissolved in 20µl DNase and RNase free water by incubating at 60°C for 10 minutes. The quality and quantity of the RNA was determined using the Nanodrop 1000. All samples had a 260/280 ratio between 1.8 and 2.0. All samples had between 9.03µg and 23.55µg total RNA. 500ng of each sample was run on a 1% agarose gel, shown in figure 2.8. Samples were stored at -80°C.

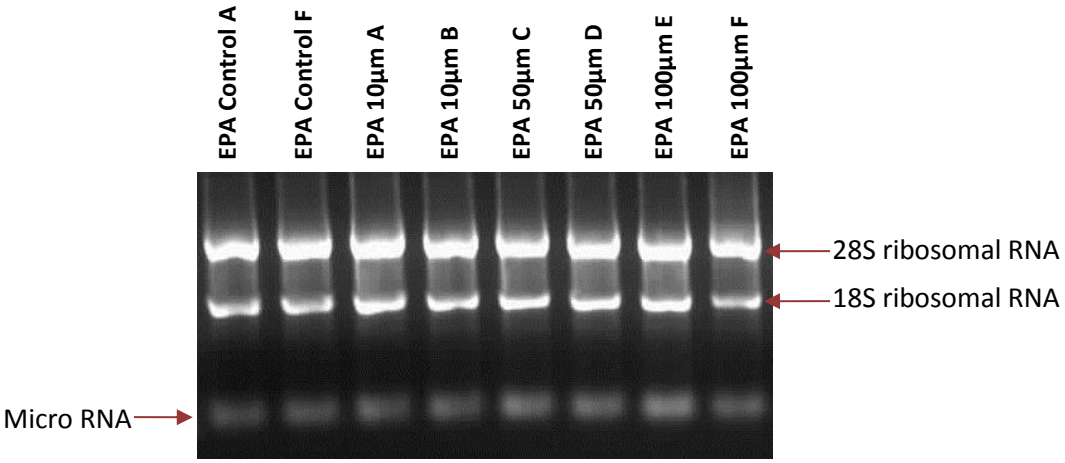


Figure 2.8- Total RNA from human liposarcoma cells supplemented with EPA. These example samples were selected to represent the range of concentrations and 260/280 ratios from all samples. Lanes are labelled with the concentration of fatty acid and the letter represents one of the sextuplicates. The ribosomal 28 and 18 subunits are labelled, along with the micro RNA.

2.2.16.3 DNase treatment and cDNA synthesis

RNA (1µg) was DNase treated before reverse transcription by adding 1µl of 10x reaction buffer and 1µl of amplification grade DNase I (1 unit/µl) to 1µg of RNA and gently mixed, before incubation at room temperature for 15 minutes. Then, 1µl of stop solution (50mM EDTA) was added to each sample to bind calcium and magnesium ions, thereby inactivating the DNase I. Samples were incubated at 70°C for 10 minutes to inactivate the DNase I, before chilling on ice.

DNase treated RNA (1µg) was converted to cDNA, as described in section 2.2.15.4, along with 10 extra samples for the standard curve. cDNA was diluted with DNase and RNase free water to a total volume of 160µl (6.25ng/µl). cDNA from the 10 extra samples was combined and mixed thoroughly. 200µl of the pooled cDNA was mixed with 600µl of DNase and RNase free water, and then serially diluted to make samples for the standard curve, as described in section 2.2.15.4. All samples were stored at -20°C.

2.2.16.4 Real time PCR using cDNA from human liposarcoma cells

To determine the optimal reference gene for human liposarcoma cells cultured with fatty acids, all 6 samples for each fatty at 100µM and 6 control samples were selected and cDNA was synthesised. These samples were chosen to represent the extremes of the experiment. Six reference genes were tested which included B2M, CYC1, GAPDH, PPIA, TOP1 and YWHAZ, and were ranked according to their expression stability. A total of 25ng of cDNA (4µl) of each sample was measured in duplicate, along with 2 negative controls where the cDNA was replaced with 4µl of DNase and RNase free water, using the conditions described in section 2.2.15.5.

A total of 25ng of cDNA was used to measure the expression of SIRT1 and PGC1α in human liposarcoma cells, using the conditions described in section 2.2.15.6. All data was normalised to cyclophillin (PPIA) as this was stably expressed, determined using the geNorm Reference Gene Selection Kit (Primerdesign).

2.2.16.5 Measuring DNA methylation in human liposarcoma cells supplemented with α linolenic acid

Because human liposarcoma cells supplemented with α linolenic acid (ALA) had a decrease in mRNA expression of PGC1α, the effect of ALA on DNA methylation of PGC1α was determined. Human liposarcoma cells, SW-872, were seeded at 1.5 x 10^5 in 6 well plates and grown overnight in 4ml of media. After 16 hours, the media was removed and replaced with media containing ALA at a final concentration of 100µM, 50µM or 10Mm, or with control media containing ethanol, in a total volume of 4ml, as described in section 2.2.16.1. After 72 hours all media was removed and cells were washed in 2ml of PBS.

Genomic DNA was extracted from human liposarcoma cells using the QIAamp DNA mini kit (Qiagen), according to the manufacturer's instructions, with 100% sample recovery efficiency. Samples were eluted in 50µl of DNase and RNase free water. The quality and quantity of the DNA was determined using the Nanodrop 1000. All samples had a 260/280 ratio between 1.8 and 2.2. All

samples had between 1.16µg and 3.78µg total DNA. 500ng of each sample was run on a 0.8% agarose gel, shown in figure 2.9 and samples were stored at -20°C.

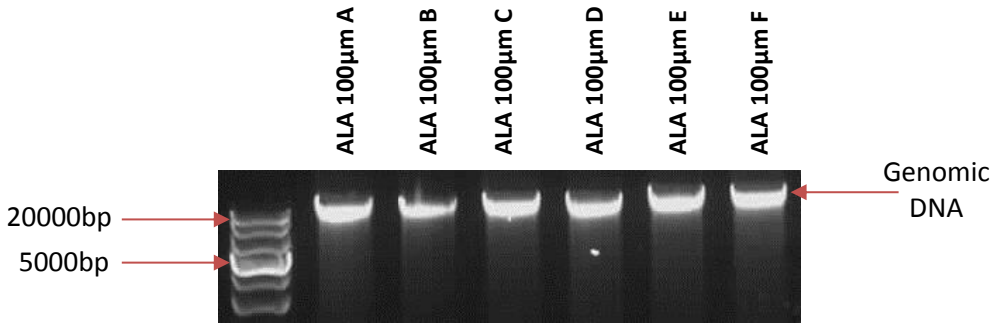


Figure 2.9- Genomic DNA from human liposarcoma cells supplemented with ALA at 100µM
Lanes are labelled with the concentration of ALA and the letter represents each of the sextuplicates.

Genomic DNA (1µg) was bisulphite treated, as described in section 2.2.3. Control samples included unmethylated DNA (Qiagen), methylated DNA (Millipore), human genomic DNA (Roche) and a no template control (NTC) where the DNA was replaced with 1µl of water. Bisulphite converted DNA was used in a PCR for all the PGC1α assays, shown in table 2.5, because only PGC1α had altered expression when cells were supplemented with α linolenic acid. An NTC was used for each assay where the DNA was replaced with 1µl of water. To confirm the product size, 10µl of each control and a random selection of 6 samples were run on a 1.5% agarose gel. The methylation of each CpG loci in PGC1α was determined by pyrosequencing using 10µl of PCR product, as described in section 2.2.5. Pass rates were 100% for each assay.

2.2.16.6 Statistical analysis of expression and methylation in fatty supplemented cells

All statistical analyses were performed using IBM SPSS Statistics version 21.0. A one way ANOVA was used to determine if there was a statistically significant difference between the means of the 4 groups for each fatty acid, when measuring expression and methylation for each CpG loci. Post-hoc analyses using tukeys' multiple comparisons test were used to test the effect of concentration on the expression and methylation of each CpG loci measured.

Chapter 3

The Stability of DNA Methylation throughout Childhood and Puberty

3.1 Introduction

3.1.1 The stability of DNA methylation

In humans, methylation patterns are set up following genome-wide demethylation and are maintained during cell division (129). There is some evidence to suggest that once methylation is established in early development, it is then maintained throughout the life course. However, this idea of stability is being challenged by a number of studies that have suggested DNA methylation is more dynamic than previously thought. For example, environmental factors such as pollution and exercise have been shown to induce changes in DNA methylation of specific loci (134)(135)(82).

There are limited data on the stability of DNA methylation during early childhood and puberty, and of the evidence that does exist there are conflicting reports. Some studies suggest that plasticity does not stop at birth and the environment continues to have an impact on long-term epigenetic regulation (136)(53)(133), whereas other data suggest that the methylation of some CpG loci is stable throughout the life course and is not influenced by future environmental challenges (132).

Some studies suggest that altered DNA methylation has the ability to act as a biomarker of disease risk (115). If altered DNA methylation were associated with a particular phenotype, it has the ability to identify individuals at increased risk of disease, allowing for potential intervention and prevention. If DNA methylation of a specific locus was predictive of future disease risk this would allow for earlier intervention and prevention. However, in order to discover potential biomarkers of disease risk, more information is required on how DNA methylation can potentially alter with disease progression.

An increased understanding of DNA methylation stability and the factors that govern stability of specific CpG loci is required. To address whether DNA methylation is stable throughout childhood and puberty, 13 genes were selected for optimisation and validation, as described in figure 3.1. Because obesity is a risk factor associated with the development of insulin resistance and T2D, with more than 80% of T2D patients being obese, genes that have been implicated in the development of obesity, insulin resistance and T2D were chosen for validation (33)(146). Genes were also selected based on previous literature to suggest they are epigenetically regulated or work within the same pathway.

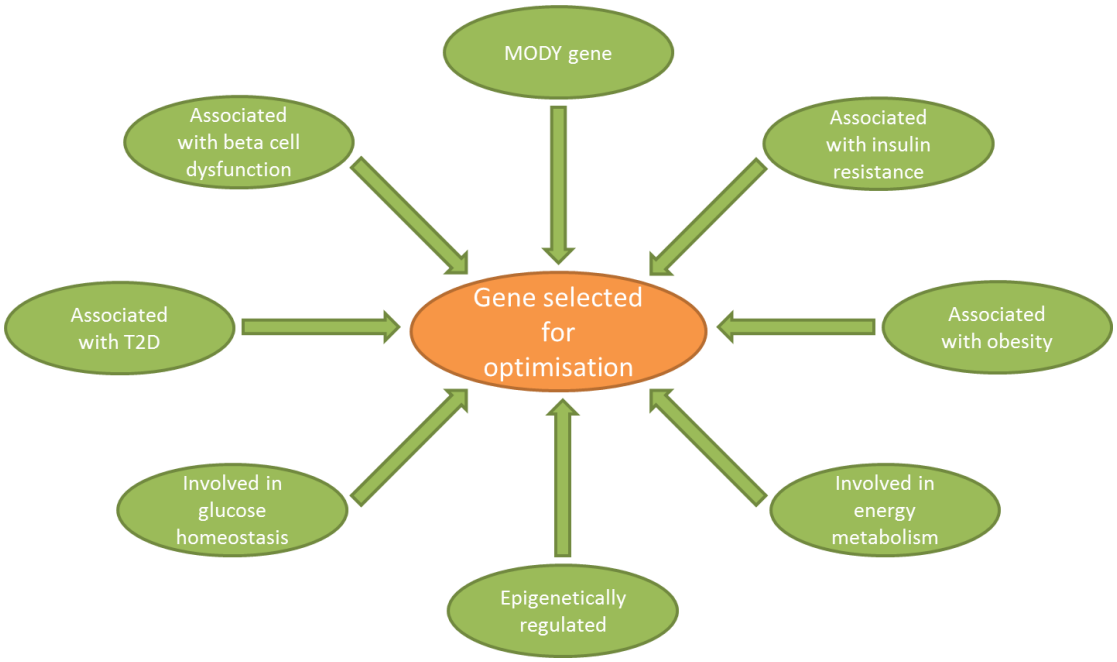


Figure 3.1- Selection criteria for gene optimisation. Candidate genes were selected based on the criteria shown, which include genes that have a biological function in the development of obesity, insulin resistance and T2D.

3.1.2 MODY genes

Mutations in specific genes give rise to a specific type of diabetes mellitus known as MODY (maturity onset diabetes of the young), which include hepatocyte nuclear factor 4 alpha (HNF4α, MODY 1), glucokinase (GCK, MODY 2), hepatocyte nuclear factor 1 alpha (HNF1α, MODY 3), pancreatic and duodenal homeobox 1 (PDX1, MODY 4) and hepatocyte nuclear factor 1 beta (HNF1β, MODY 5) (147).

HNF4α is a transcription factor expressed in many tissues that is involved in the regulation of several metabolic pathways including insulin secretion and β cell proliferation (148)(149)(150). The gene for HNF4α encodes 2 tissue specific promoters, the proximal P1 promoter expressed in the liver, kidney and intestine, and the distal P2 promoter expressed in the pancreatic β cell (151). An upstream enhancer is able to interact with both promoters via a looping mechanism, which is stabilised by transcription factors (152). Evidence from a rat model suggests that HNF4α may be epigenetically regulated when a protein restricted diet during pregnancy and lactation resulted in hypermethylation of the P2 promoter and a weakened P2 promoter-enhancer interaction in the offspring, which was associated with glucose intolerance (151). In humans, the methylation of the P1 promoter has been

shown to be increased in the subcutaneous adipose tissue from monozygotic twins with T2D (153). Einstein et al. have shown increased methylation of the proximal P1 promoter in umbilical cord blood from neonates with intrauterine growth restriction (154). This suggests that the environment can induce altered methylation of HNF4α, which may be associated with future disease risk.

Decreased GCK gene expression may be involved in the development of obesity and T2D, with increased GCK expression in animal models protecting against high fat diet induced obesity, glucose intolerance and insulin resistance (155)(156). In humans, it has been shown that obese subjects with T2D have reduced hepatic GCK expression, when compared to healthy lean subjects (155). Evidence from a rat model suggests that GCK may be epigenetically regulated when 11 CpG sites in the hepatic GCK promoter were shown to be hypermethylated in aged rats when compared to young rats, which correlated with a decrease in hepatic GCK expression, suggesting that methylation within this promoter may not be stable over time (157). Little is known about the epigenetic regulation and stability of methylation in GCK in humans.

The most common mutation in the HNF1α gene results in a truncated protein lacking the transactivation domain, but retains a fully functional DNA binding domain which competes with endogenous HNF1α for DNA targets (158). This dominant negative mutant binds directly to the promoter of the insulin gene in rats, resulting in decreased gene expression, suggesting HNF1α is important for the regulation of downstream mechanisms (158). Mutations in the HNF1β gene are less common and are characterised by impaired insulin secretion in response to glucose and a reduction in β cell mass, suggesting HNF1β may be important in maintaining β cell mass for normal insulin secretion (147). Due to technical constraints associated with assay design the methylation of the PDX1 promoter could not be examined.

3.1.3 Genes that regulate pancreatic β cell function

β cell dysfunction is known to be involved in the development of T2D, either as a result of increased insulin secretion to maintain normal glucose concentration causing a decline in β cell function, or as a result of increased apoptosis and decreased regeneration of the β cells leading to decreased β cell mass and β cell exhaustion (37)(33). Micro-RNA 34a (miR34a) expression has been shown to be increased in the islet cells of diabetic mice and transfection of a mouse pancreatic β cell line *in vitro* associated with increased apoptosis suggesting that miR34a may be involved in the development of T2D via decreased β cell mass and dysfunction (159). Enhancer of zeste homolog 2 (EZH2) is a histone methyltransferase within the Polycomb group (PcG) protein complex, which is

involved in transcriptional silencing via histone H3 methylation (160). Mutant mice where EZH2 was selectively deleted from the pancreatic β cells had reduced β cell mass and proliferation, hypoinsulinemia and diabetes, when compared to controls, suggesting EZH2 may be important in β cell proliferation (160).

3.1.4 Genes that regulate insulin sensitivity and glucose homeostasis

Insulin binds to the insulin receptor (INSR) on the surface of target cells, transmitting a signal across the plasma membrane resulting in the activation of the phosphorylation cascade. Therefore, decreased sensitivity of the insulin receptors, or decreased concentration of insulin receptors can lead to insulin resistance. For example, obese, insulin resistant mice have a decreased number of insulin receptors on their hepatocytes and adipocytes (161).

Responding to changes in glucose and insulin concentration is essential to maintain glucose homeostasis. Glucose transporter type 4 (GLUT4) is an insulin sensitive glucose transporter which transports glucose primarily into adipose tissue and skeletal muscle. GLUT4 gene expression is decreased in adipocytes from obese and T2D patients and animal models have shown overexpression of GLUT4 reverses insulin resistance in mice lacking muscle specific GLUT4 (162). Glucose 6 phosphatase (G6P) hydrolyses glucose 6 phosphate releasing phosphate and glucose, which is exported out the cell by glucose transporters. Obese, T2D patients have been shown to have increased hepatic G6P activity, compared to obese non-diabetic controls, resulting in increased glucose concentration (163).

3.1.5 Genes involved in adipogenesis and metabolism

Sirtuin 1 (SIRT1) and Peroxisomal proliferator-activated receptor gamma, coactivator 1 alpha (PGC1α) are expressed in many tissues and play a key role in many metabolic processes including insulin secretion, glucose homeostasis and adipogenesis, and are therefore often described as master regulators (63)(75)(75)(66). SIRT1 is known to positively regulate PGC1α expression via deacetylation of several lysine residues, thereby increasing the ability of PGC1α to activate target genes (67)(figure 1.2).

See section 1.1.7 for further details on these genes. Briefly, evidence from *in vivo* and *in vitro* studies suggests that the expression of SIRT1 and PGC1α changes in response to altered metabolic demand. For example, in animals and humans expression is increased in response to fasting and caloric restriction, whereas overfeeding and high fat diets reduce expression

(10)(72)(73)(74)(46)(68)(70). SIRT1 and PGC1α expression is known to be decreased in the subcutaneous adipose tissue of obese subjects, when compared to lean subjects, suggesting these genes are important in the development of obesity (72)(73)(79).

Several studies have shown that PGC1α methylation is increased in obesity and T2D, which is associated with a decrease in mRNA expression in a range of tissues (49)(79). It has also been shown that high fat diet and exercise can influence the methylation of PGC1α, which is negatively associated with gene expression, suggesting that methylation may not be stable throughout the life course and may be influenced by environmental challenges (164)(82).

Retinoid X receptor α (RXRα) is a nuclear receptor that is important in the regulation of many metabolic processes including adipogenesis (165). Animal models have shown that diet induced obesity results in decreased RXRα expression in adipose tissue (166). There is some evidence in humans to suggest that RXRα may be epigenetically regulated and that altered methylation at birth may be predictive of future childhood adiposity (115).

3.1.6 Aims

In order to test the hypothesis that the methylation of specific CpG loci in genes involved in β cell function, glucose homeostasis, adipogenesis and energy metabolism, is stable throughout childhood and puberty, pyrosequencing was used to measure the methylation in peripheral blood from 40 subjects taken annually between 5-7 years and 14 years.

3.2 Methods

40 children (20 boys and 20 girls) were selected from the EarlyBird cohort and stratified according to insulin resistance at 5 and 14 years, measured by HOMA-IR (section 2.2.1). Subject characteristics are given in table 2.3. DNA was extracted from peripheral blood taken annually between 5-7 and 14 years (section 2.2.2). Pyrosequencing was used to measure the methylation of specific CpG loci within the regulatory regions of genes involved in β cell function, glucose homeostasis, adipogenesis and energy metabolism (section 2.2.5). Data shown at 5-7 and 8-9 years is the mean methylation, which was to ensure a complete data set for longitudinal statistical analysis. The coefficient of variance of each assay is shown in section 2.2.6. Pyrosequencing was used to determine the genotype of 2 known CpG single nucleotide polymorphisms (SNPs) within RXRα and GCK using DNA from peripheral blood at age 14 years.

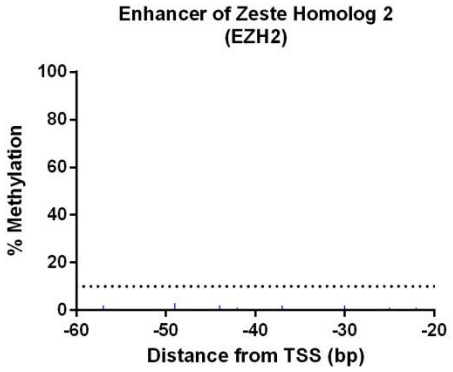
Statistical analysis was carried out by Dr. Joanne Hosking (Plymouth University Peninsula School of Medicine and Dentistry), due to confidentiality of the EarlyBird cohort. Pearson's correlation coefficient was used to calculate if there was a significant association between the % methylation of each CpG at each time point in order to determine the temporal stability of methylation throughout childhood and puberty, between 5-7 and 14 years (appendix). Longitudinal tracking co-efficients were generated to determine the association between the initial measurement of methylation (5-7 years) and all other subsequent measurements simultaneously between 8-9 and 14 years.

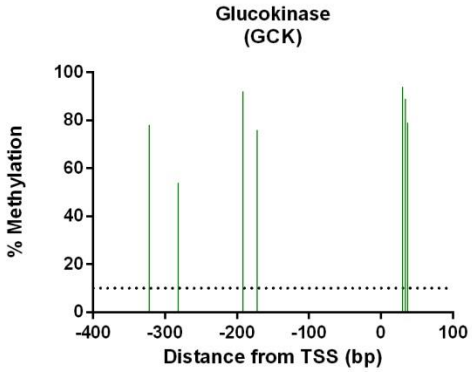
A confounding factor associated with measuring DNA methylation in peripheral blood is cellular heterogeneity (120). Therefore, pearson's correlation coefficient was used to calculate if there was a significant association between the proportions of the major circulating leukocyte populations, neutrophils and lymphocytes, and the methylation of each CpG measured at each time point. Leukocyte proportions were measured by a leukocyte differential count, which determines the percentage of each white blood cell type. The major white blood cell types found within peripheral blood in children between 6 to 18 years are neutrophils (~52%), lymphocytes (~36%), monocytes (~8.5%), eosinophils (~3%) and basophils (~0.5%), which is consistent with the proportions measured in the EarlyBird subjects (table 2.3) (121). Therefore with neutrophils and lymphocytes making up about 88% of the total white blood cell population, only the proportions of these cell types were used in the statistical analysis.

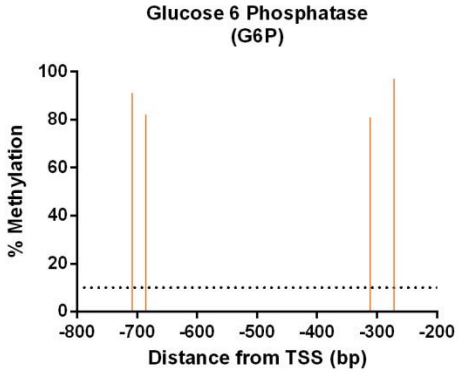
3.3 Results

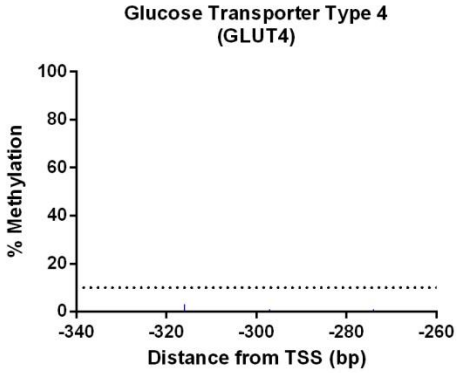
3.3.1 Optimisation and validation of CpG loci

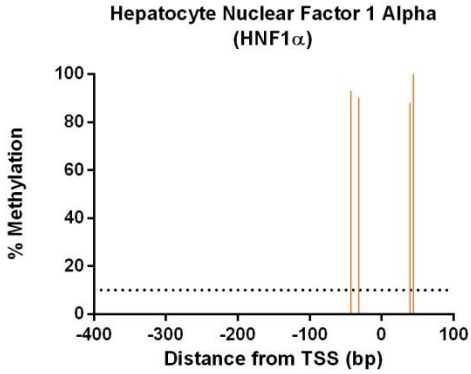
Genes were selected for validation and optimisation based on previous literature that suggests they may be important in the development of obesity, insulin resistance and T2D (148)(149)(151)(153)(155)(156)(72)(73)(79)(71)(164)(115)(82)(49)(79). Assays were designed to cover CpGs that were within the promoter or enhancer of the gene, covering putative binding sites for transcription factors that have been associated with the development of obesity or insulin resistance, or transcription factors that have been suggested to be important in the regulation of gene function (49). The methylation of some CpG loci has been shown to be associated with either obesity or T2D (82)(115)(49)(79).

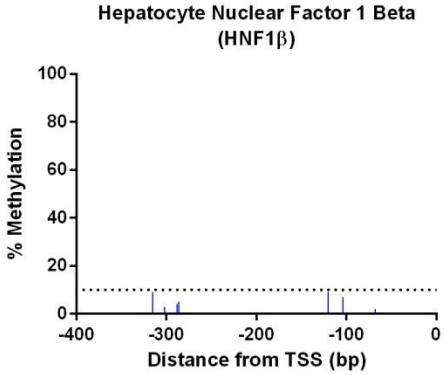

Enhancer of Zeste Homolog 2
(EZH2)
% Methylation
Distance from TSS (bp)

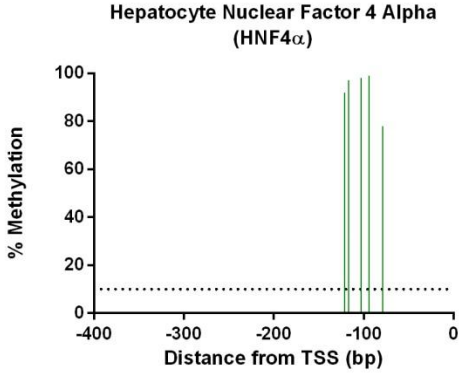

Glucokinase
(GCK)
% Methylation
Distance from TSS (bp)

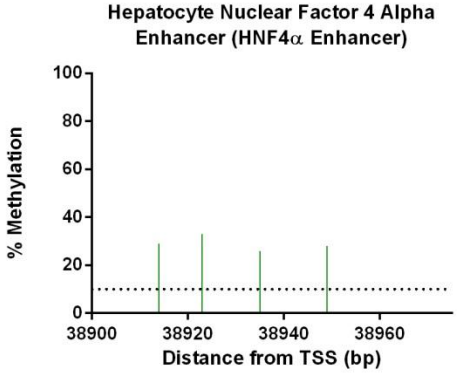

Glucose 6 Phosphatase
(G6P)
% Methylation
Distance from TSS (bp)


Glucose Transporter Type 4
(GLUT4)
% Methylation
Distance from TSS (bp)


Hepatocyte Nuclear Factor 1 Alpha
(HNF1α)
% Methylation
Distance from TSS (bp)


Hepatocyte Nuclear Factor 1 Beta
(HNF1β)
% Methylation
Distance from TSS (bp)


Hepatocyte Nuclear Factor 4 Alpha
(HNF4α)
% Methylation
Distance from TSS (bp)


Hepatocyte Nuclear Factor 4 Alpha
Enhancer (HNF4α Enhancer)
% Methylation
Distance from TSS (bp)

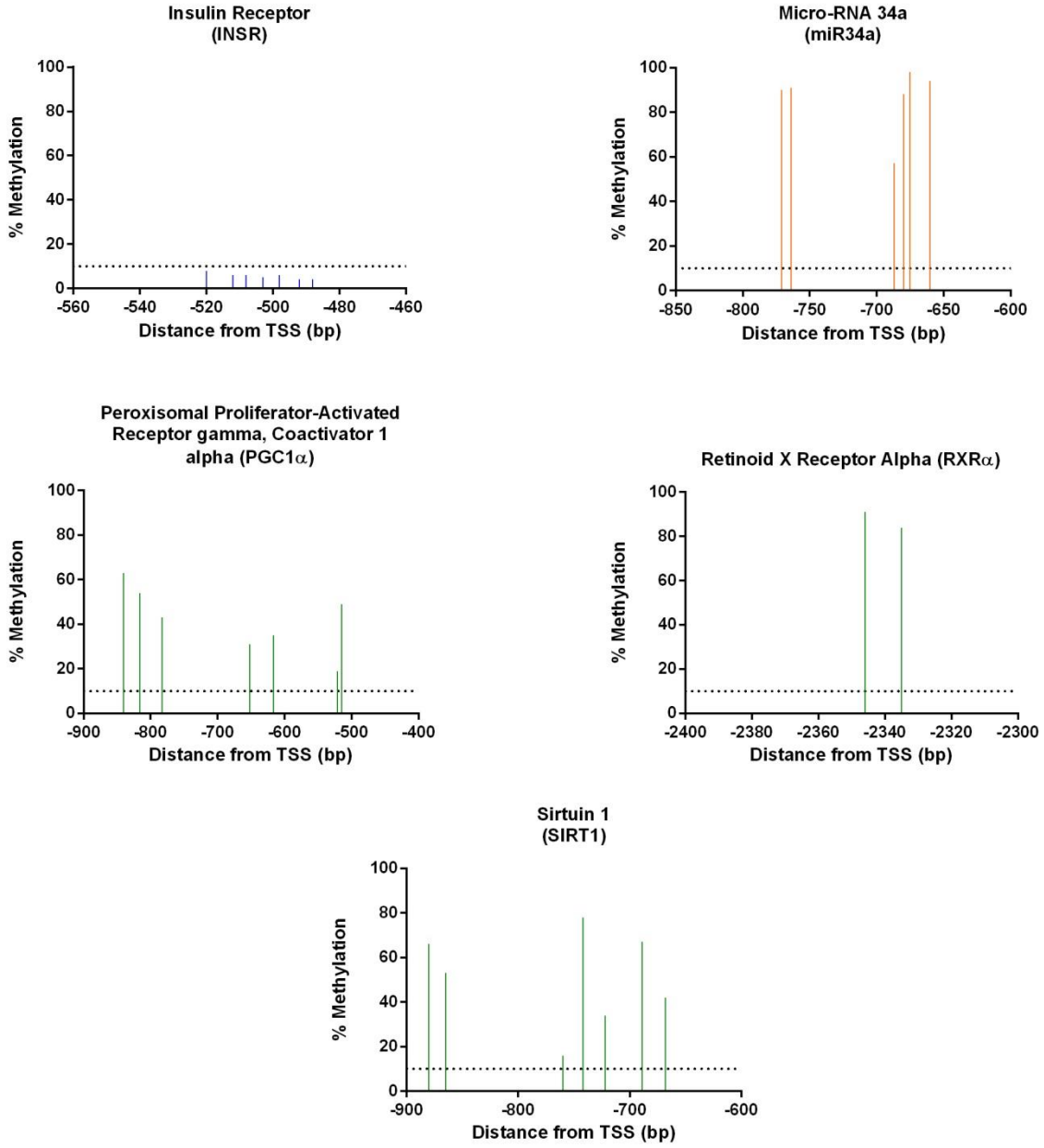


Figure 3.2- Methylation of all CpGs optimised in peripheral blood. Each panel represents the methylation of the genes chosen for optimisation, measured in control human peripheral blood DNA. Levels of methylation are representative of the methylation levels that would be measured in peripheral blood from the EarlyBird subjects. Each bar shows the level of methylation for each individual CpG loci in each gene. The dashed line represents the bisulphite conversion control threshold (10%). Genes with low methylation (<10%) are shown by blue bars. Genes with high methylation (>80%) are shown with orange bars. Genes that were selected for amplification are shown with green bars.

Gene selection criteria included those that had CpGs with a varied level of DNA methylation encompassing a transition between regions of high and low methylation and had methylation levels above the bisulphite conversion control threshold. Genes were also selected if there were known to be involved in the same pathway, which include SIRT1, PGC1α and HNF4α (67)(71). Genes that contained CpG loci where methylation has been shown to be important in gene function, or where methylation has been shown to be associated with adiposity and/or T2D, were also chosen (82)(115)(49)(79). Genes and CpG loci that met these criteria and were chosen for amplification in the EarlyBird subjects include SIRT1, PGC1α, HNF4α, RXRα and GCK (shown by the green bars in figure 3.2).

3.3.2.1 The methylation of the SIRT1 promoter in peripheral blood

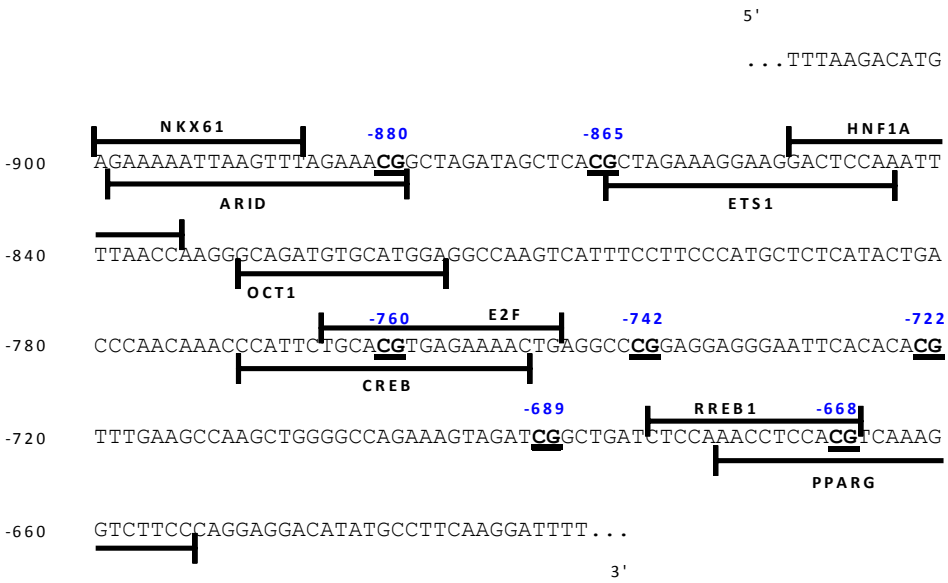


Figure 3.3- The proximal promoter of SIRT1 in humans with putative regulatory transcription factor binding sites. Putative transcription factor binding consensus sequences were determined using MatInspector (http://www.genomatix.de/cgi-bin//matinspector) and are shown by T-bars. Each CpG loci is numbered according to the distance from the transcription start site (TSS), and underlined in bold. Abbreviations are as follows:- **NKX61**, NK6 Homeobox 1; **ARID**, AT Rich Interactive Domain; **ETS1**, V-Ets Erythroblastosis Virus E26 Oncogene Homolog 1; **HNF1A**, Hepatocyte Nuclear Factor 1-Alpha; **OCT1**, Octamer-Binding Transcription Factor 1; **CREB**, CAMP Responsive Element Binding Protein 1; **E2F**, E2F Transcription factor; **RREB1**, Ras Responsive Element Binding Protein 1; **PPARG**, Peroxisome Proliferator-Activated Receptor Gamma.

Figure 3.3 shows the distance of each CpG from the transcription start site covering a total of 212bp within the SIRT1 promoter (167). To date, the methylation of these CpGs has not been shown to be involved in the regulation of gene expression, however these loci are within the promoter of SIRT1 and the promoters of genes are known to be important for transcription (95).

Of these putative transcription factor binding sequences CREB, E2F and PPARG are known to be involved in the regulation of SIRT1. An *In vitro* study showed that CREB induces SIRT1 expression in hepatic cells from fasted mice (168). E2F is involved in the regulation of apoptosis and induces SIRT1 expression by binding directly to the SIRT1 promoter (169). PPARG is known to promote cell senescence and represses SIRT1 expression (170). Therefore, the binding of these transcription factors to this region of the promoter may be important in the regulation of SIRT1 expression.

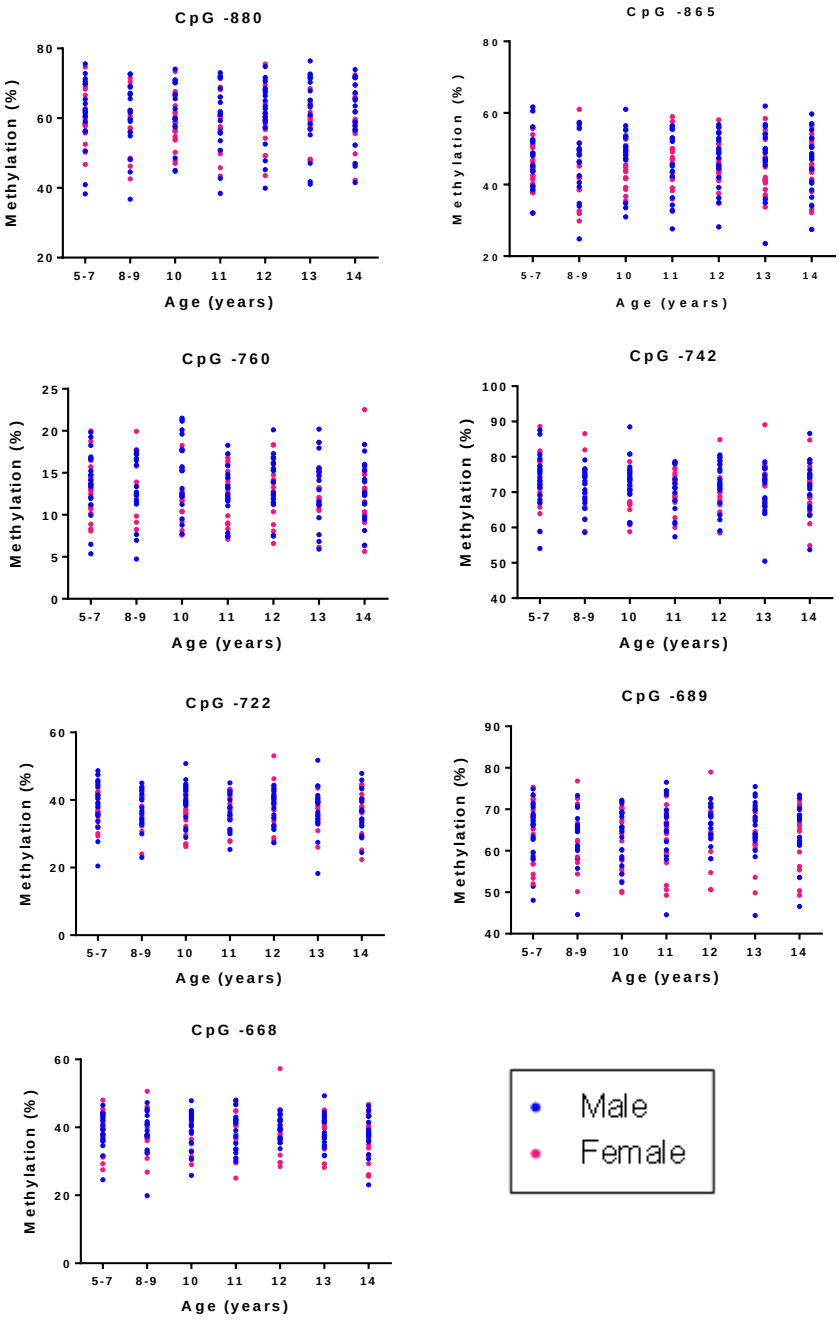


Figure 3.4- The methylation of each EarlyBird subject in the SIRT1 promoter in peripheral blood between 5-7 and 14 years. Each panel represents the methylation of each subject at every age between 5-7 and 14 years for individual CpG loci within the SIRT1 promoter. CpG loci are numbered according to the distance from the TSS. Male subjects are shown with a blue circle, and female subjects with a pink circle.

Figure 3.4 shows the methylation of the SIRT1 promoter in peripheral blood for all subjects between 5-7 and 14 years. The methylation of CpG -760 shows the least variation across all subjects and ages with a difference of 18.24% between the highest and lowest levels, whereas CpG -880 has the largest variation with a difference of 40.43%. In peripheral blood, CpG -742 has the highest level of methylation across all subjects and ages and CpG -760 has the lowest level of methylation. There were no significant differences between sex and % methylation for any CpG measured ($p>0.05$), determined using an unpaired t-test with a Bonferroni correction.

3.3.2.2 Is the methylation of the SIRT1 promoter stable between 5-7 and 14 years?

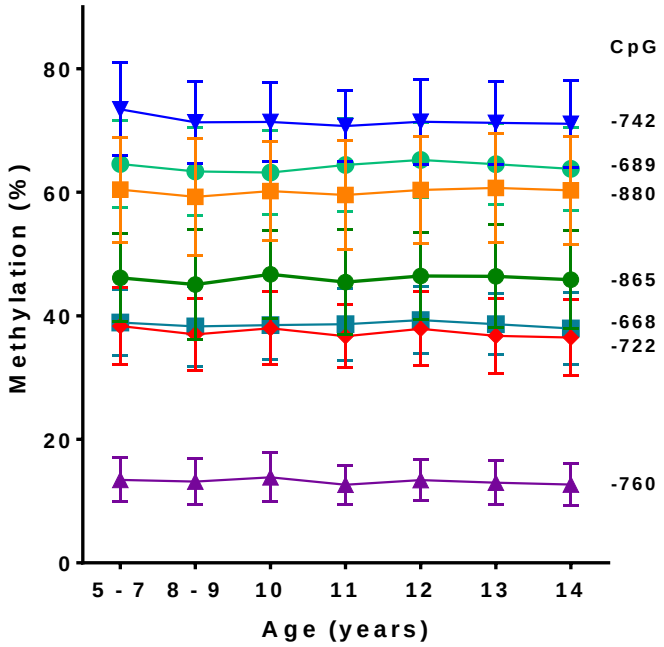


Figure 3.5- Mean methylation across the SIRT1 promoter measured in peripheral blood between 5-7 and 14 years. The mean ± SD per cent methylation for each CpG loci within the SIRT1 promoter is shown for all subjects (n=40). CpG loci are numbered relative to the TSS. Mean methylation of individual CpG loci in the SIRT1 promoter varied from 12.7 ± 3.2% (CpG -760) to 73.4 ± 7.5% (CpG -742) across all subjects and years.

Table 3.1- The stability of methylation across the SIRT1 promoter in peripheral blood between 5-7 and 14 years

CpG	Longitudinal tracking co-efficient	p-value	% of variation in methylation at 14 years explained by the methylation at 5-7 years
-880	0.92	<0.001	84.6%
-865	0.79	<0.001	62.4%
-760	0.87	<0.001	75.7%
-742	0.90	<0.001	81.0%
-722	0.80	<0.001	64.0%
-689	0.82	<0.001	67.2%
-668	0.86	<0.001	74.0%

Table 3.1 shows the stability of methylation of the SIRT1 promoter in peripheral blood between 5-7 and 14 years. Longitudinal tracking co-efficients were high for all CpG loci in the SIRT1 promoter ranging from 0.79 to 0.92, all $p<0.001$, such that the methylation at 5-7 years predicted between 64.0% and 84.6% of the variation at 14 years. Table 10.1 in the appendix shows there is a strong year-on-year correlation between % methylation of each CpG at 5-7 years and the methylation at all subsequent ages up to 14 years ($r=0.73$-0.96, $p<0.001$). Therefore, the methylation of each CpG in SIRT1 in peripheral blood is temporally stable throughout childhood.

3.3.2.3 Is the methylation of the SIRT1 promoter associated with the proportions of neutrophils or lymphocytes?

Table 3.2- Cross-sectional associations between the methylation of SIRT1 and leukocyte proportions in peripheral blood between 5-7 and 14 years

Age (years)	Correlation with neutrophils			Correlation with lymphocytes		
	r	p	n	r	p	n
	CpG -880					
5-7y	0.14	0.40	38	<-0.01	0.99	38
8-9y	-0.10	0.62	30	0.27	0.15	30
10y	-0.17	0.31	37	0.14	0.41	37
11y	0.02	0.93	35	0.09	0.61	35
12y	0.16	0.36	36	-0.16	0.36	36
13y	-0.11	0.52	35	0.10	0.56	35
14y	-0.16	0.32	39	0.27	0.09	39
	CpG -865					
5-7y	0.07	0.69	37	0.05	0.79	37
8-9y	-0.26	0.17	29	0.45	0.01	29
10y	-0.16	0.37	35	0.15	0.41	35
11y	-0.23	0.19	33	0.27	0.13	33
12y	0.10	0.58	36	-0.05	0.77	36
13y	-0.12	0.50	34	0.16	0.38	34
14y	-0.19	0.25	37	0.33	0.05	37
	CpG -760					
5-7y	0.15	0.37	37	0.05	0.77	37
8-9y	-0.20	0.33	27	0.35	0.08	27
10y	-0.15	0.40	36	0.22	0.20	36
11y	0.04	0.82	32	0.12	0.52	32
12y	0.09	0.61	34	-0.01	0.95	34
13y	-0.04	0.85	32	0.10	0.60	32
14y	0.01	0.95	38	0.22	0.18	38
	CpG -742					
5-7y	0.17	0.31	37	-0.03	0.84	37
8-9y	-0.20	0.32	27	0.32	0.10	27
10y	<0.01	0.99	36	0.09	0.62	36
11y	0.26	0.16	32	-0.04	0.83	32
12y	0.08	0.66	34	<0.01	0.99	34
13y	0.01	0.96	32	0.07	0.70	32
14y	0.09	0.61	38	0.16	0.35	38

	CpG -722					
5-7y	0.17	0.33	36	0.06	0.73	36
8-9y	-0.35	0.08	27	0.53	0.004	27
10y	-0.31	0.06	36	0.45	0.006	36
11y	-0.10	0.58	32	0.24	0.18	32
12y	-0.17	0.34	34	0.28	0.11	34
13y	-0.31	0.09	31	0.36	0.05	31
14y	-0.17	0.33	36	0.42	0.01	36
	CpG -689					
5-7y	0.22	0.19	38	-0.02	0.92	38
8-9y	-0.02	0.93	28	0.36	0.06	28
10y	-0.36	0.03	37	0.35	0.03	37
11y	0.02	0.90	34	0.09	0.63	34
12y	0.04	0.82	34	0.10	0.58	34
13y	-0.09	0.63	34	0.24	0.18	34
14y	-0.22	0.17	39	0.34	0.03	39
	CpG -668					
5-7y	0.06	0.73	36	0.11	0.53	36
8-9y	-0.26	0.19	28	0.53	0.004	28
10y	-0.35	0.04	36	0.32	0.06	36
11y	-0.27	0.13	33	0.37	0.03	33
12y	-0.28	0.11	34	0.32	0.07	34
13y	-0.17	0.34	33	0.28	0.12	33
14y	-0.32	0.05	39	0.49	0.002	39

Table 3.2 shows the cross-sectional associations between the methylation of SIRT1 and the proportion of neutrophils and lymphocytes; the major white blood cells in peripheral blood, at each age between 5-7 and 14 years. The methylation at the majority of time points for each CpG was inversely correlated with the proportion of neutrophils, whereas there was a positive correlation between methylation and the proportion of lymphocytes.

Table 3.2 shows highlighted in grey the significant associations between the methylation of CpG -689 and -668 and the proportion of neutrophils at age 10 years and also age 14 years for CpG -668 ($p<0.05$). There were significant associations between the methylation of CpG -865, -722, -689 and -668 and the proportion of lymphocytes at age 14 years ($p<0.05$), along with other associations at ages 8-9, 10, 11 and 13 years, shown highlighted in grey ($p<0.05$).

There were no significant associations between the methylation of CpG -880, -760, -742 and the proportion of neutrophils or lymphocytes between 5-7 and 14 years ($p>0.05$). Therefore, the methylation of these CpGs in peripheral blood is not influenced by the proportions of neutrophils or lymphocytes between 5-7 and 14 years.

3.3.3.1 The methylation of the PGC1α promoter in peripheral blood

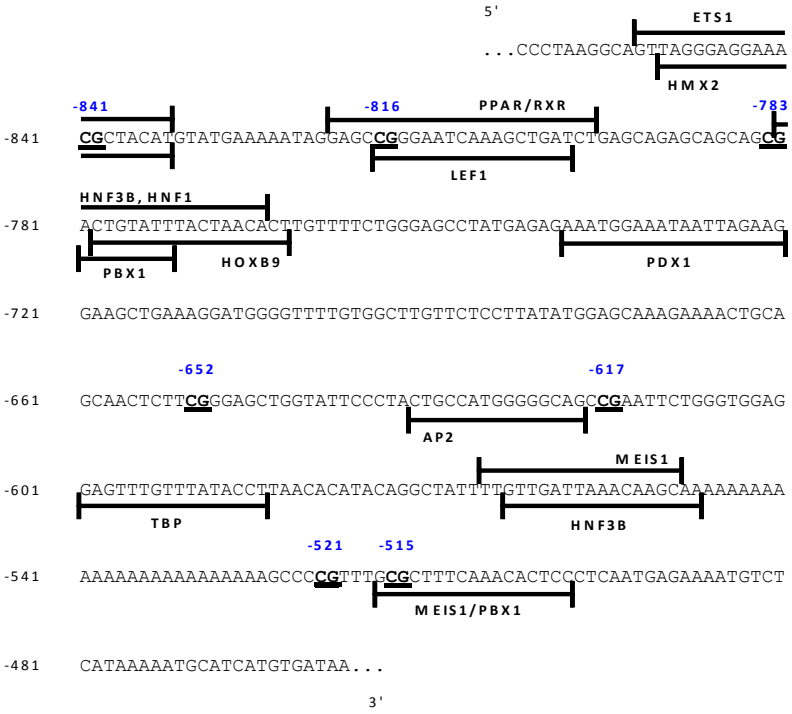


Figure 3.6- The proximal promoter of PGC1α in humans with putative regulatory transcription factor binding sites. Putative transcription factor binding consensus sequences were determined using MatInspector (http://www.genomatix.de/cgi-bin//matinspector) and are shown by T-bars. Each CpG loci is numbered according to the distance from the TSS, and underlined in bold. Abbreviations are as follows:- **ETS1**, V-Ets Erythroblastosis Virus E26 Oncogene Homolog 1; **HMX2**, H6 Family Homeobox 2; **PPAR/RXR**, Peroxisomal Proliferator-Activated Receptor/ Retinoid X Receptor heterodimer; **LEF1**, Lymphoid Enhancer-Binding Factor 1; **HNF3B**, Hepatocyte Nuclear Factor 3-Beta; **HNF1**, Hepatocyte Nuclear Factor 1; **PBX1**, Pre-B-Cell Leukemia Homeobox 1; **HOXB9**, Homeobox B9; **PDX1**, Pancreatic And Duodenal Homeobox 1; **AP2**, Activating Enhancer Binding Protein 2; **TBP**, TATA Box Binding Protein; **MEIS1**, Meis Homeobox 1.

Figure 3.6 shows the distance of each CpG from the transcription start site covering a total of 326bp within the PGC1α promoter (171). The methylation of CpGs -841, -816, -783, -652 and -617 has been shown to be increased in obesity and T2D in different tissues, which associated with a decrease in gene expression (49)(79).

Of these putative transcription factor binding sequences PPARG/RXRα is known to activate PGC1α expression which leads to the conversion of preadipocytes to brown adipocytes (75).

Therefore, binding of these transcription factors to this region of the promoter may be important in the regulation of PGC1α expression.

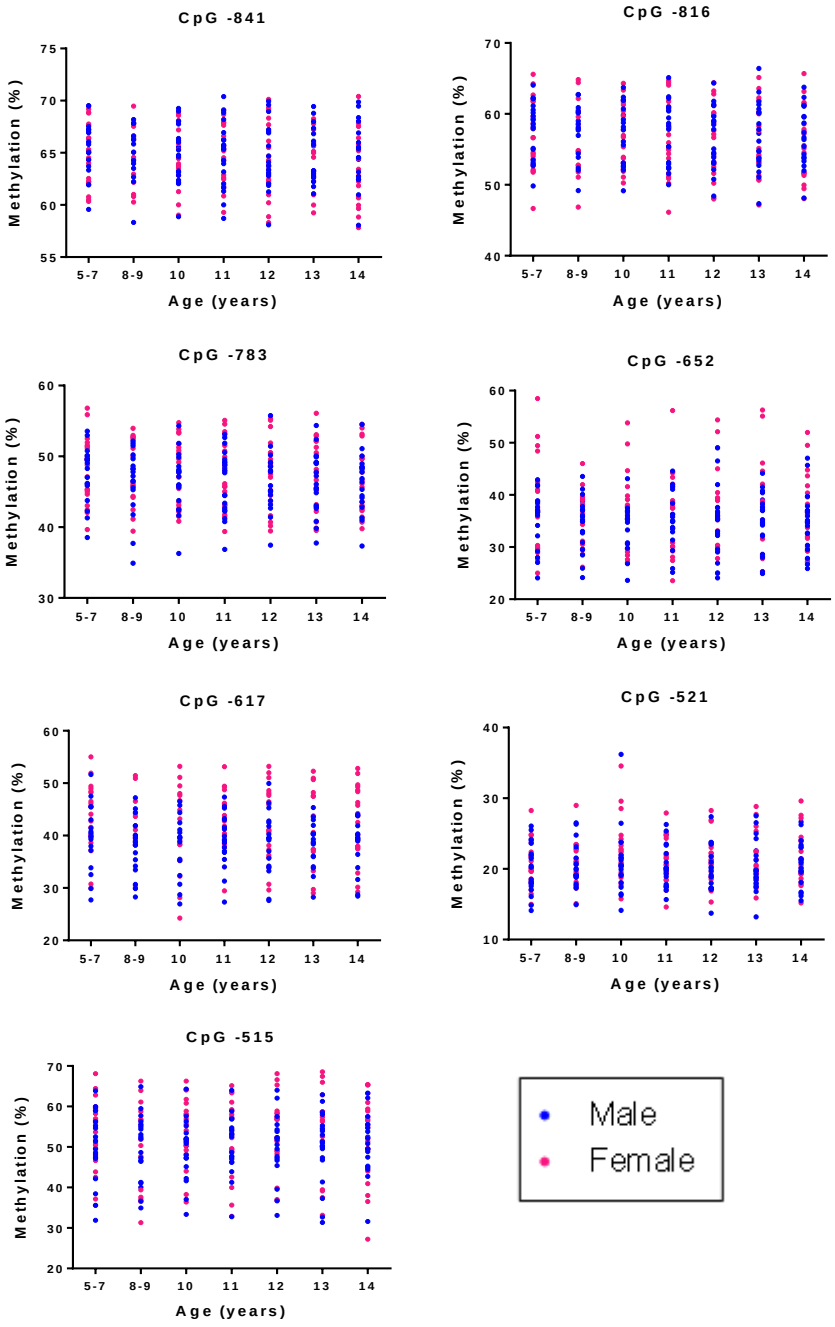


Figure 3.7- The methylation of each EarlyBird subject in the PGC1α promoter in peripheral blood between 5-7 and 14 years. Each panel represents the methylation of each subject at every age between 5-7 and 14 years for individual CpG loci within the PGC1α promoter. CpG loci are numbered according to the distance from the TSS. Male subjects are shown with a blue circle, and female subjects with a pink circle.

Figure 3.7 shows the methylation of the PGC1α promoter in peripheral blood for all subjects between 5-7 and 14 years. The methylation of CpG -841 shows the least variation across all subjects and ages with a difference of 12.59% between the highest and lowest levels, whereas CpG -515 has the largest variation with a difference of 41.33%. In peripheral blood, CpG -841 has the highest level of methylation across all subjects and ages and CpG -521 has the lowest level of methylation. There were no significant differences between sex and % methylation for any CpG measured ($p>0.05$), determined using an unpaired t-test with a Bonferroni correction.

3.3.3.2 Is the methylation of the PGC1α promoter stable between 5-7 and 14 years?

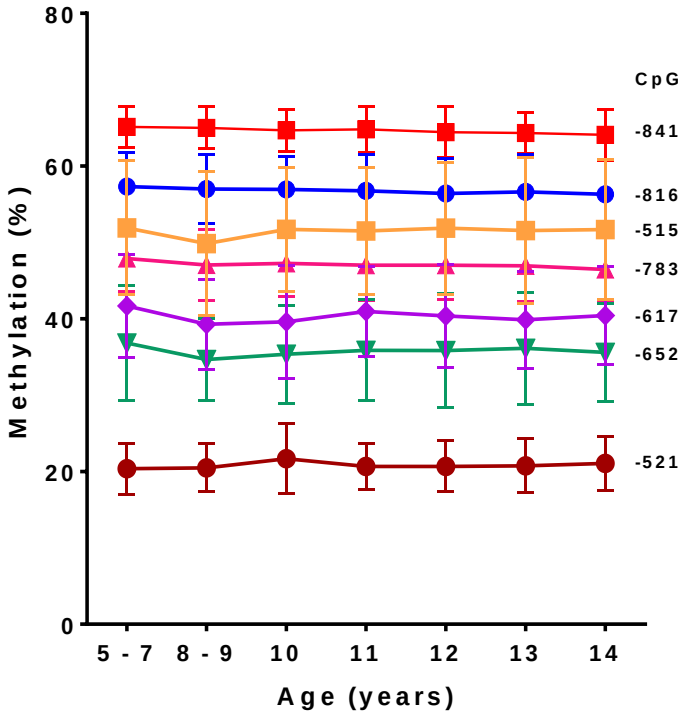


Figure 3.8- Mean methylation across the PGC1α promoter measured in peripheral blood between 5-7 and 14 years. The mean ± SD per cent methylation for each CpG loci within the PGC1α promoter is shown for all subjects (n=40). CpG loci are numbered relative to the TSS. Mean methylation of individual CpG loci in the PGC1α promoter varied from 20.4 ± 3.5% (CpG -521) to 64.9 ± 2.8% (CpG -841) across all subjects and years.

Table 3.3- The stability of methylation across the PGC1α promoter in peripheral blood between 5-7 and 14 years

CpG	Longitudinal tracking co-efficient	p-value	% of variation in methylation at 14 years explained by the methylation at 5-7 years
-841	0.91	<0.001	82.8%
-816	0.96	<0.001	92.2%
-783	0.95	<0.001	90.3%
-652	0.99	<0.001	98.0%
-617	0.97	<0.001	94.1%
-521	0.83	<0.001	68.9%
-515	0.93	<0.001	86.5%

Table 3.3 shows the stability of methylation of the PGC1α promoter in peripheral blood between 5-7 and 14 years. Longitudinal tracking co-efficients were high for all CpG loci in the PGC1α promoter ranging from 0.83 to 0.99, all $p<0.001$, such that the methylation at 5-7 years predicted between 68.9% and 98.0% of the variation at 14 years. Table 10.2 in the appendix shows there is a strong year-on-year correlation between % methylation of each CpG at 5-7 years and the methylation at all subsequent ages up to 14 years ($r=0.65$-0.97, $p<0.001$). Therefore, the methylation of each CpG in PGC1α in peripheral blood is temporally stable throughout childhood.

3.3.3.3 Is the methylation of the PGC1α promoter associated with the proportions of neutrophils or lymphocytes?

Table 3.4- Cross-sectional associations between the methylation of PGC1α and leukocyte proportions in peripheral blood between 5-7 and 14 years

Age (years)	Correlation with neutrophils			Correlation with lymphocytes		
	r	p	n	r	p	n
	CpG -841					
5-7y	0.04	0.81	36	-0.06	0.72	36
8-9y	0.34	0.07	29	-0.24	0.21	29
10y	0.14	0.42	36	-0.10	0.58	36
11y	0.15	0.36	37	-0.23	0.17	37
12y	0.13	0.44	37	-0.21	0.22	37
13y	-0.20	0.27	33	0.11	0.55	33
14y	0.04	0.80	35	0.03	0.87	35
	CpG -816					
5-7y	0.07	0.68	38	-0.14	0.39	38
8-9y	0.19	0.31	30	-0.20	0.28	30
10y	0.10	0.58	37	-0.04	0.83	37
11y	0.12	0.46	39	-0.18	0.29	39
12y	0.13	0.46	38	-0.15	0.37	38
13y	-0.19	0.26	37	0.13	0.46	37
14y	-0.002	0.99	37	0.04	0.84	37
	CpG -783					
5-7y	0.09	0.59	38	-0.13	0.45	38
8-9y	0.12	0.51	32	-0.23	0.21	32
10y	0.26	0.12	38	-0.20	0.22	38
11y	0.11	0.51	39	-0.14	0.40	39
12y	0.05	0.78	39	-0.09	0.60	39
13y	-0.26	0.12	38	0.10	0.56	38
14y	-0.08	0.65	38	0.11	0.53	38
	CpG -652					
5-7y	-0.03	0.84	37	0.01	0.96	37
8-9y	-0.02	0.91	33	0.01	0.95	33
10y	0.10	0.59	35	-0.14	0.44	35
11y	-0.06	0.73	38	0.02	0.90	38
12y	-0.28	0.09	38	0.26	0.12	38
13y	-0.26	0.12	37	0.18	0.29	37
14y	-0.22	0.17	40	0.22	0.18	40

	CpG -617					
5-7y	-0.25	0.15	34	0.20	0.27	34
8-9y	-0.04	0.86	30	0.10	0.60	30
10y	0.11	0.54	31	-0.10	0.61	31
11y	-0.05	0.77	32	0.09	0.62	32
12y	-0.25	0.14	36	0.20	0.24	36
13y	-0.31	0.09	32	0.15	0.41	32
14y	-0.23	0.18	36	0.21	0.23	36
	CpG -521					
5-7y	0.13	0.44	38	-0.02	0.90	38
8-9y	0.14	0.43	33	-0.05	0.79	33
10y	0.13	0.44	39	0.03	0.86	39
11y	0.26	0.12	38	-0.10	0.57	38
12y	-0.15	0.39	37	0.23	0.18	37
13y	-0.10	0.55	38	0.12	0.49	38
14y	0.11	0.52	39	-0.08	0.65	39
	CpG -515					
5-7y	0.06	0.71	38	0.04	0.81	38
8-9y	0.37	0.04	33	-0.14	0.39	38
10y	0.13	0.42	38	-0.23	0.16	39
11y	-0.06	0.72	38	0.05	0.75	39
12y	-0.06	0.72	37	0.01	0.98	39
13y	-0.09	0.61	38	-0.04	0.79	39
14y	-0.13	0.44	39	0.02	0.89	39

Table 3.4 shows the cross-sectional associations between the methylation of PGC1α and the proportion of neutrophils and lymphocytes in peripheral blood, at each age between 5-7 and 14 years. The methylation of CpG -841, -816 and -783 at the majority of time points was positively correlated with the proportion of neutrophils and inversely correlated with the proportion of lymphocytes. The methylation of CpG -652 and -617 was inversely correlated with the proportion of neutrophils and positively correlated with the proportion of lymphocytes, excluding age 10 years. There is no consistent relationship in the direction of the correlation between the methylation of CpG -521 and -515 and the proportion of neutrophils and lymphocytes, however when there is a positive correlation with neutrophils there is an inverse correlation with lymphocytes.

There were no significant associations between the methylation of any of the CpGs and the proportion of neutrophils between the ages of 5-7 and 14 years ($p>0.07$), apart from an association between the methylation of CpG -515 at age 8-9 years ($p=0.04$). However, the number of subjects is reduced at this time point ($n=33$) compared to other measurements, which may explain the significant association. Therefore, any outliers in methylation or the proportion of neutrophils measured at this time point may influence the association and may generate a false positive. This

would need to be repeated for all 40 subjects in order to determine if the proportion of neutrophils influences methylation of this particular locus at this age, in peripheral blood.

There were no significant associations between the methylation of any of the CpGs and the proportion of lymphocytes between the ages of 5-7 and 14 years ($p>0.12$). Therefore, the methylation of each CpG in peripheral blood, except CpG -515, is not influenced by the proportions of neutrophils or lymphocytes between 5-7 and 14 years.

3.3.4.1 The methylation of the HNF4α distal/P2 promoter in peripheral blood

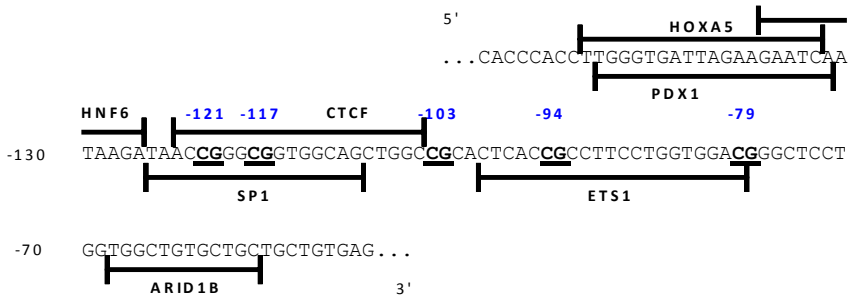


Figure 3.9- The distal/P2 promoter of HNF4α in humans with putative regulatory transcription factor binding sites. Putative transcription factor binding consensus sequences were determined using MatInspector (http://www.genomatix.de/cgi-bin//matinspector) and are shown by T-bars. Each CpG loci is numbered according to the distance from the transcription start site (TSS), and underlined in bold. Abbreviations are as follows:- **HOXA5**, Homeobox A5; **PDX1**, Pancreatic And Duodenal Homeobox 1; **HNF6**, Hepatocyte Nuclear Factor 6; **SP1**, Specificity Protein 1; **CTCF**, CCCTC-Binding Factor; **ETS1**, V-Ets Erythroblastosis Virus E26 Oncogene Homolog 1; **ARID1B**, AT Rich Interactive Domain 1B.

Figure 3.9 shows the distance of each CpG from the transcription start site covering a total of 42bp within the HNF4α distal/P2 promoter (150)(http://refgene.com/gene/3172). The distal/P2 promoter was selected as previous evidence in animal studies suggests it may be epigenetically altered by diet and may regulate gene expression via interaction with an upstream enhancer (151). However, the methylation of these CpGs has not been shown to be important in the regulation of gene expression in humans.

There is no literature to suggest that the binding of these putative transcription factors are important in the regulation of HNF4α.

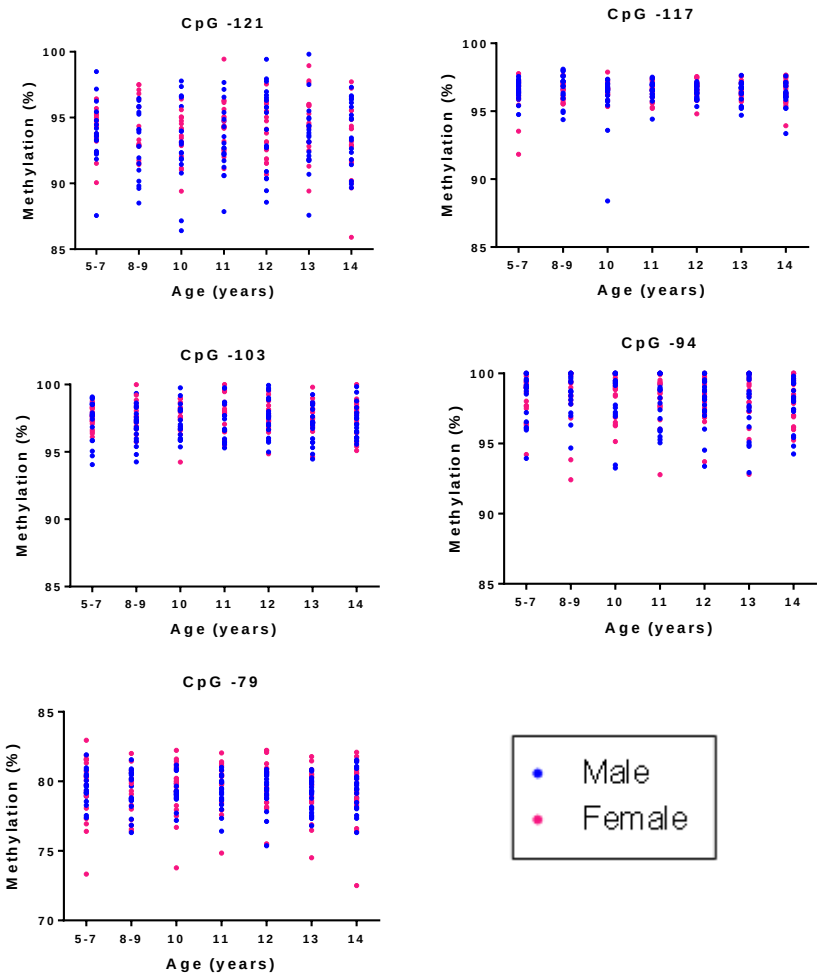


Figure 3.10- The methylation of each EarlyBird subject in the HNF4α distal/P2 promoter in peripheral blood between 5-7 and 14 years. Each panel represents the methylation of each subject at every age between 5-7 and 14 years for individual CpG loci within the HNF4α distal/P2 promoter. CpG loci are numbered according to the distance from the TSS. Male subjects are shown with a blue circle, and female subjects with a pink circle.

Figure 3.10 shows the methylation of the HNF4α distal/P2 promoter in peripheral blood for all subjects between 5-7 and 14 years. The methylation of CpG -103 shows the least variation across all subjects and ages with a difference of 5.94% between the highest and lowest levels, whereas CpG -121 has the largest variation with a difference of 13.92%. In peripheral blood, CpG -94 has the highest level of methylation across all subjects and ages and CpG -79 has the lowest level of methylation. There were no significant differences between sex and % methylation for any CpG measured ($p>0.05$), determined using an unpaired t-test with a Bonferroni correction.

3.3.4.2 Is the methylation of the HNF4α distal/P2 promoter stable between 5-7 and 14 years?

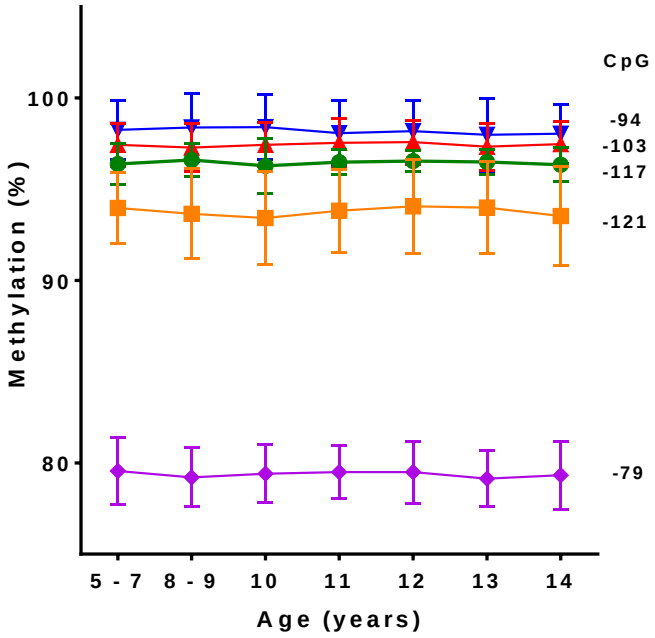


Figure 3.11- Mean methylation across the HNF4α distal/P2 promoter measured in peripheral blood between 5-7 and 14 years. The mean ± SD per cent methylation for each CpG loci within HNF4α distal/P2 promoter is shown for all subjects (n=40). CpG loci are numbered relative to the TSS. Mean methylation of individual CpG loci in the HNF4α distal/P2 promoter varied from 79.1 ± 1.6% (CpG -79) to 98.4 ± 1.8% (CpG -94) across all subjects and years.

Table 3.5- The stability of methylation across the HNF4α distal/P2 promoter in peripheral blood between 5-7 and 14 years

CpG	Longitudinal tracking co-efficient	p-value	% of variation in methylation at 14 years explained by the methylation at 5-7 years
-121	0.60	<0.001	36.0%
-117	0.17	0.01	2.9%
-103	0.20	0.09	4.0%
-94	0.68	<0.001	46.2%
-79	0.75	<0.001	56.3%

Table 3.5 shows the stability of methylation of the HNF4α distal/P2 promoter in peripheral blood between 5-7 and 14 years. Longitudinal tracking co-efficients were high for CpGs -121, -94 and -79 ranging from 0.60 to 0.75, all p<0.001, such that the methylation of these CpGs at 5-7 years predicted between 36.0% and 56.3% of the variation at 14 years. Table 10.3 in the appendix supports

this as there is a strong year-on-year correlation between % methylation of CpGs -121, -94 and -79 at 5-7 years and the methylation at all subsequent ages up to 14 years (r=0.51-0.84, p=<0.001-0.003). Therefore, the methylation of CpGs -121, -94 and -79 in the HNF4α distal/P2 promoter in peripheral blood is temporally stable throughout childhood.

The longitudinal tracking co-efficient for CpG -117 was significant (p=0.01), however the methylation of CpG -117 and -103 at 5-7 years did not predict the methylation at 14 years (<5%). The % methylation of CpGs -117 and -103 at 5-7 years was not correlated with the methylation at all subsequent ages up to 14 years (p>0.05), as shown in table 10.3 in the appendix. Therefore, the methylation of CpGs -117 and -103 in the HNF4α distal/P2 promoter in peripheral blood is not temporally stable throughout childhood.

3.3.4.3 Is the methylation of the HNF4α distal/P2 promoter associated with the proportions of neutrophils or lymphocytes?

Table 3.6- Cross-sectional associations between the methylation of HNF4α and leukocyte proportions in peripheral blood between 5-7 and 14 years

Age (years)	Correlation with neutrophils			Correlation with lymphocytes		
	r	p	n	r	p	n
	CpG -121					
5-7y	-0.19	0.25	37	0.28	0.09	37
8-9y	-0.15	0.40	32	0.03	0.87	32
10y	0.03	0.85	37	0.18	0.28	37
11y	0.10	0.57	38	-0.01	0.96	38
12y	0.36	0.03	39	-0.16	0.33	39
13y	0.01	0.97	38	0.09	0.61	38
14y	0.24	0.14	39	-0.02	0.91	39
	CpG -117					
5-7y	-0.42	0.01	37	0.40	0.02	37
8-9y	-0.28	0.12	32	0.36	0.04	32
10y	-0.13	0.44	37	0.23	0.18	37
11y	0.19	0.25	38	-0.06	0.74	38
12y	-0.30	0.07	39	0.28	0.09	39
13y	-0.17	0.32	38	0.20	0.24	38
14y	-0.19	0.24	39	0.16	0.32	39
	CpG -103					
5-7y	-0.08	0.63	37	0.19	0.26	37
8-9y	0.00	1.00	32	0.03	0.88	32
10y	0.37	0.03	37	-0.20	0.13	37
11y	0.08	0.64	38	-0.17	0.30	38
12y	0.08	0.62	39	0.03	0.86	39
13y	-0.14	0.40	38	0.12	0.47	38
14y	-0.05	0.75	39	0.11	0.52	39
	CpG -94					
5-7y	-0.02	0.90	37	-0.04	0.84	37
8-9y	-0.21	0.25	32	0.24	0.19	32
10y	0.13	0.44	37	-0.09	0.61	37
11y	0.05	0.77	38	0.01	0.94	38
12y	-0.14	0.39	39	0.09	0.59	39
13y	-0.25	0.13	38	0.24	0.16	38
14y	-0.23	0.17	39	0.25	0.12	39

	CpG -79					
5-7y	0.16	0.34	37	0.01	0.95	37
8-9y	0.07	0.72	32	0.08	0.66	32
10y	0.24	0.16	37	-0.18	0.28	37
11y	0.02	0.89	38	0.07	0.66	38
12y	-0.03	0.85	39	0.04	0.82	39
13y	0.07	0.68	38	-0.18	0.27	38
14y	-0.10	0.54	39	0.05	0.76	39

Table 3.6 shows the cross-sectional associations between the methylation of HNF4α and the proportion of neutrophils and lymphocytes in peripheral blood, at each age between 5-7 and 14 years. There is no consistent relationship between the proportion of neutrophils and lymphocytes and the level of methylation, however there is an inverse correlation between the neutrophils and lymphocytes for each CpG. This means that generally when the neutrophils are positively associated with methylation the lymphocytes are negatively associated with methylation, however this pattern is not always true for each CpG or each age measured.

Table 3.6 shows highlighted in grey the significant associations between the methylation of CpG -121, -117, and -103 and the proportion of neutrophils at ages 12, 5-7 and 10 years respectively ($p<0.05$). There was a significant association between the methylation of CpG -117 and the proportion of lymphocytes at ages 5-7 and 8-9 years, shown highlighted in grey ($p<0.05$).

There were no significant associations between the methylation of CpG -94 and -79 and the proportion of neutrophils between the ages of 5-7 and 14 years ($p>0.05$). There were no significant associations between the methylation of CpG -121, -103, -94 and -79 and the proportion of lymphocytes between the ages of 5-7 and 14 years ($p>0.05$). Therefore the methylation of CpG -94 and -79 in the HNF4α distal/P2 promoter in peripheral blood is not influenced by the proportions of neutrophils or lymphocytes between 5-7 and 14 years.

3.3.5.1 The methylation of RXRα in peripheral blood

Figure 3.12- An upstream region of the RXRα gene in humans with putative regulatory transcription factor binding sites. Putative transcription factor binding consensus sequences were determined using MatInspector (http://www.genomatix.de/cgi-bin//matinspector) and are shown by T-bars. Each CpG loci is numbered according to the distance from the transcription start site (TSS), and underlined in bold. Abbreviations are as follows:- **ZIC2**, ZIC Family Member 2; **HDBP1,** Huntington Disease Gene Regulatory Region-Binding Protein 1; **MAF**, Musculoaponeurotic Fibrosarcoma Oncogene Homolog; **EGR1**, Early Growth Response 1.

Figure 3.12 shows the distance of each CpG from the transcription start site covering a total of 11bp within the RXRα gene (http://refgene.com/gene/6256). This region of the RXRα gene was selected because the methylation of CpG -2346 in umbilical cord has been shown to be positively associated with childhood adiposity (115). However, this region of the RXRα gene has not been shown to be important in the regulation of gene expression in humans.

There is no literature to suggest that the binding of these putative transcription factors are important in the regulation of RXRα.

3.3.5.1.1 Are there any SNPs that modify the cytosine residue of the CpG dinucleotide measured?

CpG -2335 in RXRα contains a known polymorphism within the CpG dinucleotide, where the cytosine residue becomes a thymine, thereby removing the CpG dinucleotide and affecting DNA methylation. All subjects selected from the Earlybird cohort are Caucasian European. The frequency of this SNP in European populations has been reported by Ensembl (http://www.ensembl.org) as T-9% and C-91%, and T is the minor allele. The reference SNP number is **RXRα (rs11185648)** (http://www.ncbi.nlm.nih.gov/projects/SNP).

Table 3.7- The identification of subjects with a CpG-SNP in CpG -2335 of RXRα

	Subject ID	Genotype
RXRα CpG -2335	57	T/T
	209	C/T
	266	C/T
	267	C/T
	292	C/T

Table 3.7 identifies subjects with a known polymorphism located within the cytosine residue of CpG -2335 in RXRα. Each subject is heterozygous C/T containing one allele where the cytosine residue is replaced by a thymine residue, apart from subject 57 who is homozygous T/T and therefore does not have any methylation data available for this CpG. The % methylation of these subjects is shown at each time point in figure 3.13, where the methylation is half that of subjects without this SNP. Cytosine is the major allele within the 40 Earlybird children measured with an allele frequency of 87.5% for CpG -2335. These values are lower than those reported by Ensembl for European populations, which is likely explained by a small number of subjects (n=40). Because there were only 5 subjects confirmed to have a CpG-SNP and these subjects have decreased DNA methylation, they were excluded from further analysis of CpG -2335 in RXRα (n=35).

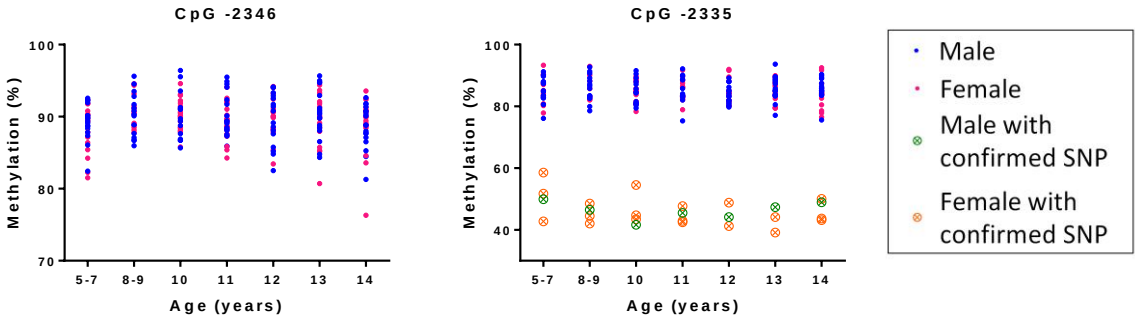


Figure 3.13- The methylation of each EarlyBird subject in the RXRα gene in peripheral blood between 5-7 and 14 years. Each panel represents the methylation of each subject at every age between 5-7 and 14 years for individual CpG loci within the SIRT1 promoter. CpG loci are numbered according to the distance from the TSS. Male subjects are shown with a blue circle, and female subjects with a pink circle. Male subjects with a confirmed SNP in CpG -2335 are shown with a green crossed circle, and females with an orange crossed circle.

Figure 3.13 shows the methylation of the RXRα gene in peripheral blood for all subjects between 5-7 and 14 years. CpG -2346 shows the least variation across all subjects and ages with a difference of 20.09% between the highest and lowest levels. CpG -2335 has the largest variation with a difference of 54.57%; however this is due to the presence of a confirmed SNP within 4 subjects. In peripheral blood, CpG -2346 has the highest level of methylation across all subjects and ages and CpG -2335 has the lowest level of methylation. There were no significant differences between sex and % methylation for either CpG measured ($p>0.05$), determined using an unpaired t-test with a Bonferroni correction.

3.3.5.2 Is the methylation within RXRα stable between 5-7 and 14 years?

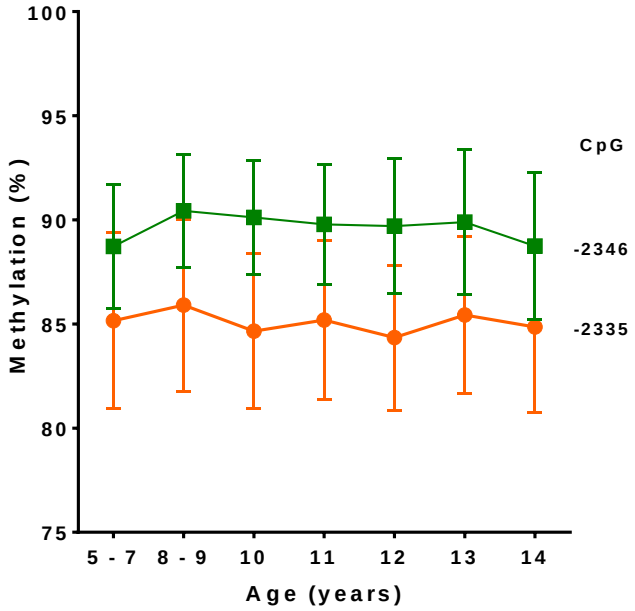


Figure 3.14- Mean methylation within RXRα measured in peripheral blood between 5-7 and 14 years. The mean ± SD per cent methylation for each CpG loci within RXRα is shown for all subjects. CpG -2335 has five subjects removed because these subjects are heterozygous or homozygous for a C>T SNP within the C of the CpG dinucleotide. Therefore, CpG -2346 (n=40) and CpG -2335 (n=35). CpG loci are numbered relative to the TSS. Mean methylation of individual CpG loci within RXRα varied from 84.3 ± 3.5% (CpG -2335) to 90.4 ± 2.7% (CpG -2346) across all subjects and years.

Table 3.8- The stability of methylation in the RXRα gene in peripheral blood between 5-7 and 14 years

CpG	Longitudinal tracking co-efficient	p-value	% of variation in methylation at 14 years explained by the methylation at 5-7 years
-2346	0.02	0.82	0.0%
-2335	0.50	<0.001	25.0%

Table 3.8 shows the stability of methylation within RXRα in peripheral blood between 5-7 and 14 years. The longitudinal tracking co-efficient was high for CpG -2335; 0.50, p<0.001, such that the methylation at 5-7 years predicted 25.0% of the variation at 14 years. Table 10.4 in the appendix shows there is a strong year-on-year correlation between % methylation of CpG -2335 at 5-7 years and the methylation at all subsequent ages up to 14 years (r=0.44-0.68, p=0.05-0.001). Therefore, the methylation of CpG -2335 in RXRα in peripheral blood is temporally stable throughout childhood.

The longitudinal tracking co-efficient was not significant for CpG -2346 ($p>0.05$) and the methylation at 5-7 years did not predict the methylation at 14 years. The % methylation of CpG -2346 at 5-7 years was not correlated with the methylation at all subsequent ages up to 14 years ($p>0.05$), as shown in table 10.4 in the appendix. Therefore, the methylation of CpG -2346 in RXRα in peripheral blood is not temporally stable throughout childhood.

3.3.5.3 Is the methylation within RXRα associated with the proportions of neutrophils or lymphocytes?

Table 3.9- Cross-sectional associations between the methylation of RXRα and leukocyte proportions in peripheral blood between 5-7 and 14 years

Age (years)	Correlation with neutrophils			Correlation with lymphocytes		
	r	p	n	r	p	n
	CpG -2346					
Age (years)	r	p	n	r	p	n
5-7y	0.01	0.96	32	-0.08	0.65	32
8-9y	-0.03	0.90	26	0.11	0.59	26
10y	-0.21	0.26	32	0.14	0.45	32
11y	0.12	0.52	33	-0.11	0.55	33
12y	-0.17	0.36	32	0.15	0.42	32
13y	0.13	0.46	34	-0.09	0.60	34
14y	-0.32	0.06	35	0.21	0.22	35
	CpG -2335					
5-7y	-0.08	0.68	27	0.17	0.40	27
8-9y	0.10	0.67	21	-0.05	0.83	21
10y	-0.17	0.38	28	0.27	0.17	28
11y	0.05	0.81	28	0.03	0.88	28
12y	-0.10	0.61	29	0.23	0.22	29
13y	0.10	0.60	31	0.02	0.93	31
14y	0.11	0.57	30	-0.11	0.55	30

Table 3.9 shows the cross-sectional associations between methylation of RXRα and the proportion of neutrophils and lymphocytes in peripheral blood, at each age between 5-7 and 14 years. There is no consistent relationship between the correlation of methylation and the proportion of neutrophils and lymphocytes; however, there is an inverse correlation between methylation and neutrophils and lymphocytes for each CpG. This means that when the neutrophils are positively associated with methylation the lymphocytes are negatively associated with methylation; however this pattern is not always true for each CpG or each age measured and changes over time. For

example for CpG -2346 at age 5-7 years there is a positive correlation between the proportion of neutrophils and methylation, however at age 8-9 years there is a negative correlation.

There were no significant associations between the methylation of CpG -2346 and -2335 and the proportions of neutrophils or lymphocytes between the ages of 5-7 and 14 years ($p>0.05$). Therefore the methylation of these CpGs in RXRα in peripheral blood is not influenced by the proportions of neutrophils and lymphocytes between 5-7 and 14 years.

3.3.6.1 The methylation of the GCK promoter in peripheral blood

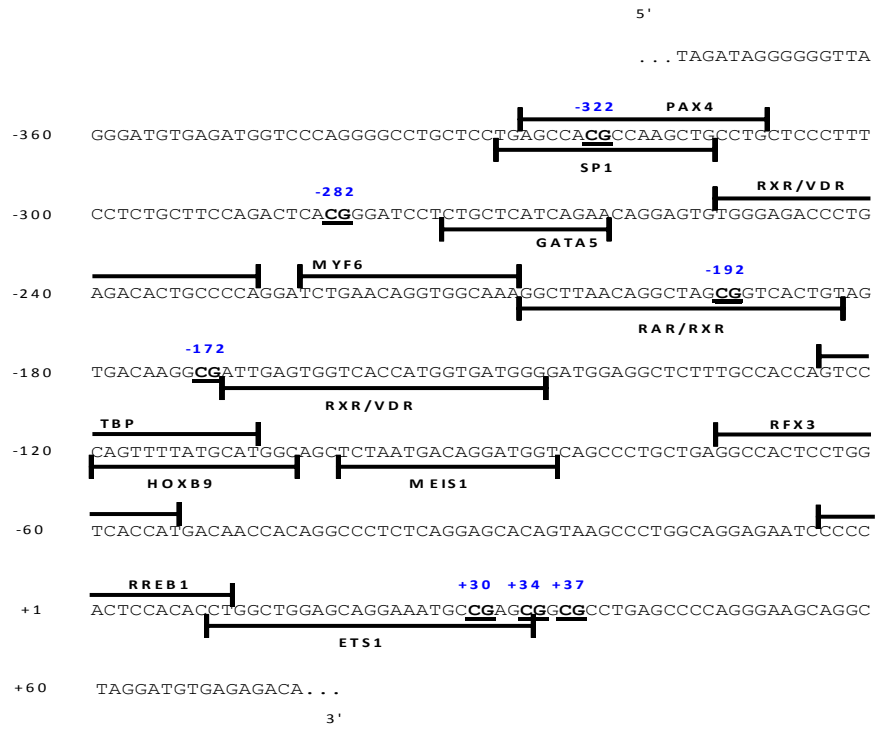


Figure 3.15- The proximal promoter of GCK in humans with putative regulatory transcription factor binding sites. Putative transcription factor binding consensus sequences were determined using MatInspector (http://www.genomatix.de/cgi-bin//matinspector) and are shown by T-bars. Each CpG loci is numbered according to the distance from the transcription start site (TSS), and underlined in bold. Abbreviations are as follows:- **SP1**, Specificity Protein 1; **PAX4**, Paired Box 4; **GATA5**, GATA Binding Protein 5; **RXR/VDR**, Retinoid X Receptor/Vitamin D Receptor heterodimer; **MYF6**, Myogenic Factor 6; **RAR/RXR**, Retinoic Acid Receptor/ Retinoid X Receptor heterodimer; **TBP**, TATA Box Binding Protein; **HOXB9**, Homeobox B9; **MEIS1**, Meis Homeobox 1; **RFX3**, Regulatory Factor X,3; **RREB1**, Ras Responsive Element Binding Protein 1; **ETS1**, V-Ets Erythroblastosis Virus E26 Oncogene Homolog 1.

Figure 3.15 shows the distance of each CpG from the transcription start site covering a total of 359bp within the GCK promoter (http://refgene.com/gene/2645). The proximal promoter of GCK was selected because evidence from a rat model suggests that methylation may not be stable over time (157). Whilst the methylation of these CpGs has not been shown to be important in the regulation of gene expression in humans, these loci are within the proximal promoter and the promoters of genes are known to be important for transcription (95).

Of these putative transcription factor binding sequences RFX3 is known to upregulate pancreatic GCK expression (172). Therefore, binding of RFX3 to this region of the GCK promoter may be important in the regulation of GCK expression.

3.3.6.1.1 Are there any SNPs that modify the cytosine residue of the CpG dinucleotide measured?

CpG -282 in GCK contains a known polymorphism within the CpG dinucleotide, where the cytosine residue becomes a thymine, thereby removing the CpG dinucleotide and affecting DNA methylation. The frequency of this SNP in European populations has been reported by Ensembl (http://www.ensembl.org) as T-7% and C-93%, and T is the minor allele. The reference SNP number is **GCK (rs35670475)** (http://www.ncbi.nlm.nih.gov/SNP).

Table 3.10- The identification of subjects with a CpG-SNP in CpG -282 of GCK

	Subject ID	Genotype
GCK CpG -282	54	C/T
	85	C/T
	130	C/T
	217	C/T
	254	C/T
	299	C/T

Table 3.10 identifies subjects with a known polymorphism located within the cytosine residue of CpG -282 GCK. Each subject is heterozygous C/T containing one allele where the cytosine residue is replaced by a thymine residue. The % methylation of these subjects is shown at each time point in figure 3.16, where the methylation is half that of subjects without this SNP. Cytosine is the major allele within the 40 Earlybird children measured with an allele frequency of 85.0%. These values are lower than those reported by Ensembl for European populations, which is explained by a small number of subjects (n=40). Because there were only 6 subjects confirmed to have a CpG-SNP and these subjects have decreased DNA methylation, they were excluded from further analysis of CpG -282 in GCK (n=34).

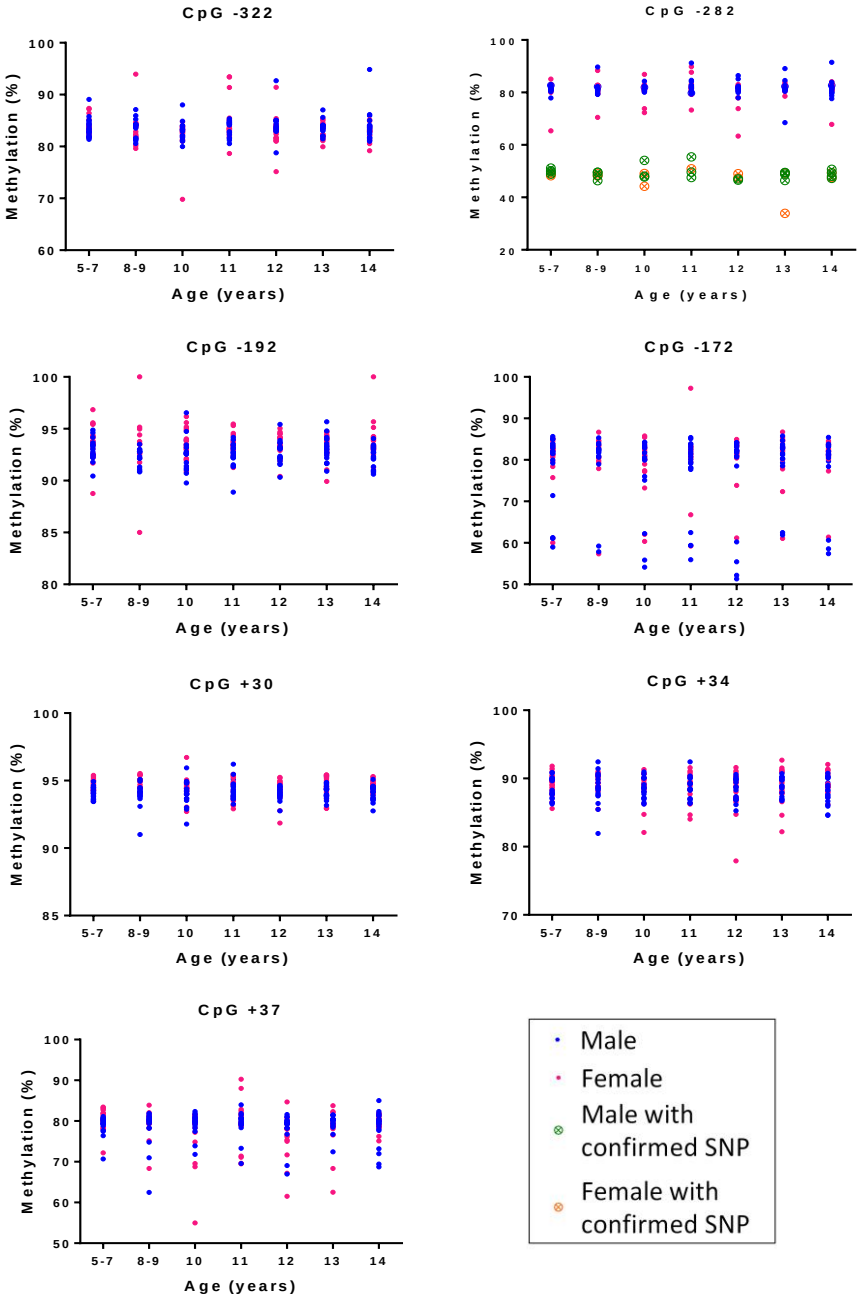


Figure 3.16- The methylation of each EarlyBird subject in the GCK promoter in peripheral blood between 5-7 and 14 years. Each panel represents the methylation of each subject at every age between 5-7 and 14 years for individual CpG loci within the GCK promoter. CpG loci are numbered according to the distance from the TSS. Male subjects are shown with a blue circle, and female subjects with a pink circle. Male subjects with a confirmed SNP in CpG -2335 are shown with a green crossed circle, and females with an orange crossed circle.

Figure 3.16 shows the methylation of the GCK promoter in peripheral blood for all subjects between 5-7 and 14 years. The methylation of CpG +30 shows the least variation across all subjects and ages with a difference of 5.72% between the highest and lowest levels, whereas CpG -282 has the largest variation with a difference of 66.67%; however this is due to the presence of a confirmed SNP within 6 subjects. In peripheral blood, CpG +30 has the highest level of methylation across all subjects and ages and CpG -282 has the lowest level of methylation. There was a significant difference between sex and % methylation of CpG -282 ($p=0.02$) and +37 ($p=0.02$) determined using an unpaired t-test with a Bonferroni correction. However, when the 6 subjects containing the SNP in CpG -282 were removed, there was no significant interaction between sex and % methylation ($p=0.13$). For CpG +37, the range of methylation for the boys is smaller than for the girls, at each age between 5-7 years and 14 years, which may explain why the methylation is significantly different between sexes. There were no significant differences between sex and % methylation of CpG -322, -192, -172, +30 and +34 ($p>0.05$) determined using an unpaired t-test with a Bonferroni correction.

3.3.6.2 Is the methylation of the GCK promoter stable between 5-7 and 14 years?

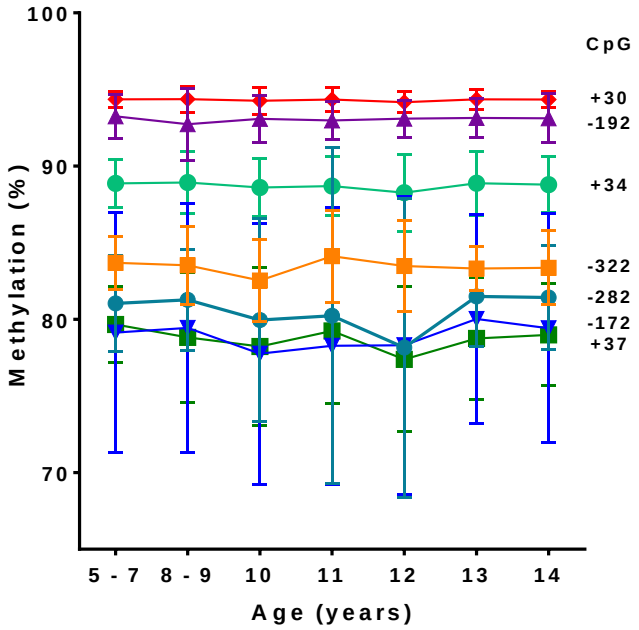


Figure 3.17- Mean methylation across the GCK promoter measured in peripheral blood between 5-7 and 14 years. The mean ± SD per cent methylation for each CpG loci within the GCK promoter is shown for all subjects. CpG -282 has six subjects removed because these subjects are heterozygous for a C>T SNP within the C of the CpG dinucleotide. Therefore, CpG -282 (n=34) and all other loci (n=40). CpG loci are numbered relative to the TSS. Mean methylation of individual CpG loci in the GCK promoter varied from 77.4 ± 4.7% (CpG +37) to 94.4 ± 0.8% (CpG +30) across all subjects and years.

Table 3.11- The stability of methylation across the GCK promoter in peripheral blood between 5-7 and 14 years

CpG	Longitudinal tracking co-efficient	p-value	% of variation in methylation at 14 years explained by the methylation at 5-7 years
-322	0.26	0.01	6.8%
-282	-0.02	0.72	0.0%
-192	0.41	<0.001	16.8%
-172	0.89	<0.001	79.2%
+30	0.43	<0.001	18.5%
+34	0.61	<0.001	37.2%
+37	0.13	0.18	1.7%

Table 3.11 shows the stability of methylation of the GCK promoter in peripheral blood between 5-7 and 14 years. Longitudinal tracking co-efficients were high for CpGs -192, -172, +30 and +34 ranging from 0.41 to 0.89, all $p<0.001$, such that the methylation at 5-7 years predicted between 16.8% to 79.2% of the variation at 14 years. Table 10.5 in the appendix shows there is a strong year-on-year correlation between % methylation of CpGs -172, +30 and +34 at 5-7 years and the methylation at all subsequent ages up to 14 years ($r=0.39$-0.97, $p=0.02$-<0.001). Table 10.5 in the appendix shows the % methylation of CpG -192 at 5-7 years strongly correlates with the methylation up to 12 years only ($r>0.58$, $p<0.001$). Therefore, the methylation of CpGs -172, +30 and +34 in the GCK promoter in peripheral blood is temporally stable throughout childhood, with the methylation of CpG -192 being less stable.

The longitudinal tracking co-efficient for CpG -322 was significant ($p=0.01$), however the methylation at 5-7 years only predicted 6.8% of the methylation at 14 years. The longitudinal tracking co-efficient was not significant for CpGs -282 and +37 ($p>0.05$) and the methylation at 5-7 years did not predict the methylation at 14 years. The % methylation of CpGs -322, -282 and +37 was not correlated with the methylation at all subsequent ages up to 14 years ($p>0.05$), as shown in table 10.5 in the appendix. Therefore, the methylation of CpGs -322, -282 and +37 in the GCK promoter in peripheral blood is not temporally stable throughout childhood.

3.3.6.3 Is the methylation of the GCK promoter associated with the proportions of neutrophils or lymphocytes?

Table 3.12- Cross-sectional associations between the methylation of GCK and leukocyte proportions in peripheral blood between 5-7 and 14 years

Age (years)	Correlation with neutrophils			Correlation with lymphocytes		
	r	p	n	r	p	n
	CpG-322					
5-7y	0.13	0.43	37	0.02	0.89	37
8-9y	0.20	0.28	31	0.01	0.95	31
10y	-0.02	0.93	34	-0.03	0.89	34
11y	-0.06	0.74	37	0.26	0.12	37
12y	0.49	0.003	34	-0.44	0.009	34
13y	0.08	0.64	33	0.03	0.87	33
14y	-0.24	0.15	37	0.24	0.15	37
	CpG -282					
5-7y	<0.01	0.99	30	<-0.01	0.98	30
8-9y	0.26	0.23	24	-0.17	0.44	24
10y	0.22	0.29	26	-0.32	0.11	26
11y	0.13	0.49	30	-0.13	0.50	30
12y	-0.07	0.73	28	-0.03	0.89	28
13y	-0.29	0.15	26	0.15	0.45	26
14y	-0.37	0.04	31	0.23	0.21	31
	CpG-192					
5-7y	0.06	0.72	38	-0.07	0.68	38
8-9y	0.08	0.69	26	0.06	0.77	26
10y	0.06	0.72	36	0.22	0.21	36
11y	0.12	0.51	35	<-0.01	0.96	35
12y	-0.01	0.94	35	0.11	0.52	35
13y	0.21	0.25	33	-0.09	0.61	33
14y	0.07	0.68	38	<0.01	0.96	38
	CpG -172					
5-7y	-0.06	0.75	37	0.29	0.08	37
8-9y	0.14	0.50	25	0.03	0.87	25
10y	-0.03	0.89	34	0.00	1.00	34
11y	0.03	0.87	34	-0.08	0.65	34
12y	-0.11	0.54	34	<0.01	0.99	34
13y	0.03	0.90	30	0.11	0.57	30
14y	0.02	0.91	35	-0.03	0.87	35

	CpG +30					
5-7y	0.09	0.58	38	-0.01	0.95	38
8-9y	-0.17	0.37	31	0.17	0.35	31
10y	-0.06	0.73	38	0.23	0.17	38
11y	<-0.01	1.00	38	0.12	0.47	38
12y	0.22	0.21	36	-0.12	0.49	36
13y	<0.01	0.97	36	0.04	0.81	36
14y	<0.01	0.91	39	0.05	0.78	39
	CpG +34					
5-7y	0.23	0.16	38	-0.28	0.09	38
8-9y	0.31	0.09	31	-0.33	0.07	31
10y	-0.12	0.48	36	0.20	0.25	36
11y	0.19	0.29	34	-0.08	0.65	34
12y	0.26	0.14	34	-0.33	0.06	34
13y	0.02	0.93	33	0.09	0.62	33
14y	0.03	0.86	38	0.02	0.88	38
	CpG +37					
5-7y	-0.04	0.81	38	0.04	0.81	38
8-9y	0.18	0.33	32	-0.18	0.34	32
10y	-0.28	0.10	36	0.35	0.03	36
11y	0.15	0.41	34	<-0.01	0.97	34
12y	0.38	0.02	35	-0.45	0.007	35
13y	-0.18	0.30	34	0.31	0.08	34
14y	-0.02	0.89	39	-0.01	0.95	39

Table 3.12 shows the cross-sectional associations between methylation of GCK and the proportion of neutrophils and lymphocytes in peripheral blood, at each age between 5-7 and 14 years. There is no consistent relationship between the proportion of neutrophils and lymphocytes and the level of methylation, however there is an inverse correlation between the neutrophils and lymphocytes for each CpG. This means that when the neutrophils are positively correlated with methylation the lymphocytes are negatively correlated with methylation; however this pattern is not always true for each CpG or each age measured and changes over time. For example for CpG +37 at all ages between 5-7 and 13 years when there is a positive correlation between methylation and the proportion of neutrophils, there is a negative correlation with the proportion of lymphocytes, and vice versa.

Table 3.12 shows highlighted in grey the significant associations between the methylation of CpG -322, -282 and +37 and the proportion of neutrophils at ages 12, 14 and 12 years respectively ($p<0.04$). There were significant associations between the methylation of CpG -322, and +37 and the proportion of lymphocytes at age 12 years, and also age 10 years for CpG +37, shown highlighted in grey ($p<0.03$).

There were no significant associations between the methylation of CpG -192, -172, +30 and +34 and the proportions of neutrophils between the ages of 5-7 and 14 years ($p>0.05$). There were no significant associations between the methylation of CpG -282, -192, -172, +30 and +34 and the proportion of lymphocytes between the ages of 5-7 and 14 years ($p>0.05$). Therefore the methylation of CpGs -192, -172, +30 and +34 in the GCK promoter in peripheral blood is not influenced by the proportions of neutrophils or lymphocytes between 5-7 and 14 years.

3.4 Discussion

3.4.1 SNPs that modify the cytosine residue of a CpG dinucleotide

Single nucleotide polymorphisms (SNPs) are common in the human genome, with the most common SNP being a C to T SNP (173). This is because methylated cytosines are frequently deaminated to thymine, which is often unrecognised and as a result is not repaired by DNA repair enzymes (173). The HapMap database has identified 2.3 million SNPs with 34% of these SNPs being located within a CpG site (173). Therefore, CpG-SNPs are common within the genome and have the ability to alter DNA methylation, which affects molecular mechanisms including the binding of transcription factors and methyl binding proteins. This can lead to altered gene expression thereby influencing a particular phenotype, which could have an impact on the development of disease. For example, data from genome wide association studies (GWASs) suggests that the presence of CpG-SNPs is associated with risk of disease, including T2D (173). Dayeh et al. have shown previously that 19 of the 40 SNPs identified by GWAS that are associated with T2D either introduce or remove a CpG site, and the presence of these SNPs was confirmed to affect methylation in pancreatic islet cells, suggesting that CpG-SNPs may be informative of future disease risk (173).

Whilst CpG-SNPs may be involved in the development of particular diseases such as obesity, methylation data for CpG -2335 in RXRα and CpG -282 in GCK was excluded for subjects confirmed to have a CpG-SNP, for several reasons. Because subjects who were heterozygous for a CpG-SNP within the cytosine residue of CpG -2335 in RXRα and CpG -282 in GCK, the level of methylation was reduced to half that of the subjects without these SNPs. Therefore, separate statistical analysis would be required for homozygous and heterozygous subjects as methylation was different between these two groups. This was not possible due to a small number of subjects containing these SNPs which would make the analyses statistically underpowered.

A common G>A SNP at -258 in the GCK promoter has been linked with insulin resistance in a population of Asian Indians, which is located 24bp downstream of the CpG-SNP measured in the EarlyBird cohort (155). Therefore, this region of the GCK promoter may be susceptible to polymorphisms, which may alter transcription factor binding and gene expression, thereby influencing a particular phenotype such as insulin resistance.

These findings have shown that the methylation of the majority of CpG loci tested was not affected by the presence of a CpG-SNP. For those CpG loci where methylation was affected by a CpG-SNP, the frequency of subjects that were heterozygous was less than 10% suggesting that T is the minor allele in European populations.

3.4.2 Stability of DNA methylation in the EarlyBird cohort

There is a lack of information about the stability of DNA methylation across the life course, in particular the stability of DNA methylation throughout childhood and puberty, which have been proposed to be periods of increased epigenetic change, where insulin resistance and hormones levels fluctuate (95)(12). Environmental factors such as pollution and exercise have been shown to be associated with altered DNA methylation of specific loci, with the methylation changing again once the environmental factor is removed (134)(135)(82). Therefore, challenges that occur during childhood and puberty such as infection or stress may have an impact on the stability of DNA methylation.

Of the information that does exist regarding the stability of DNA methylation, there are conflicting findings (133)(136)(132). For example, data from a human twin study has shown that younger monozygotic twins who had spent their lives together had methylation patterns more identical than older twins who had spent time in different environments (133). Another study by Wong et al. showed that the methylation of specific CpG loci in genes associated with neurological function in buccal cells was unstable between 5 and 10 years of age in monozygotic and dizygotic twins (136). However, whilst the methylation of individual CpG loci was measured for each gene, the average DNA methylation of multiple CpG sites was used in the statistical analysis for this study. Therefore, these data are unreliable as the methylation of a specific CpG locus is not necessarily associated with the methylation of the remaining CpG loci within a genomic region.

There is also some evidence to suggest that the methylation of specific loci may be stable throughout the life course and not influenced by future environmental challenges (132). For example, a study by Wang et al. showed the methylation levels of 95.5% of CpGs from a 27K array were stable from birth throughout the first 2 years of life, measured in umbilical cord blood leukocytes and peripheral blood leukocytes (132). However, a 27K array measures less than 1% of the total CpGs within the human genome and this study did not validate the findings of the array making these findings unreliable.

Therefore, to address if DNA methylation is stable throughout childhood and puberty, the methylation of 5 genes that have been implicated in the development of obesity, insulin resistance and T2D was examined annually between 5 and 14 years. These findings have shown that the methylation of some CpG loci is stable throughout childhood and puberty up to 14 years, and is unaffected by future stochastic and environmental challenges, or by hormonal changes that occur during puberty. The methylation of each CpG measured in SIRT1 was temporally stable between the

ages of 5-7 and 14 years in peripheral blood. SIRT1 down regulates P53 activity by deacetylation thereby reducing cellular senescence and is known as the 'longevity gene' (174). SIRT1 has been shown to positively regulate telomere length *in vivo* and reduce telomere shortening that occurs with aging (175). Therefore, if methylation of these CpGs were shown to have a functional effect on SIRT1 gene expression, methylation could be a potential marker of aging.

The methylation of each CpG measured in PGC1α was temporally stable between the ages of 5-7 and 14 years in peripheral blood. This in contrast to a study by Barrès et al. where acute exercise resulted in hypomethylation of PGC1α in skeletal muscle after only 3 hours, which associated with a significant increase in gene expression (82). However, the methylation of these 7 CpG loci in the PGC1α promoter was not measured in the Barrès et al. study. Instead, the methylation was measured by methylated DNA immunoprecipitation (MeDIP) which measures the relative enrichment of DNA rather than absolute DNA methylation and therefore, methylation changes could be very small, and the methylation of these 7 CpG loci may be unaffected by exercise. Another consideration is that the methylation was measured in skeletal muscle, which is a highly adaptive and plastic tissue that is known to respond to environmental challenges, both structurally and metabolically (82). Whilst the immune system is also dynamic and adaptive, the methylation of these loci were shown to be unaffected by the proportions of neutrophils and lymphocytes (table 3.4), suggesting that the immune system may not have an impact on the stability of these loci between 5-7 and 14 years. Brøns et al have shown that the methylation of CpGs -841, -816, -783 and -652 increases following a high fat diet for 5 days in skeletal muscle from normal birth weight men (164). However, the methylation of these CpG loci was determined using bisulphite sequencing data rather than pyrosequencing, which is a less accurate method for measuring DNA methylation. Also, the methylation was measured in skeletal muscle, suggesting that the stability of methylation of PGC1α may differ across tissue types, which may be important for tissue specific gene expression and function.

These findings have shown that the methylation of 3 CpG loci in HNFα and 4 CpG loci in GCK was temporally stable between the ages of 5-7 and 14 years in peripheral blood, however the remaining CpGs measured were not stable. This suggests that not all CpG loci within the same region of a gene are coordinated and may not influence gene expression equally. For example, methylation is usually associated with a reduction in transcription factor binding as the methyl group physically blocks the binding site, or prevents the transcriptional machinery from binding (104). However, methylation can also enhance the binding of some transcription factors (105)(106). Therefore,

depending on the stability of methylation, the binding of certain transcription factors may be altered over time which may alter gene expression and influence a particular phenotype.

The methylation of CpG -2335 in the RXRα gene is temporally stable between the ages of 5-7 and 13 years in peripheral blood, however the correlation was lost at age 14 suggesting the methylation may be affected by puberty, which has been proposed to be a period of increased epigenetic change, where insulin resistance and hormones levels fluctuate (95)(12).

Evidence suggests that the epigenome is most susceptible to environmental factors during periods of rapid cell division throughout early development when methylation patterns are set up following genome-wide demethylation (129)(176). Therefore, the prenatal and early life environment can potentially induce differentially methylated loci, which, if associated with a particular disease has the ability to act as a biomarker of disease risk (177). Whilst Godfrey et al. have shown previously that the methylation of CpG -2346 in RXRα is positively associated with future adiposity in childhood in umbilical cord; these findings have shown the methylation of this loci is unstable in peripheral blood throughout childhood between 5-7 years and 14 years (115). However, the stability of DNA methylation at this locus may vary across different tissue types.

Whilst it may be preferential that the methylation of potential biomarkers of disease risk is stable over time so they could be measured throughout the life course, an association between methylation and disease at any time point is useful. It is also unknown if the methylation of these potential biomarkers of disease may change over time as disease progression occurs. Unstable DNA methylation may be informative of epigenetic dysregulation that may be associated with a particular disease.

These findings suggest that, at least for some loci, methylation patterns are established during early childhood before the age of 5-7 years and are not influenced by environmental challenges such as puberty, up to the age of 14 years. The methylation of these loci was measured in peripheral blood, which is a heterogeneous mix of white blood cell populations, which each have their own specific DNA methylation levels. Therefore, the association between leukocyte proportions and methylation was determined for each CpG between 5-7 and 14 years.

3.4.3 Association between leukocyte proportions and DNA methylation

Epidemiological studies investigating the effect of environment on DNA methylation have used peripheral blood as a surrogate for DNA from target tissues due to the ease of collection from large numbers of subjects and because samples can be taken repeatedly throughout a longitudinal study (119). However, peripheral blood is a heterogeneous mix of blood cell populations, including leukocytes which have their own methylation levels, and has been identified as a potential confounder when measuring DNA methylation in peripheral blood (120). The relative leukocyte proportions may show intra-individual variation, which is particularly important in longitudinal studies because changes in DNA methylation over time may be explained by changes to the relative proportions of different white blood cell types (119).

Relatively few studies have addressed whether leukocyte proportions can influence the levels of DNA methylation measured in peripheral blood, with conflicting findings from those studies that have (120)(119). For example, the proportion of neutrophils explained 50% of the variation in DNA methylation of IL10 measured in peripheral blood from adults aged between 21 and 73 years old (119). However, the variation in methylation that was explained by the proportion of neutrophils was less ($p>0.05$) for the remaining genes studied, which included both imprinted (IGF2, 3.5%) and non-imprinted genes (FTO, 0.1%) (119). A caveat to this study is the small sample size (n=30), compared with a large age range (52 years) and data may need to be taken with caution. Similar results were found in a study by Adalsteinsson et al., where cellular heterogeneity explained 40% of the variation in methylation measured in a CpG island in HHEX ($p<0.0001$), measured in peripheral blood (120). However, for the remaining CpG islands measured in 3 different genes, the variation in methylation was not explained by cellular heterogeneity (120). Whilst the sample size was larger for this study, the age range of the subjects was between 22 to 96 years. Data was not corrected for age suggesting these results may not be statistically accurate.

These findings in the EarlyBird subjects support findings from previous studies which suggest that cellular heterogeneity does not affect DNA methylation equally across the epigenome. The methylation of PGC1α was not affected by the proportion of neutrophils or lymphocytes, apart from CpG -515, in peripheral blood. Whilst the methylation of CpG -515 in the PGC1α promoter significantly associated with the proportion of neutrophils, the association was at 8-9 years only where the number of subjects was reduced from 40 to 33. Therefore, any outliers in the methylation or neutrophil proportions at this age may have influenced the association, and this may be a false positive. The methylation of each subject and the proportion of neutrophils would need to be

available for all subjects in order to determine whether methylation of this locus is truly influenced by the proportion of neutrophils in peripheral blood during childhood and puberty.

The methylation of RXRα was not affected by the proportion of neutrophils or lymphocytes at any age between 5-7 and 14 years, in peripheral blood. RXRα is known to be important in the regulation and function of the immune system and therefore it is perhaps surprising that the methylation of these loci was not influenced by neutrophils or lymphocytes, which are an essential part of the immune system (178). This may be because the level of methylation of these loci is high (>75%) for all subjects and the proportions of leukocytes may influence the methylation of CpG loci elsewhere within the RXRα gene where the methylation is within a smaller range (e.g. <25%).

For the remaining genes SIRT1, HNF4α and GCK, the methylation of specific CpGs were found to be significantly associated with the proportion of neutrophils or lymphocytes. These findings suggest that cellular heterogeneity affects DNA methylation in a locus-specific manner, and the methylation of CpGs within the same region of a gene may not be equally affected by leukocyte proportions. The relative size of the variation in methylation explained by cellular heterogeneity compared to the variation in methylation that is explained by other factors such as transcription factor binding will also be influenced by the location of the specific locus within the gene (120).

The methylation of specific CpG loci in SIRT1, HNF4α and GCK was affected by leukocyte proportions at particular ages only, and none of these loci were significantly associated with the proportion of neutrophils or lymphocytes at all ages between 5-7 and 14 years. This could be explained by the time of year when the peripheral blood samples were taken. Leukocyte proportions vary seasonally for example with hay fever in the summer compared to winter months, and also with illness such as a common cold. Unfortunately, it is unknown when these samples were taken each year and whether the subjects were ill or recovering from illness. Therefore, it was not possible to correct for seasonality or illness in the statistical analysis, which could explain why the association between leukocyte proportions and the methylation of specific CpG loci in SIRT1, HNF4α and GCK occurs at certain time points only.

3.5 Summary

These data support the hypothesis that the methylation of specific CpG loci in genes involved in β cell function, glucose homeostasis, adipogenesis and energy metabolism, is stable throughout childhood and puberty. These findings suggest that, at least for some loci, methylation patterns are established during early childhood before the age of 5-7 years and are not influenced by environmental challenges such as puberty, up to the age of 14 years. These findings also suggest that cellular heterogeneity does not affect DNA methylation equally across the epigenome.

Therefore, methylation of these temporally stable loci may be set up *in utero* or during early life. An environmental challenge that occurs during these periods of development could permanently alter epigenetic mechanisms, which may increase the risk of disease later in life (179). Therefore, if the methylation of these temporally stable loci associated with a particular phenotype, they may have utility as potential epigenetic biomarkers of disease risk.

Chapter 4

DNA Methylation of Metabolic Genes in Early Life can be a Biomarker of Future Adiposity

4.1 Introduction

4.1.1 Altered DNA methylation can be a potential biomarker of disease risk

A biomarker is broadly defined as 'a characteristic that is objectively measured and evaluated as an indicator of normal biological processes or pharmacologic responses to a therapeutic intervention' (100). Therefore, if altered DNA methylation were associated with a particular phenotype, it has the ability to act as a biomarker of disease risk and could be used to identify those individuals at increased risk of disease who may benefit from targeted intervention.

There is evidence from animal and human studies that suggests altered DNA methylation of specific CpG loci is associated with risk of non-communicable diseases later in life. For example, it has been shown that increased methylation of exon 3 of POMC (proopiomelanocortin) in umbilical cord blood is associated with lower birth weight and higher insulin concentrations in peripheral blood in childhood, suggesting that methylation in umbilical cord blood may be predictive of future risk of insulin resistance (122). Another study has shown that the methylation of specific CpG loci in CDKN1C (cyclin-dependent kinase inhibitor 1C), a gene involved in cellular senescence, and EPHA1 (ephrin type-A receptor), a gene involved in cell proliferation, measured in umbilical cord blood is associated with BMI and fat mass at 9 years of age (116). For each 1% increase in methylation BMI increased by 2.08% and 0.80%, and fat mass increased by 5.16% and 1.84%, respectively (116). Godfrey et al. showed increased methylation of a specific CpG in RXRα in umbilical cord associated with increased adiposity in childhood at age 6 and 9 years, in two separate cohorts, such that methylation at birth explained more than 25% of the variation in adiposity in childhood (115). Taken together, these data suggest that measuring the methylation of specific loci at birth in easy to access tissues may be useful for identifying individuals with an increased risk of obesity and metabolic syndrome.

This supports the DOHaD concept which suggests that the early life environment can alter the epigenetic regulation of genes, which leads to a change in later phenotype. Maternal pre-pregnancy BMI and gestational weight gain have been suggested to increase the risk of disease in the offspring, and there is evidence to suggest that maternal body composition can influence the methylation of specific CpG loci in the offspring (171)(118). Therefore, maternal body composition may be a factor in determining the methylation of the offspring, which in turn may influence the risk of disease. For example, Gemma et al. showed a positive association between maternal BMI and the methylation of 3 CpG loci in the PGC1α promoter, measured in umbilical cord DNA, and the methylation of one of these loci has also been shown to be hypermethylated in subcutaneous adipose tissue from obese subjects, compared to lean (171)(79). Morales et al. have shown that increased gestational weight

gain during early pregnancy is associated with increased methylation of a CpG loci in MMP7 (matrix metallopeptidase 7) in umbilical cord blood, a gene involved in embryonic development and extracellular matrix remodelling that occurs during adipose tissue formation (118). These data suggest that maternal body composition may be involved in determining the methylation levels of these potential epigenetic biomarkers that associated with future adiposity. Therefore, maternal body composition may be the driver of these associations between methylation and adiposity in the offspring.

However, there are lots of studies in animals and humans to suggest that maternal diet is also important in influencing the epigenetic regulation of genes that are associated with risk of disease in the offspring (114)(113)(117). For example, data from the Dutch Hunger Winter Famine has shown that individuals exposed during early gestation had increased methylation of specific CpG loci in the IGF2 gene and these children had an increased susceptibility to obesity, T2D and cardiovascular disease later in life (117)(55). Therefore, maternal diet may be important in determining the level of methylation of these potential biomarkers of disease risk.

Several studies have shown that altered methylation occurs in diseases such as T2D (49)(180). For example, specific CpG loci in the PGC1α promoter have been shown to be hypermethylated in the islet cells from overweight T2D subjects, compared to normal weight, healthy controls, which associated with a decrease in mRNA expression and impaired glucose-mediated insulin secretion (49). Whilst the methylation of these loci is associated with disease risk, it may be more beneficial to human health to discover potential epigenetic biomarkers that are able to predict future disease risk, allowing for earlier intervention and prevention.

More evidence is required in humans to determine if altered DNA methylation has utility as a potential biomarker of disease risk. For example, the methylation of some loci may change over time as a particular disease progresses. Whilst it is not essential that the methylation of these potential biomarkers is stable throughout the life course, it would be preferable to discover a biomarker that could predict future risk of disease when measured at any age. It would also be beneficial to discover a biomarker of disease risk that could be measured in easy to access tissues such as peripheral blood or fetal tissues that would reflect the methylation in harder to access tissues.

The results from chapter 3 have shown that the methylation of specific CpG loci in genes involved in the development of obesity, insulin resistance and T2D is unaffected by the presence of a SNP or cellular heterogeneity, and the methylation was temporally stable throughout childhood, up to 14 years. Therefore, if the methylation of these CpG loci were associated with future phenotype,

which in this case is adiposity and insulin resistance during childhood, they may be beneficial as potential biomarkers of future risk of metabolic syndrome.

4.1.2 Aims

In order to test the hypothesis that DNA methylation is predictive of future adiposity and/or insulin resistance, the association between methylation status of individual CpG loci and adiposity (% fat mass) and insulin resistance (HOMA-IR) between 5-7 years and 14 years was examined. Identification of these potential biomarkers of future adiposity and/or insulin resistance in peripheral blood would increase the number of individuals in whom such associations could be tested due to ease of collection.

4.2 Methods

After determining that the methylation of specific CpG loci was unaffected by the presence of a SNP, cellular heterogeneity and was stable between 5-7 years and 14 years, the association between methylation and adiposity, and methylation and insulin resistance was examined. Statistical analysis was carried out by Dr. Joanne Hosking (Plymouth University Peninsula School of Medicine and Dentistry) due to confidentiality of the EarlyBird database. Data shown is for boys and girls combined, as there were no significant associations between methylation and sex for any of the CpG loci shown. Adiposity was determined by % fat mass measured annually by dual-energy X-ray absorptiometry (DEXA). Insulin resistance was determined by homeostasis model assessment (HOMA2-IR) measured annually in peripheral blood.

The association between the methylation of each CpG and adiposity (DEXA % fat mass) and insulin resistance (HOMA2-IR) was determined for each corresponding year, shown as a cross sectional association. This determines if the methylation at a specific time point is significantly associated with % fat mass or HOMA2-IR at the same age. The association between the methylation of each CpG at 5-7 years and future adiposity (DEXA % fat mass) and future insulin resistance (HOMA2-IR) between 8-9 years and 14 years was determined, shown as a time-lagged association. This determines if the methylation at 5-7 years is significantly associated with and able to predict future % fat mass and/or insulin resistance between 8-9 years and 14 years.

Mixed effects modelling was used to assess the association between the methylation of each CpG loci at 5-7 years and adiposity (% fat mass) and insulin resistance (HOMA2-IR) between 9-14 years, which takes into account the repeated measures on the same subjects. Age was included in these models as a categorical variable, and covariates in the model included sex, age at peak height velocity (APHV)(indicates pubertal timing) and minutes per week spent in moderate-to-vigorous physical activity (MVPA).

4.3 Results

4.3.1 Methylation and adiposity

4.3.1.1 Is the methylation of specific CpG loci in the SIRT1 promoter associated with adiposity?

Table 4.1- The association between methylation of SIRT1 and adiposity

Age (years)	Cross sectional association between % methylation and adiposity (DEXA % fat)			Time-lagged association between % methylation at 5-7 years and future adiposity (DEXA % fat)		
	r	p	n	r	p	n
	CpG -880					
5-7y	-0.31	0.06	39	-	-	-
8-9y	-0.52	0.004	29	-0.29	0.08	37
10y	-0.41	0.01	37	-0.36	0.03	39
11y	-0.41	0.02	31	-0.35	0.04	34
12y	-0.34	0.05	36	-0.36	0.03	39
13y	-0.45	0.008	34	-0.29	0.08	38
14y	-0.28	0.09	39	-0.29	0.07	39
	CpG -760					
5-7y	-0.26	0.12	38	-	-	-
8-9y	-0.25	0.21	27	-0.12	0.47	37
10y	-0.35	0.04	36	-0.16	0.35	38
11y	-0.28	0.14	30	-0.11	0.55	33
12y	-0.24	0.18	34	-0.14	0.39	38
13y	-0.26	0.15	31	-0.16	0.34	37
14y	-0.18	0.29	38	-0.21	0.20	38
	CpG -742					
5-7y	-0.17	0.30	38	-	-	-
8-9y	-0.22	0.27	27	-0.01	0.96	18
10y	-0.11	0.53	36	-0.04	0.87	19
11y	-0.21	0.27	30	-0.09	0.73	17
12y	-0.17	0.33	34	0.01	0.98	19
13y	-0.13	0.50	31	0.01	0.97	19
14y	0.001	0.99	38	-0.03	0.91	19

Table 4.1 shows that the methylation of each CpG analysed for SIRT1 negatively correlated with adiposity (% fat mass). The methylation of CpG -880 at all years significantly associated with adiposity (% fat mass) ($p<0.05$) at each corresponding year, except 5-7 and 14 years where there was a trend ($p<0.10$). There were no significant associations between the methylation of CpGs -760 and -742 and adiposity (% fat mass) at the same corresponding year, except at 10 years for CpG -760 ($p=0.04$).

The methylation of CpG -880 at 5-7 years significantly associated with future adiposity (% fat mass) at 10, 11 and 12 years ($p<0.05$), and showed a trend toward significance at 8-9, 13 and 14 years ($p<0.10$). The methylation of CpGs -760 and -742 at 5-7 years was not significantly associated with future adiposity (% fat mass) ($p>0.05$).

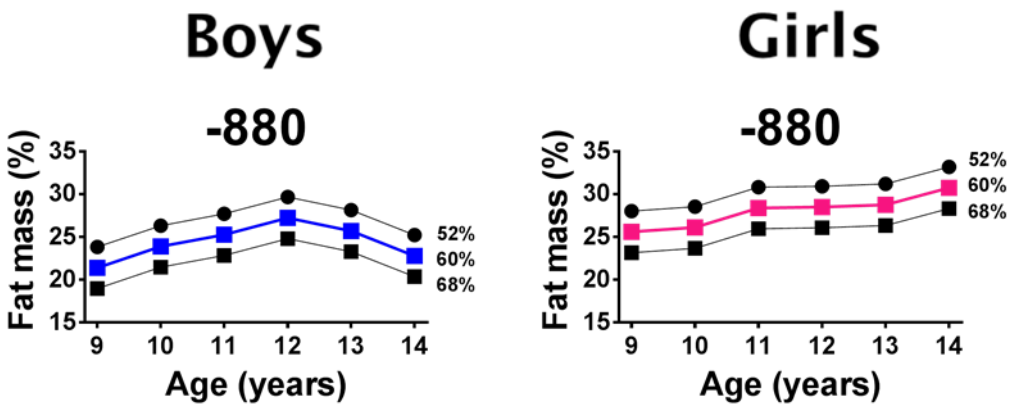


Figure 4.1- Modelled trajectories of methylation at 5-7 years and adiposity in boys and girls between 9 to 14 years in SIRT1. The mean methylation for the boys at 5-7 years is shown against percentage fat mass from 9-14 years with blue squares. The mean methylation for the girls at 5-7 years is shown against percentage fat mass from 9-14 years with pink squares. The mean methylation at 5-7 years +1SD is shown against percentage fat mass from 9-14 years with black circles, for boys and girls separately. The mean methylation at 5-7 years -1SD is shown against percentage fat mass from 9-14 years with black squares, for boys and girls separately. CpG -880 is numbered according to the distance from the transcription start site (TSS).

Using a mixed effects model, the methylation of CpG -880 at 5-7 years was negatively associated with % fat mass between 9 and 14 years, shown in figure 4.1. This was true for boys and girls, despite a difference in % fat mass between these ages for each sex. For each 10% difference in methylation at 5-7 years, per cent body fat differed by -3.0% (95% CI: -6.0 to -0.1) at CpG -880, ($P=0.01$); between 9 and 14 years. There were no significant associations between methylation at 5-7 years and future adiposity for CpGs -760 and -742 when using a mixed effects model ($p>0.05$).

4.3.1.2 Is the methylation of specific CpG loci in the PGC1α promoter associated with adiposity?

Table 4.2- The association between methylation of PGC1α and adiposity

Age (years)	Cross sectional association between % methylation and adiposity (DEXA % fat)			Time-lagged association between % methylation at 5-7 years and future adiposity (DEXA % fat)		
	r	p	n	r	p	n
	CpG -841					
5-7y	0.24	0.15	37	-	-	-
8-9y	0.29	0.13	29	0.24	0.16	35
10y	0.29	0.09	36	0.31	0.07	37
11y	0.24	0.19	33	0.28	0.12	32
12y	0.28	0.09	37	0.34	0.04	37
13y	0.16	0.37	32	0.22	0.21	36
14y	0.11	0.55	35	0.15	0.39	37
	CpG -816					
5-7y	0.33	0.04	39	-	-	-
8-9y	0.27	0.16	30	0.28	0.09	37
10y	0.34	0.04	37	0.34	0.03	39
11y	0.29	0.09	34	0.30	0.09	34
12y	0.36	0.03	38	0.35	0.03	39
13y	0.27	0.12	36	0.29	0.07	38
14y	0.22	0.20	37	0.22	0.18	39
	CpG -783					
5-7y	0.32	0.05	39	-	-	-
8-9y	0.26	0.16	31	0.25	0.13	37
10y	0.35	0.03	38	0.33	0.04	39
11y	0.32	0.06	34	0.27	0.13	34
12y	0.30	0.06	39	0.32	0.05	39
13y	0.23	0.18	37	0.27	0.11	38
14y	0.13	0.46	38	0.24	0.15	39
	CpG -652					
5-7y	0.17	0.32	38	-	-	-
8-9y	0.17	0.34	32	0.14	0.41	36
10y	0.40	0.02	35	0.18	0.29	38
11y	0.08	0.67	33	0.10	0.56	33
12y	0.11	0.50	38	0.10	0.54	38
13y	-0.02	0.92	36	0.16	0.35	37
14y	0.13	0.41	40	0.19	0.25	38

	CpG -617					
5-7y	0.37	0.03	35	-	-	-
8-9y	0.16	0.40	30	0.30	0.09	33
10y	0.52	0.003	31	0.34	0.05	35
11y	0.26	0.16	31	0.27	0.14	32
12y	0.15	0.39	36	0.23	0.19	35
13y	0.14	0.47	31	0.22	0.21	34
14y	0.23	0.17	36	0.24	0.16	35
	CpG -521					
5-7y	0.30	0.07	39	-	-	-
8-9y	0.02	0.93	32	0.23	0.17	37
10y	0.05	0.78	39	0.32	0.05	39
11y	0.17	0.33	34	0.30	0.09	34
12y	0.26	0.12	37	0.29	0.08	39
13y	0.23	0.18	37	0.36	0.03	38
14y	0.22	0.18	39	0.27	0.10	39

Table 4.2 shows the methylation of each CpG analysed for PGC1α positively correlated with adiposity (% fat mass). The methylation of CpGs -816, -783, -652 and -617 significantly associated with adiposity (% fat mass) at 5-7 years and 10 years, and also 12 years for CpG -816 ($p<0.05$). There were no significant associations between methylation and adiposity at any other years, apart from a trend toward significance at 11 and 12 years for CpG -783 ($p=0.06$). There were no significant associations between the methylation of CpGs -841 and -521 and adiposity (% fat mass) at the same corresponding year ($p>0.05$).

The methylation of CpG -816 significantly associated with future adiposity (% fat mass) at 10 and 12 years ($p<0.05$), and showed a trend toward significance at 8-9, 11 and 13 years ($p<0.10$). The methylation of CpG -783, -617 and -521 at 5-7 years significantly associated with future adiposity (% fat mass) at 10 years ($p<0.05$), with the methylation of CpG -783 also associating with adiposity at 12 years ($p=0.05$), and CpG -521 at 13 years ($p=0.03$). The methylation of CpG -841 at 5-7 years significantly associated with future adiposity (% fat mass) at 12 years only ($p<0.05$). The methylation of CpG -652 at 5-7 years was not significantly associated with future adiposity at any age (% fat mass) ($p>0.05$).

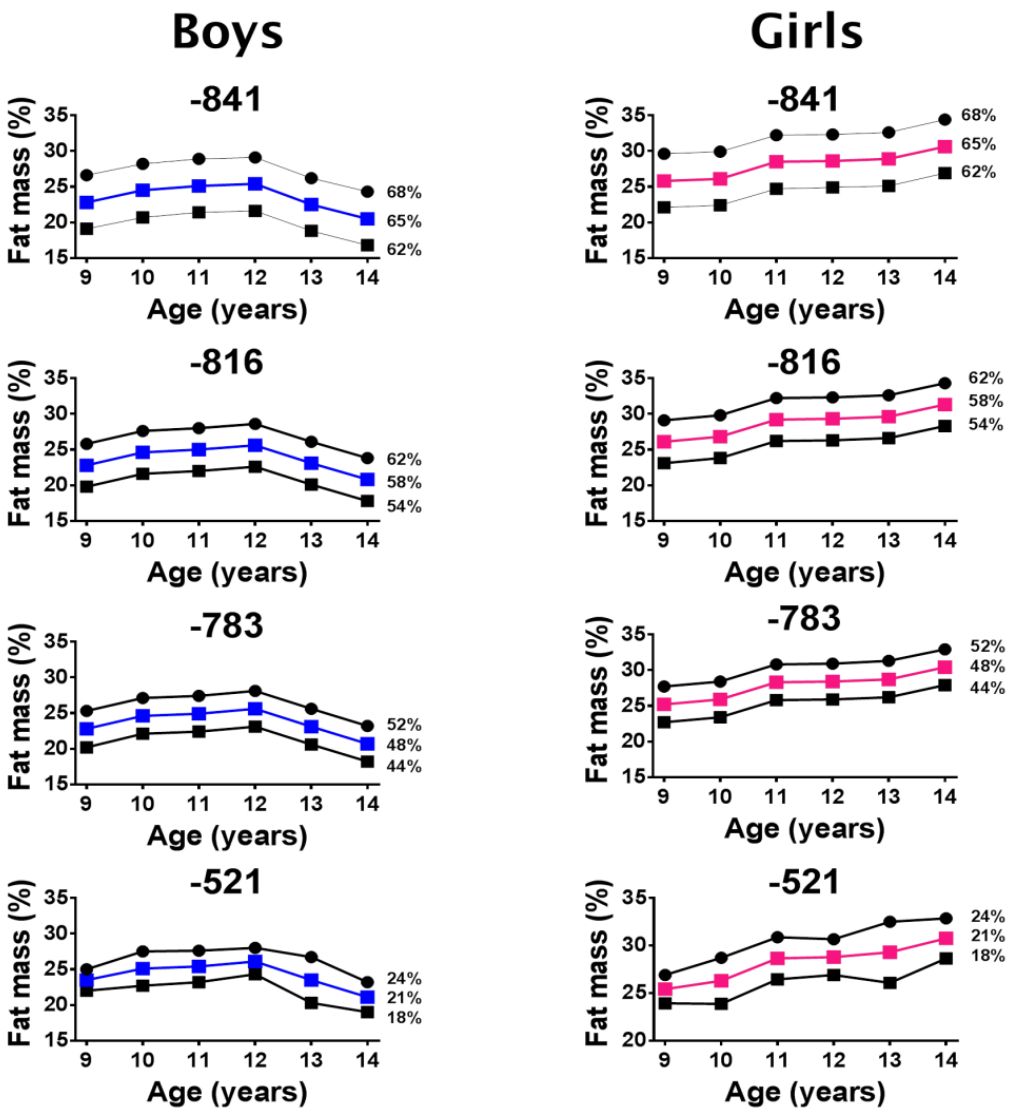


Figure 4.2- Modelled trajectories of methylation at 5-7 years and adiposity in boys and girls between 9 to 14 years in PGC1α. The mean methylation for the boys at 5-7 years is shown against percentage fat mass from 9-14 years with blue squares, for each CpG loci. The mean methylation for the girls at 5-7 years is shown against percentage fat mass from 9-14 years with pink squares, for each CpG loci. The mean methylation at 5-7 years +1SD is shown against percentage fat mass from 9-14 years with black circles, for boys and girls separately. The mean methylation at 5-7 years -1SD is shown against percentage fat mass from 9-14 years with black squares, for boys and girls separately. CpG loci are numbered according to the distance from the TSS.

Using a mixed effects model, the methylation of CpGs -841, -816, -783 and -521 was positively associated with % fat mass between 9 and 14 years, shown in figure 4.2. This was true for boys and girls, despite a difference in % fat mass between these ages for each sex. For each 10% difference in methylation at 5-7 years, per cent body fat differed by 12.7% (95% CI: 3.4-22.0) at CpG -841, (P=0.01); 7.5% (95% CI: 2.2-12.8) at CpG -816, (p=0.009); 6.4% (95% CI: 0.7-12.1) at CpG -783, (p=0.04) and 7.4% (95% CI: 0.01-14.8) at CpG -521, (p=0.05), between 9 and 14 years. There were no significant associations between the methylation of CpGs -652 and -617 at 5-7 years and future adiposity when using a mixed effects model (p>0.05).

4.3.1.3 Is the methylation of specific CpG loci in the HNF4α distal/P2 promoter associated with adiposity?

Table 4.3- The association between methylation of HNF4α and adiposity

Age (years)	Cross sectional association between % methylation and adiposity (DEXA % fat)			Time-lagged association between % methylation at 5-7 years and future adiposity (DEXA % fat)		
	r	p	n	r	p	n
	CpG -94					
5-7y	-0.05	0.76	38	-	-	-
8-9y	-0.28	0.12	31	-0.09	0.66	36
10y	-0.05	0.78	37	-0.12	0.48	38
11y	-0.13	0.48	33	-0.11	0.54	33
12y	-0.19	0.25	39	-0.11	0.52	38
13y	-0.02	0.92	37	0.08	0.66	37
14y	0.06	0.74	39	0.08	0.65	38
	CpG -79					
5-7y	0.05	0.75	38	-	-	-
8-9y	0.24	0.19	31	-0.11	0.51	36
10y	-0.07	0.70	37	-0.16	0.33	38
11y	-0.11	0.56	33	-0.30	0.09	33
12y	-0.23	0.17	39	-0.26	0.12	38
13y	-0.17	0.33	37	-0.31	0.07	37
14y	-0.14	0.39	39	-0.24	0.15	38

Table 4.3 shows the methylation of each CpG analysed for HNF4α negatively correlated with adiposity (% fat mass) for most years. There were no significant associations between the methylation of CpGs -94 or -79 and adiposity (% fat mass) at the same corresponding year (p>0.05). The methylation of CpGs -94 and -79 at 5-7 years was not significantly associated with future adiposity (% fat mass) at any age (p>0.05).

When a mixed effects model was used, the methylation of each CpG at 5-7 years was not significantly associated with future adiposity between 9 and 14 years ($p>0.05$).

4.3.1.4 Is the methylation of specific CpG loci in the GCK promoter associated with adiposity?

Table 4.4- The association between methylation of GCK and adiposity

Age (years)	Cross sectional association between % methylation and adiposity (DEXA % fat)			Time-lagged association between % methylation at 5-7 years and future adiposity (DEXA % fat)		
	r	p	n	r	p	n
	CpG -192					
5-7y	0.06	0.70	39	-	-	-
8-9y	0.09	0.68	25	0.001	0.99	37
10y	0.28	0.10	36	0.07	0.69	39
11y	0.15	0.44	30	0.04	0.82	34
12y	0.11	0.53	35	0.09	0.58	39
13y	0.24	0.19	32	0.12	0.49	38
14y	0.08	0.65	38	0.16	0.33	39
	CpG -172					
5-7y	-0.17	0.31	38	-	-	-
8-9y	-0.11	0.60	24	-0.24	0.15	36
10y	-0.24	0.18	34	-0.37	0.02	38
11y	-0.16	0.41	29	-0.46	0.007	33
12y	-0.13	0.47	34	-0.49	0.002	38
13y	-0.27	0.15	29	-0.46	0.005	37
14y	-0.24	0.18	35	-0.42	0.008	38
	CpG +30					
5-7y	-0.05	0.75	39	-	-	-
8-9y	0.10	0.61	30	0.06	0.72	37
10y	0.21	0.20	38	-0.05	0.76	39
11y	0.30	0.10	33	-0.02	0.90	34
12y	0.09	0.61	36	-0.004	0.98	39
13y	0.22	0.20	35	0.01	0.96	38
14y	-0.10	0.54	39	0.08	0.63	39
	CpG +34					
5-7y	0.05	0.77	39	-	-	-
8-9y	-0.01	0.97	30	0.11	0.52	37
10y	0.14	0.42	36	0.06	0.72	39
11y	0.09	0.65	29	0.09	0.60	34
12y	0.12	0.50	34	0.15	0.35	39
13y	0.11	0.56	32	0.07	0.66	38
14y	0.12	0.49	38	0.07	0.68	39

Table 4.4 shows the methylation of CpGs -192, +30 and +34 in GCK positively correlated with adiposity (% fat mass). The methylation of CpG -172 in GCK negatively correlated with adiposity (% fat mass). There were no significant associations between the methylation of any of the CpGs and adiposity (% fat mass) at the same corresponding year ($p>0.05$).

The methylation of CpG -172 at 5-7 years significantly associated with future adiposity (% fat mass) between 10 and 14 years ($p<0.05$). The methylation of CpGs -192, +30 and +34 at 5-7 years was not significantly associated with future adiposity (% fat mass) at any age ($p>0.05$).

When a mixed effects model was used, the methylation of each CpG at 5-7 years was not significantly associated with adiposity between 9 and 14 years ($p>0.05$).

4.3.2 Methylation and insulin resistance

4.3.2.1 Is the methylation of specific CpG loci in the SIRT1 promoter associated with insulin resistance?

Table 4.5- The association between methylation of SIRT1 and insulin resistance

Age (years)	Cross sectional association between % methylation and insulin resistance (HOMA2-IR)			Time-lagged association between % methylation at 5-7 years and future insulin resistance (HOMA2-IR)		
	r	p	n	r	p	n
	CpG -880					
5-7y	0.10	0.54	39	-	-	-
8-9y	-0.28	0.13	31	-0.19	0.25	39
10y	-0.33	0.04	37	-0.36	0.02	39
11y	-0.14	0.42	35	-0.07	0.68	39
12y	-0.33	0.05	36	-0.33	0.04	39
13y	-0.20	0.24	35	-0.23	0.16	39
14y	-0.42	0.008	39	-0.48	0.002	39
	CpG -760					
5-7y	-0.17	0.31	38	-	-	-
8-9y	-0.18	0.36	28	-0.14	0.40	38
10y	-0.26	0.13	36	-0.24	0.15	38
11y	0.02	0.91	32	-0.02	0.91	38
12y	-0.47	0.005	34	-0.26	0.11	38
13y	-0.06	0.73	32	-0.08	0.62	38
14y	-0.42	0.009	38	-0.39	0.02	38
	CpG -742					
5-7y	-0.14	0.41	38	-	-	-
8-9y	-0.32	0.10	28	-0.26	0.12	38
10y	-0.07	0.70	36	-0.30	0.66	38
11y	-0.02	0.91	32	-0.04	0.79	38
12y	-0.32	0.06	34	-0.25	0.13	38
13y	0.12	0.53	32	-0.05	0.77	38
14y	-0.26	0.12	38	-0.39	0.02	38

Table 4.5 shows the methylation of each CpG analysed for SIRT1 negatively correlated with insulin resistance (HOMA2-IR). The methylation of CpG -880 and -760 significantly associated with insulin resistance (HOMA2-IR) at 12 and 14 years ($p<0.05$), with an association at 10 years also for CpG -880 ($p=0.04$). There was no significant association between the methylation of CpG -742 and insulin resistance (HOMA2-IR) at the same corresponding year ($p>0.05$).

The methylation of CpG -880 at 5-7 years significantly associated with future insulin resistance (HOMA2-IR) at 10, 12 and 14 years only ($p<0.05$). The methylation of CpGs -760 and -742 at 5-7 years significantly associated with future insulin resistance (HOMA2-IR) ($p=0.02$) at 14 years only.

When a mixed effects model was used, the methylation of each CpG at 5-7 years was not significantly associated with insulin resistance between 9 and 14 years (p>0.05).

4.3.2.2 Is the methylation of specific CpG loci in the PGC1α promoter associated with insulin resistance?

Table 4.6- The association between methylation of PGC1α and insulin resistance

Age (years)	Cross sectional association between % methylation and insulin resistance (HOMA2-IR)			Time-lagged association between % methylation at 5-7 years and future insulin resistance (HOMA2-IR)		
	r	p	n	r	p	n
	CpG -841					
5-7y	0.14	0.40	37	-	-	-
8-9y	0.14	0.47	30	0.22	0.19	37
10y	0.38	0.02	36	0.31	0.07	37
11y	0.13	0.44	37	0.20	0.23	37
12y	0.17	0.31	37	0.26	0.12	37
13y	-0.09	0.62	33	0.08	0.64	37
14y	0.21	0.23	35	0.25	0.14	37
	CpG -816					
5-7y	0.11	0.50	39	-	-	-
8-9y	0.22	0.24	31	0.21	0.20	39
10y	0.35	0.03	37	0.35	0.03	39
11y	0.17	0.29	39	0.20	0.23	39
12y	0.21	0.22	38	0.18	0.28	39
13y	0.05	0.75	37	0.13	0.45	39
14y	0.30	0.07	37	0.30	0.06	39
	CpG -783					
5-7y	0.08	0.64	39	-	-	-
8-9y	0.24	0.18	33	0.24	0.15	39
10y	0.36	0.03	38	0.33	0.04	39
11y	0.22	0.18	39	0.18	0.28	39
12y	0.22	0.17	39	0.19	0.26	39
13y	0.10	0.56	38	0.07	0.69	39
14y	0.17	0.30	38	0.23	0.16	39

	CpG -652					
5-7y	0.12	0.48	38	-	-	-
8-9y	0.38	0.03	34	0.33	0.04	38
10y	0.36	0.03	35	0.42	0.008	38
11y	-0.02	0.89	38	0.04	0.80	38
12y	-0.06	0.74	38	0.02	0.92	38
13y	-0.08	0.65	37	0.08	0.62	38
14y	0.29	0.07	40	0.34	0.04	38
	CpG -617					
5-7y	0.16	0.35	35	-	-	-
8-9y	0.35	0.06	31	0.36	0.04	35
10y	0.51	0.004	31	0.51	0.002	35
11y	0.19	0.31	32	0.21	0.24	35
12y	0.08	0.63	36	0.13	0.47	35
13y	0.04	0.83	32	0.11	0.55	35
14y	0.32	0.06	36	0.34	0.05	35
	CpG -521					
5-7y	0.004	0.98	39	-	-	-
8-9y	0.33	0.06	34	0.37	0.02	39
10y	0.21	0.20	39	0.41	0.01	39
11y	0.14	0.40	38	0.13	0.45	39
12y	0.12	0.48	37	0.04	0.80	39
13y	0.01	0.94	38	0.11	0.51	39
14y	0.24	0.15	39	0.23	0.16	39

Table 4.6 shows the methylation of each CpG analysed for PGC1α positively correlated with insulin resistance (HOMA2-IR). The methylation of CpGs -841, -816, -783, -652 and -617 significantly associated with insulin resistance (HOMA2-IR) at 10 years, and also at 8-9 years for CpG -652 ($p<0.05$). There was no significant association between the methylation of CpG -521 and insulin resistance (HOMA2-IR) at the same corresponding year ($p>0.05$).

The methylation of all CpGs at 5-7 years significantly associated with future insulin resistance (HOMA2-IR) at 10 years ($p<0.05$), except for CpG -841. The methylation of CpGs -652, -617 and -521 at 5-7 years also significantly associated with future insulin resistance (HOMA2-IR) at 8-9 years, and 14 years for CpG -652 and -617 ($p<0.05$). The methylation of CpG -841 at 5-7 years was not significantly associated with future insulin resistance (HOMA2-IR) at any age ($p>0.05$).

When a mixed effects model was used, the methylation of each CpG at 5-7 years was not significantly associated with insulin resistance between 9 and 14 years ($p>0.05$).

4.3.2.3 Is the methylation of specific CpG loci in the HNF4α distal/P2 promoter associated with insulin resistance?

Table 4.7- The association between methylation of HNF4α and insulin resistance

Age (years)	Cross sectional association between % methylation and insulin resistance (HOMA2-IR)			Time-lagged association between % methylation at 5-7 years and future insulin resistance (HOMA2-IR)		
	r	p	n	r	p	n
	CpG -94					
5-7y	-0.09	0.61	38	-	-	-
8-9y	-0.41	0.02	33	-0.16	0.35	38
10y	-0.32	0.05	37	-0.22	0.19	38
11y	-0.06	0.70	38	0.01	0.95	38
12y	-0.11	0.53	39	-0.03	0.86	38
13y	-0.06	0.72	38	0.09	0.59	38
14y	-0.12	0.48	39	-0.17	0.32	38
	CpG -79					
5-7y	0.07	0.68	38	-	-	-
8-9y	0.61	<0.001	33	0.20	0.22	38
10y	0.28	0.10	37	0.12	0.46	38
11y	0.23	0.16	38	-0.10	0.54	38
12y	0.10	0.56	39	-0.05	0.76	38
13y	-0.23	0.17	38	-0.29	0.08	38
14y	-0.04	0.82	39	-0.10	0.56	38

Table 4.7 shows the methylation of CpG -94 in HNF4α negatively correlated with insulin resistance (HOMA2-IR), whereas the methylation of CpG -79 positively correlated with insulin resistance (HOMA2-IR). The methylation of CpGs -94 and -79 significantly associated with insulin resistance (HOMA2-IR) at 8-9 years, and also 10 years for CpG -94 ($p<0.05$). The methylation of CpGs -94 and -79 at 5-7 years was not significantly associated with future insulin resistance (HOMA2-IR) at any age ($p>0.05$).

When a mixed effects model was used, the methylation of each CpG at 5-7 years was not significantly associated with insulin resistance between 9 and 14 years ($p>0.05$).

4.3.2.4 Is the methylation of specific CpG loci in the GCK promoter associated with insulin resistance?

Table 4.8- The association between methylation of GCK and insulin resistance

Age (years)	Cross sectional association between % methylation and insulin resistance (HOMA2-IR)			Time-lagged association between % methylation at 5-7 years and future insulin resistance (HOMA2-IR)		
	r	p	n	r	p	n
	CpG -192					
5-7y	-0.26	0.11	39	-	-	-
8-9y	0.04	0.83	27	-0.01	0.97	39
10y	0.14	0.42	36	0.06	0.72	39
11y	0.07	0.70	35	0.16	0.34	39
12y	0.26	0.14	35	0.25	0.12	39
13y	0.15	0.41	33	0.09	0.59	39
14y	-0.25	0.13	38	-0.03	0.85	39
	CpG -172					
5-7y	0.24	0.14	38	-	-	-
8-9y	0.10	0.64	26	0.14	0.39	38
10y	0.10	0.59	34	-0.01	0.94	38
11y	-0.16	0.36	34	-0.18	0.29	38
12y	-0.02	0.92	34	-0.35	0.03	38
13y	-0.12	0.53	30	-0.28	0.10	38
14y	-0.27	0.12	35	-0.33	0.04	38
	CpG +30					
5-7y	-0.17	0.31	39	-	-	-
8-9y	0.04	0.83	32	-0.09	0.57	39
10y	0.06	0.73	38	-0.19	0.25	39
11y	0.16	0.34	38	-0.04	0.80	39
12y	0.18	0.29	36	0.20	0.22	39
13y	0.27	0.11	36	0.14	0.40	39
14y	-0.44	0.005	39	-0.16	0.32	39
	CpG +34					
5-7y	-0.26	0.11	39	-	-	-
8-9y	-0.44	0.01	32	-0.25	0.12	39
10y	-0.24	0.16	36	-0.23	0.15	39
11y	0.15	0.41	34	0.02	0.90	39
12y	0.05	0.80	34	0.18	0.28	39
13y	0.16	0.36	33	0.14	0.40	39
14y	-0.28	0.09	38	-0.25	0.13	39

Table 4.8 shows the methylation of each CpG analysed for GCK positively correlated with insulin resistance (HOMA2-IR) at most years. The methylation of CpGs +30 and +34 significantly associated with insulin resistance (HOMA2-IR) at 14 years and 8-9 years respectively ($p<0.05$). There

were no significant associations between the methylation of CpGs -192 and -172 and insulin resistance (HOMA2-IR) at the same corresponding year ($p>0.05$).

The methylation of CpG -172 at 5-7 years significantly associated with future insulin resistance (HOMA2-IR) ($p<0.05$) at 12 and 14 years only. The methylation of CpGs -192, +30 and +34 at 5-7 years was not significantly associated with future insulin resistance (HOMA2-IR) at any age ($p>0.05$).

When a mixed effects model was used, the methylation of each CpG at 5-7 years was not significantly associated with insulin resistance between 9 and 14 years ($p>0.05$).

4.4 Discussion

4.4.1 The methylation of specific CpG loci can predict future adiposity but not insulin resistance

4.4.1.1 SIRT1 methylation, adiposity and insulin resistance

Altered DNA methylation has the ability to act as a biomarker of disease risk if it is associated with a particular phenotype. Biomarkers can be predictive of future disease risk if altered methylation occurs before the onset of disease and could be beneficial in identifying individuals that may benefit from targeted intervention. Mixed effects modelling was used to investigate whether differential methylation of CpG loci that were temporally stable between 5-7 and 14 years was related to future adiposity and/or insulin resistance in childhood.

These findings have shown that the methylation of CpG -880 in the SIRT1 promoter was inversely associated with adiposity at the same age during childhood, and the methylation at 5-7 years was significantly associated with future adiposity up to the age of 14 years. Therefore, increased methylation of CpG -880 is associated with a decrease in percentage fat mass in these subjects during childhood. DNA methylation within the promoter of a gene is generally associated with gene silencing by recruiting MeCP2 and preventing the transcriptional machinery from binding, shown in figure 1.4 (62). Whilst no studies have determined if altered methylation of this specific CpG has an effect on gene expression, if methylation were to cause gene silencing then subjects with higher methylation and lower fat mass would have decreased SIRT1 gene expression. However, SIRT1 has been shown to inhibit adipogenesis by repressing PPARγ in adipocytes and increasing fat mobilisation (63). Evidence from previous studies have shown that SIRT1 expression and protein levels are decreased in the subcutaneous adipose tissue from obese humans, when compared to lean, suggesting that SIRT1 expression is decreased in obesity (73)(72). Therefore, you would expect the subjects with higher methylation and lower fat mass to have increased SIRT1 gene expression. This could be explained by the methylation of CpG -880 affecting the binding of repressive transcription factors to their recognition site. For example, NKX61 which binds upstream of CpG -880 has been shown previously to be a transcriptional repressor *in vitro* (181)(figure 3.3). Whilst methylation has not been shown to block the binding of NKX61 specifically, methylation has been shown to block the binding of other transcription factors (182). Therefore, methylation of CpG -880 may promote gene expression if the binding of a repressor is blocked. Alternatively, methylation of this CpG may simply act a biomarker of adiposity in peripheral blood and may not be involved in altering gene expression or in the causal pathway of adiposity.

Using a mixed effects model, the methylation of CpG -880 in SIRT1 at 5-7 years predicted future adiposity between 9 and 14 years, independent of sex, age, pubertal timing and physical activity, with a 10% increase in methylation predicting a 3.0% decrease in percentage fat mass. This was true for boys and girls, despite percent body fat decreasing for the boys and increasing for the girls during the peri-pubertal period, shown in figure 4.1. These findings suggest that the methylation status of CpG -880 in the SIRT1 promoter may be suitable as a potential biomarker of future adiposity when measured in peripheral blood.

There was a weak inverse association between the methylation of CpGs -880, -760 and -742 in the SIRT1 promoter and insulin resistance at several ages during childhood, and the methylation at 5-7 years significantly associated with future insulin resistance at 14 years. However, when using a mixed effects model correcting for several factors including pubertal timing, methylation of these CpG loci did not predict future insulin resistance. Data was corrected for pubertal timing as insulin resistance is known to increase in puberty (183)(184)(185). Insulin secretion increases during puberty, which is thought to be a compensatory mechanism to temporally increase insulin levels to overcome this insulin resistance, which explains why insulin resistance is not necessarily maintained into adulthood (183)(184)(185). Because these effects are temporary and both insulin levels and insulin resistance decrease after puberty, peripheral blood samples after the age of 14 years would be required to determine if the weak association between methylation of these loci and insulin resistance remain into adulthood. Because the association was lost after correcting for pubertal timing, this suggests that the association between methylation of these loci and insulin resistance is due to pubertal timing.

The methylation of CpG -880 in the SIRT1 promoter at 5-7 years inversely associated with adiposity between 9 and 14 years, and insulin resistance at 10, 12 and 14 years. Obesity and insulin resistance often occur together, with weight loss known to improve insulin resistance (1)(2)(33). This is thought to occur as a result of large hypertrophic adipocytes undergoing increased lipolysis via hormone-sensitive lipase, which releases excess non-esterified fatty acids (NEFAs) into the plasma (39). The Randle hypothesis states that NEFAs compete with glucose to be the major energy substrate in muscle and adipose tissue, which results in decreased glucose uptake (27). NEFAs are taken up into skeletal muscle and are transported to the mitochondria, where they undergo β oxidation to produce acetyl CoA that enters the tricarboxylic acid (TCA) cycle in order to release adenosine triphosphate (ATP) (40). However, accumulation of acetyl CoA inhibits pyruvate dehydrogenase, which in turn leads to an excess of glucose-6-phosphate (G6P) and inhibition of hexokinase (40). This reduces

glucose uptake into the skeletal muscle via the glucose transporter GLUT4, leading to increased plasma glucose concentration and increased insulin secretion from the pancreatic β cells (8)(41)(42). Excess NEFAs are also stored in the pancreas, known as lipotoxicity, which results in reduced insulin secretion and the development of insulin resistance (8)(9). Excess NEFAs also increase hepatic gluconeogenesis, which increases circulating glucose concentrations further and increases the demand for insulin secretion from the pancreatic β cells (27). Therefore, decreased methylation of CpG -880 in SIRT1 in peripheral blood may be a marker of future risk of developing metabolic syndrome.

4.4.1.2 PGC1α methylation, adiposity and insulin resistance

These findings have shown that the methylation of CpGs -816, -783, -652 and -617 in the PGC1α promoter was positively associated with adiposity at the same age during childhood, and the methylation at 5-7 years was significantly associated with future adiposity at 10, 12 and 13 years, except for CpG -652. Therefore, increased methylation was associated with an increase in percentage fat mass in these subjects during childhood. As discussed previously, methylation of a gene promoter is generally associated with gene silencing, which may suggest that subjects with increased methylation and adiposity may have decreased PGC1α gene expression (62). This would support previous evidence that suggests increased methylation of PGC1α is associated with decreased gene expression. For example, it has been shown that 3 of these CpG loci were hypermethylated in subcutaneous adipose tissue from obese subjects, which associated with a decrease in PGC1α mRNA expression, when compared to lean subjects (79). Ling et al. have also shown that 3 of these CpG loci were hypermethylated in the pancreatic islet cells of overweight T2D patients, which associated with a decrease in PGC1α mRNA expression, when compared to healthy controls (49). These data suggest that increased methylation of these loci occurs in obesity and is associated with a decrease in gene expression in different tissue types. This might suggest that increased methylation of these loci in peripheral blood may also result in decreased PGC1α expression.

Using a mixed effects model, the methylation of CpG -841, -816, -783 and -521 in PGC1α at 5-7 years predicted future adiposity between 9 and 14 years, independent of sex, age, pubertal timing and activity, with a 10% increase in methylation at these loci predicting between 6.4% and 12.7% increase in percentage fat mass. This was true for boys and girls, despite percent body fat decreasing for the boys and increasing for the girls during the peri-pubertal period, shown in figure 4.2. This

suggests that measuring the methylation of these loci in peripheral blood during childhood may be beneficial for predicting future risk of adiposity.

The methylation of these CpG loci may alter transcription factor binding, which may alter gene expression. CpG -816 lies in a putative binding site for PPARG/RXRα, which has been shown to activate PGC1α expression and increase differentiation of preadipocytes to adipocytes, and CpG -783 lies in a putative binding site for PBX1, which is known to play a role in adipogenesis (75)(186)(figure 3.6). Therefore, altered methylation of these loci may have a functional impact on gene expression and may be involved in the causal pathway of adiposity, which could be tested using electrophoretic mobility shift assays shift assays or western blotting. Or, altered methylation of these loci in peripheral blood may simply be a biomarker of future adiposity and may not influence gene expression, which would still be beneficial for predicting those individuals at an increased risk of later adiposity.

The methylation of each CpG loci in the PGC1α promoter at 10 years positively associated with insulin resistance at 10 years, except for CpG -521, and the methylation of each CpG loci at 5-7 years positively associated with future insulin resistance at several ages during childhood, except for CpG -841. However, when using a mixed effects model correcting for pubertal timing and physical activity, methylation of these CpG loci did not predict future insulin resistance. This suggests that pubertal timing and/or physical activity may be the driver of these associations between methylation and insulin resistance. It is known that insulin resistance temporally increases during puberty, which is associated with an increase in insulin secretion as a compensatory mechanism (183)(184)(185). It is also known that physical activity increases insulin sensitivity in healthy subjects as well as those that are insulin resistant or have T2D (183)(184)(185)(187). Another consideration is that the methylation of these loci may be related to different functions of PGC1α besides insulin secretion and glucose homeostasis, as it is involved in many different pathways.

Other hormonal changes occur during puberty, including increased growth hormone secretion, which has been shown to decrease insulin sensitivity (183). Growth hormone, along with other hormones such as oestrogen and testosterone were not measured in the peripheral blood of these subjects. Therefore, hormone levels could not be corrected for in the statistical analysis. The levels of these hormones may be associated with the methylation of these loci and, if corrected for may alter the association between methylation and insulin resistance.

Whilst the methylation of CpGs -841, -816, -783 and -521 in PGC1α at 5-7 years predicted future adiposity between 9 and 14 years, methylation did not predict future insulin resistance after

correcting for pubertal timing and physical activity. This supports previous data that suggests obesity is not always associated with insulin resistance. For example, it has been shown that the development of insulin resistance during puberty is not associated with changes in body fat or visceral fat, suggesting that these diseases can occur separately during childhood and puberty (188)(185). In order to determine that the methylation of these loci is not suitable as a marker of insulin resistance, peripheral blood samples after the age of 14 years would be required to measure insulin resistance in adulthood. Another consideration is that the number of subjects (n=40) might be underpowered to detect a significant association between methylation of these loci and insulin resistance.

These findings have shown the methylation of specific CpG loci in the SIRT1 and PGC1α promoters in peripheral blood at 5-7 years associated with future adiposity in childhood, up to the age of 14 years, albeit a negative association for SIRT1 and a positive association for PGC1α. SIRT1 is known to positively regulate PGC1α activity via deacetylation of several lysine residues, thereby increasing the ability of PGC1α to activate target genes (67). Both SIRT1 and PGC1α are expressed in many different tissues and have a role in many pathways including adipogenesis (63)(75). Because SIRT1 positively regulates PGC1α activity and both genes are involved in the same pathways it may be expected that the association between methylation and future adiposity would be in the same direction. However, as discussed previously there is a predicted binding site for NKX61 just upstream of CpG -880 in the SIRT1 promoter, which is known to be a transcriptional repressor *in vitro* (181). Because methylation is known to block the binding of many transcription factors, this may result in the blocking of a repressive transcription factor, thereby increasing gene expression meaning that expression of both genes would be decreased in adiposity.

However, whilst the methylation of both of these genes in peripheral blood was associated with future adiposity, the functional consequence of altered methylation in peripheral blood is not known. Therefore, the effect of differential methylation of these CpG loci needs to be further explored in order to determine if methylation has any functional significance. More information is also required on how the methylation of these loci is determined. For example, there is now evidence to suggest that the methylation of some of these loci in PGC1α may be influenced by maternal body composition and diet, suggesting environmental factors could be causing these associations between methylation and adiposity in the offspring (171)(164).

4.5 Summary

These findings support the hypothesis that DNA methylation is predictive of future adiposity, with the methylation of a single CpG in SIRT1 and 4 CpG loci in PGC1α in peripheral blood at 5-7 years predicting future adiposity up the age of 14 years, irrespective of pubertal timing and physical activity. This suggests that measuring the methylation of these loci in peripheral blood during early childhood may be suitable as a biomarker of future risk of adiposity, allowing for potential intervention and prevention. Whilst identification of potential biomarkers in blood is advantageous as it is an easy method of sampling large populations and may increase the number of individuals in whom such associations can be tested, more information is required to determine if the methylation of these loci is altered in different tissues as SIRT1 and PGC1α are involved in many different pathways. The effect of differential methylation of SIRT1 and PGC1α gene expression needs to be determined as altered methylation of these loci may not be involved in the causal pathway of obesity.

Chapter 5

The Methylation of CpG -783 in the PGC1α Promoter Affects Transcription Factor Binding

5.1 Introduction

5.1.1 Functional significance of altered methylation on PGC1α expression

There is evidence from human studies that shows altered methylation of PGC1α associates with a change in gene expression in several different tissues from obese and T2D patients (189)(49)(79). For example, Barrès et al. showed using a genome-wide approach that the PGC1α promoter was hypermethylated in the skeletal muscle of T2D patients, which associated with a decrease in PGC1α mRNA, when compared to controls (189). Chen et al. have shown that the mean methylation of 5 CpG loci in the PGC1α promoter was increased in the subcutaneous adipose tissue of obese subjects, compared to lean subjects, and obese subjects also had a decrease in PGC1α expression (79). Whilst these studies demonstrate an association between altered methylation and PGC1α gene expression, these associations occur after the onset of disease. Therefore, it is important to determine if differential methylation of PGC1α is involved in the causal pathway of altered gene expression.

5.1.2 The implications of a potential biomarker of adiposity measured in peripheral blood

The results from chapter 4 have shown that the methylation of 4 CpG loci in the PGC1α promoter at 5-7 years in peripheral blood predicts future adiposity in childhood. However, because the methylation was measured in peripheral blood, which is a proxy tissue, this may not reflect the level of methylation in different tissues such as adipose tissue. An environmental challenge early in development could alter the methylation of these loci equally across the different germline layers and, therefore, the methylation of these loci in a proxy tissue such as peripheral blood may reflect the methylation of these loci in many different tissues. This is supported by evidence from studies that have shown the methylation of specific CpG loci is conserved across different tissues that originate from different germline layers (128)(119). However, an environmental challenge that occurs after cellular differentiation could affect the methylation of these loci in a tissue specific manner. This is supported by findings from studies that have shown methylation of specific loci varies across different types (126)(127).

It has been shown that pharmacological inhibition of PGC1α in preadipocytes *in vitro* results in increased fat storage during adipocyte differentiation, whereas pharmacological activation inhibits fat storage in preadipocytes and increases fat mobilisation in mature adipocytes, suggesting PGC1α negatively regulates fat storage in adipose tissue (78). Therefore, it is important to determine if the methylation of these loci in peripheral blood is simply a biomarker of future adiposity or if altered

methylation of PGC1α affects gene expression and might have a causal role in the regulation of adipose tissue mass.

5.1.3 Aims

In order to test the hypothesis that differential methylation of CpG -783 in the PGC1α promoter alters transcription factor binding, electrophoretic mobility shift assays were used. CpG -783 was selected because the methylation of this CpG was associated with future adiposity in the EarlyBird cohort, where for each 10% difference in methylation at 5-7 years, % body fat differed by 6.4% (p=0.003) up to 14 years (Chapter 4). CpG -783 also lies within several putative transcription factor binding sites, that have previously been associated with adipogenesis, adipocyte metabolism and adipocyte differentiation (190)(186)(191). The functional significance of methylation of CpG -783 in the PGC1α promoter was examined in the adipose derived cell line human liposarcoma cells, which are a relevant cell type because they originate from the same cell lineage as pre-adipocytes.

5.2 Methods

Electrophoretic mobility shift assays (EMSAs) were used to determine whether differential methylation of CpG -783 in the PGC1α promoter altered transcription factor binding. A biotin-labelled double-stranded oligonucleotide probe corresponding to a region of the PGC1α promoter from -797 to -764 containing either an unmethylated or methylated cytosine base at -783 was incubated with nuclear extracts from the human liposarcoma cell line SW-872 (section 2.2.10). These cells are undifferentiated and represent a preadipocyte cell model with high proliferative capacity (192).

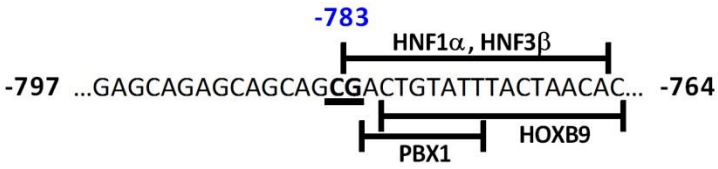


Figure 5.1- The region of the PGC1α promoter used in electrophoretic mobility shift assays. The probe contained the region corresponding to -797 to -764 within the PGC1α promoter is shown with putative transcription factor binding consensus sequences (http://www.genomatix.de/cgi-bin//matinspector). Abbreviations are as follows:- **HNF1α**, Hepatocyte Nuclear Factor 1 Alpha; **HNF3B**, Hepatocyte Nuclear Factor 3-Beta; **PBX1**, Pre-B-Cell Leukemia Homeobox 1; **HOXB9**, Homeobox B9.

CpG -783 is located within a putative transcription factor binding site for hepatocyte nuclear factor 1 beta (HNF3β), hepatocyte nuclear factor 1 alpha (HNF1α), homeobox B9 (HOXB9) and pre B cell leukemia homeobox 1 (PBX1), as shown in figure 5.1. To determine the identity of the protein(s) binding to this sequence, the unmethylated biotin-labelled probe was incubated with nuclear extracts from liposarcoma cells along with a 500-fold excess of an unlabelled oligonucleotide containing the consensus binding sequence for HNF3β and HNF1α. The unmethylated and methylated biotin-labelled probes were incubated with nuclear extracts from liposarcoma cells along with a 100 and 500-fold excess of an unlabelled oligonucleotide containing the consensus binding sequence for HOXB9-PBX1, as these transcriptions factors are known to bind as heterodimers (143).

To confirm the identity of the protein(s) binding to the unmethylated and methylated sequence, antibodies specific to HOXB9 and PBX1 were incubated with nuclear extracts from liposarcoma cells prior to the addition of the biotin-labelled probes.

To determine if PBX1 has an effect on PGC1α gene expression, human liposarcoma cells were co-transfected, in triplicate, with the PGC1α promoter (CpG -783 unmethylated) and increasing

concentrations of PBX1. In order to maintain the total amount of transfected DNA all co-transfections were equalised with empty plasmid containing the same CMV-promoter but lacking the PBX1 cDNA sequence. The total concentration of PBX1 plus empty plasmid was 1000ng for each condition, which was co-transfected with 1000ng of PGC1α-PGL3-basic plasmid, and 1.25ng of the renilla CMV control plasmid (section 2.2.12). The level of firefly luciferase activity was normalised to renilla luciferase activity which removes sample-to-sample variability due to differences in transfection efficiencies, cell numbers and pipetting errors.

5.3 Results

5.3.1 Does the methylation of CpG -783 in the PGC1α promoter alter the binding of transcription factors?

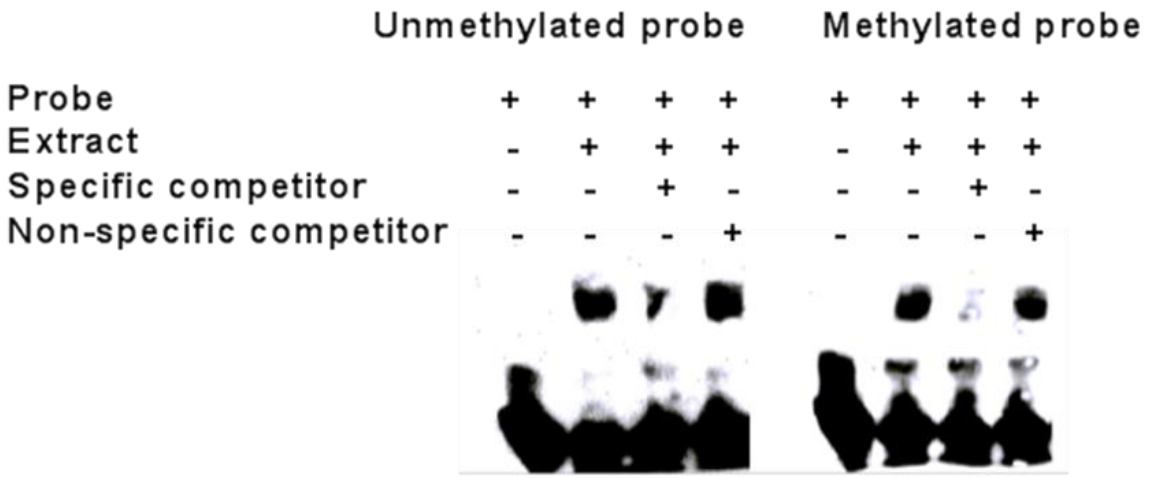


Figure 5.2- Testing the specificity of protein binding. The left panel shows protein binding to the unmethylated biotin-labelled probe, whereas the right panel shows binding to the methylated biotin-labelled probe. The addition of probe, extract, specific competitor and non-specific is indicated by a '+' for each lane, whereas a lack of these is indicated by a '-'.

Figure 5.2 shows a DNA/protein complex of similar size with both the unmethylated and methylated biotin-labelled probe. Binding was markedly reduced with incubation with a 500-fold excess of specific competitor, which was the same sequence as the unmethylated probe but was not labelled with biotin. However, binding was not reduced with incubation with a 500-fold excess of unlabelled non-specific competitor, which was a random sequence of DNA. These findings confirm specific protein binding to this region of the PGC1α promoter.

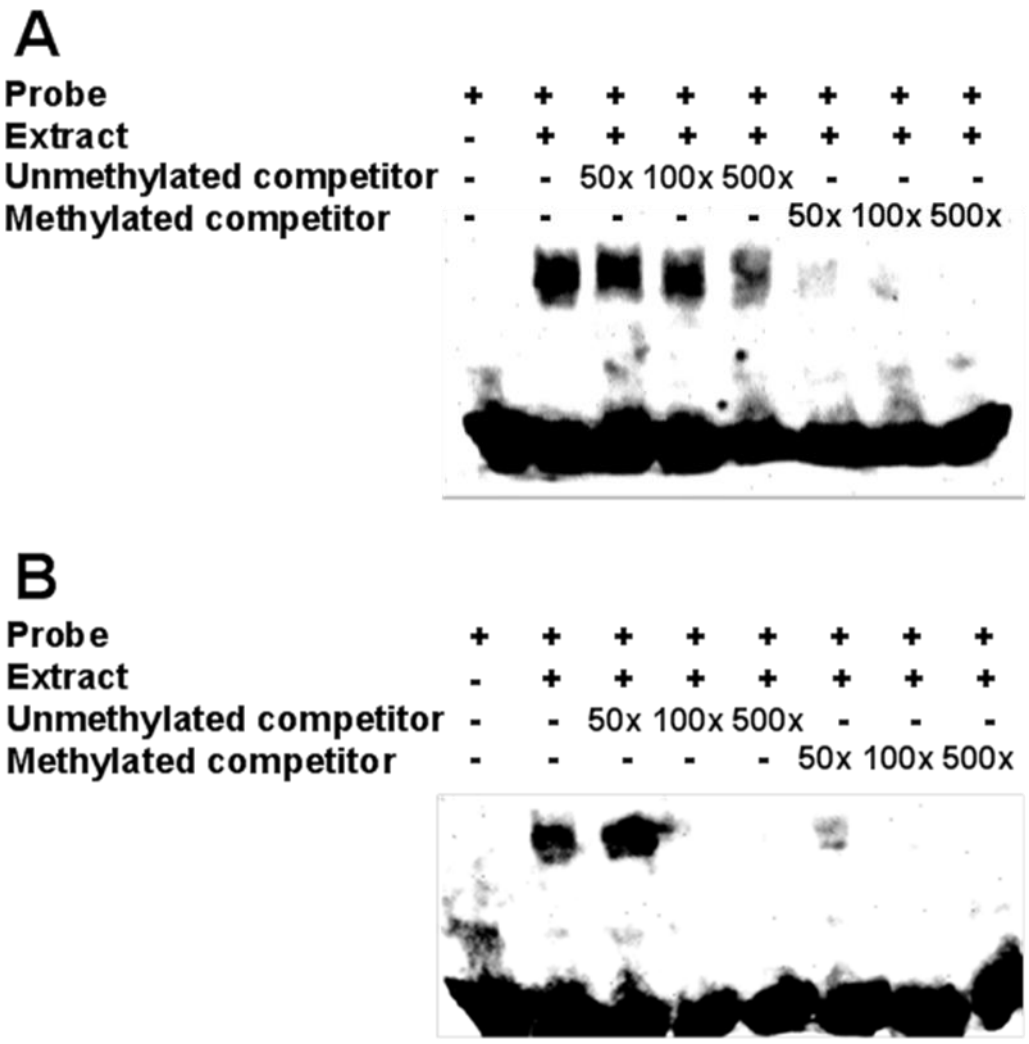


Figure 5.3- The effect of methylation of CpG -783 in PGC1α on transcription factor binding. Figure A shows protein binding to the unmethylated biotin-labelled probe in the presence or absence of nuclear extract, unmethylated competitor or methylated competitor, indicated by a '+' or '-' for each lane. An increasing fold excess of each unlabelled competitor is indicated by 50x, 100x or 500x.

Figure B shows protein binding to the methylated biotin-labelled probe in the presence or absence of nuclear extract, unmethylated competitor or methylated competitor, indicated by a '+' or '-' for each lane. An increasing fold excess of each unlabelled competitor is indicated by 50x, 100x or 500x.

To determine whether methylation of CpG -783 affected protein binding to this sequence, both the unmethylated and methylated biotin-labelled probe were incubated with nuclear extracts

along with a 50, 100 and 500-fold excess of either the unmethylated or methylated unlabelled specific competitor. Figure 5.3A shows that binding to the unmethylated biotin-labelled probe was reduced in the presence of a 500-fold excess of unmethylated unlabelled specific competitor, whereas only a 50-fold excess of methylated unlabelled specific competitor was required to compete out binding. Figure 5.3B shows that binding to the methylated biotin-labelled probe was completely diminished in the presence of a 100-fold excess of unmethylated unlabelled specific competitor, showing binding was competed out more effectively than when the probe was methylated. Binding to the methylated biotin-labelled probe was still reduced in the presence of only a 50-fold excess of methylated unlabelled specific competitor. These findings suggest that methylation of CpG -783 enhances protein binding to the PGC1α promoter sequence.

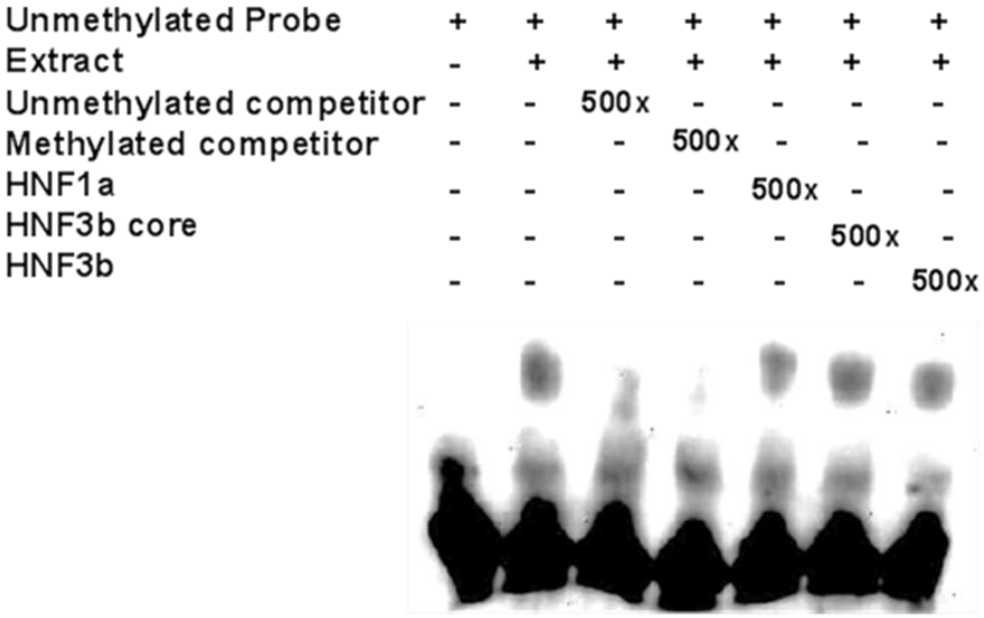


Figure 5.4- HNF3β and HNF1α do not bind to CpG -783 in PGC1α. Protein binding to the unmethylated biotin-labelled probe in the presence or absence of nuclear extract, unmethylated competitor or methylated competitor is shown in the first four lanes, indicated by a '+' or '-'. A 500-fold excess of each competitor was used, indicated by 500x.

Protein binding to the unmethylated biotin-labelled probe in the presence or absence of an unlabelled oligonucleotide containing the consensus binding sequence for HNF1α, HNF3β core, or HNF3β is shown in the last three lane, indicated by a '+' or '-'. A 500-fold excess of each competitor was used, indicated by 500x.

Figure 5.4 shows that binding to the unmethylated biotin-labelled probe was reduced in the presence of a 500-fold excess of unmethylated and methylated unlabelled specific competitor, more effectively when the competitor was methylated, as discussed previously. Binding was unaltered by co-incubation with a 500-fold excess of unlabelled HNF1α, or HNF3β consensus sequence, suggesting that these transcription factors do not bind to this sequence of the PGC1α promoter.

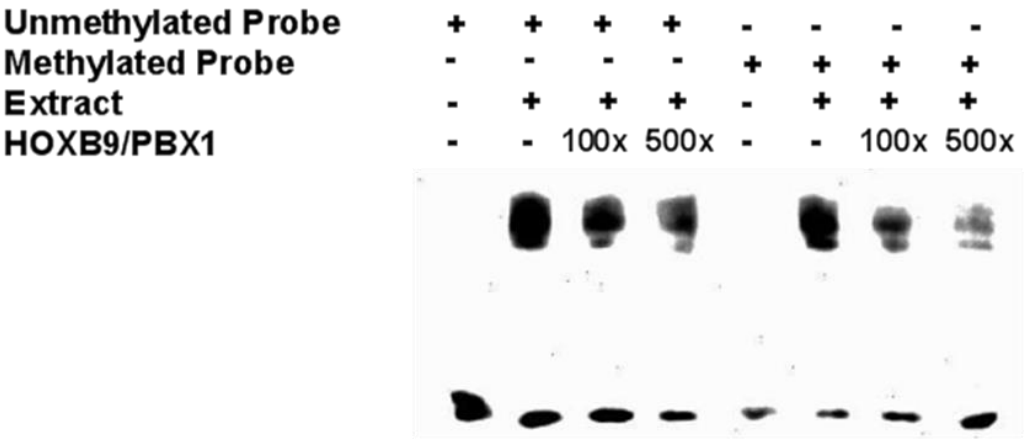


Figure 5.5- The HOXB9-PBX1 heterodimer binds to CpG -783 in PGC1α. Protein binding to the unmethylated biotin-labelled probe in the presence or absence of nuclear extract and unlabelled oligonucleotide containing the consensus binding sequence for the HOXB9-PBX1 heterodimer is shown in the first four lanes, indicated by a '+' or '-'. An increasing fold excess of unlabelled HOXB9-PBX1 is indicated by 100x or 500x.

Protein binding to the methylated biotin-labelled probe in the presence or absence of nuclear extract and unlabelled oligonucleotide containing the consensus binding sequence for the HOXB9-PBX1 heterodimer is shown in the last four lanes, indicated by a '+' or '-'. An increasing fold excess of unlabelled HOXB9-PBX1 is indicated by 100x or 500x.

Figure 5.5 shows that binding to the methylated biotin-labelled probe was reduced in the presence of 100 and 500-fold excess of unlabelled HOXB9-PBX1 heterodimer consensus sequence, whilst binding to the unmethylated biotin-labelled probe was only reduced by a 500-fold excess of unlabelled HOXB9-PBX1 heterodimer consensus sequence. These data suggest that the HOXB9-PBX1 heterodimer binds to this sequence of the PGC1α promoter, and methylation of CpG -783 enhances binding.

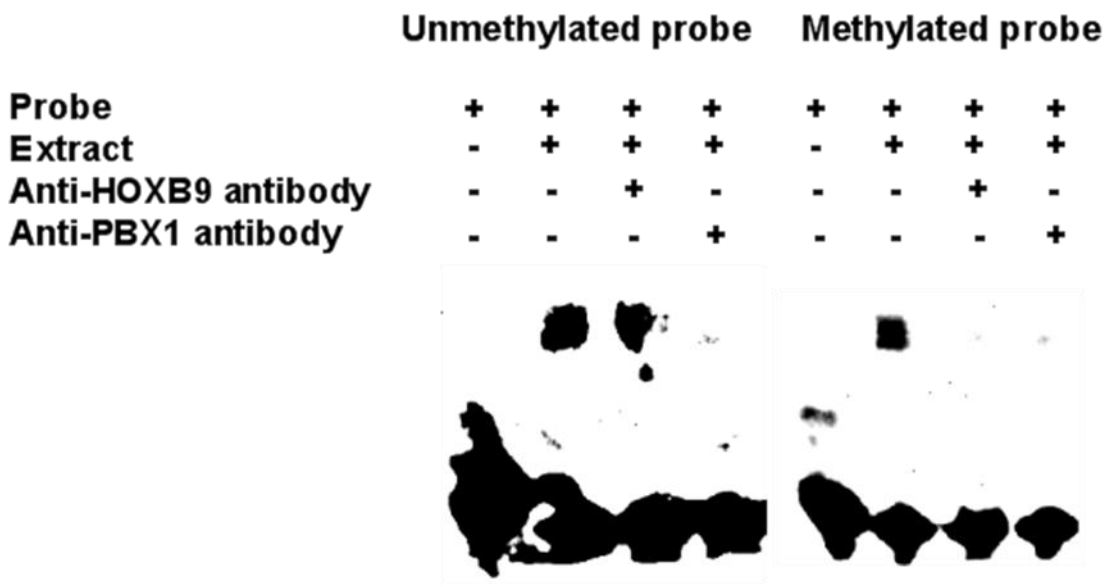


Figure 5.6- Differential methylation of CpG -783 in PGC1α alters the binding of a heterodimeric complex. The left panels shows protein binding to the unmethylated biotin-labelled probe, whereas the right panel shows binding to the methylated biotin-labelled probe. The addition of probe, extract, anti-HOXB9 antibody and anti-PBX1 antibody is indicated by a '+' for each lane, whereas a lack of these is indicated by a '-'.

Figure 5.6 shows that the addition of anti-PBX1 antibody diminished protein binding to both the unmethylated and methylated probe, whereas the anti-HOXB9 antibody only diminished protein binding to the methylated probe, with little effect on protein binding to the unmethylated probe. These data suggest that the HOXB9-PBX1 heterodimer binds to this sequence only when CpG -783 is methylated, and when unmethylated PBX1 binds with an unidentified partner.

5.3.2 Does PBX1 alter PGC1α promoter activity?

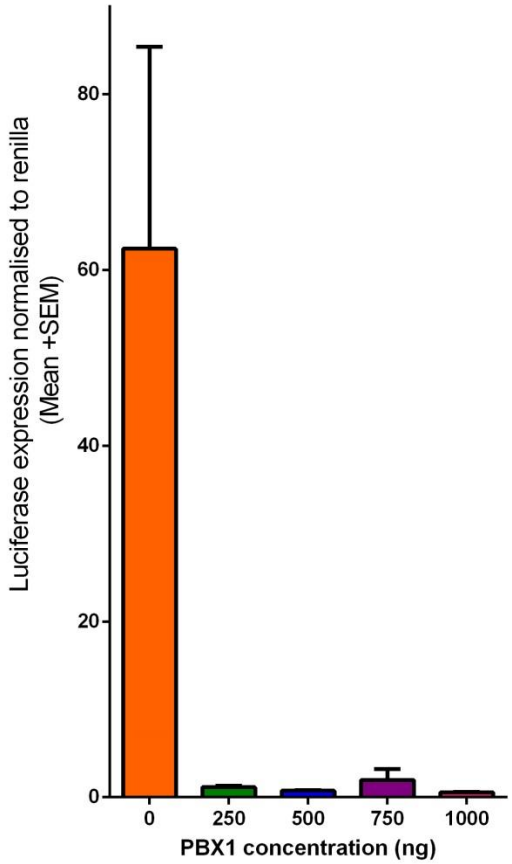


Figure 5.7- PBX1 decreases PGC1α gene expression. The effect of co-transfection of unmethylated PGC1α promoter with an increasing concentration of PBX1 on luciferase expression is shown. For each concentration the mean plus the standard error of the mean (SEM) is shown against luciferase expression normalised to renilla.

There was a high level of luciferase expression when the unmethylated PGC1α promoter was transfected without PBX1; however expression was completely diminished when PBX1 was co-transfected, even at 250ng. These data suggest that when CpG -783 is unmethylated, PBX1 negatively regulates PGC1α gene expression.

5.4 Discussion

5.4.1 Methylation of CpG -783 in the PGC1α promoter enhances the binding of the HOXB9-PBX1 heterodimer

The functional significance of methylation of CpG -783 in the PGC1α promoter was examined in adipose derived cells by determining the effect of methylation on transcription factor binding. Whilst altered methylation of this CpG may not be causally involved in the regulation of adipose tissue, as an initial investigation a cell line that originates from the same cell lineage as adipocytes was used because the methylation of CpG –783 in peripheral blood associated with future risk of adiposity in childhood (chapter 4), and lies within the putative binding site of transcription factors known to be involved in adipogenesis (190)(186)(191).

These findings show that in human liposarcoma cells, two complexes of similar mobility bound specifically to the region of the PGC1α promoter corresponding to -797 to -764. However, protein binding was enhanced when the cytosine of CpG -783 was methylated. The methylation of DNA is generally associated with a reduction in transcription factor binding, which occurs as a result of the methyl group physically blocking the a transcription factor binding site or the recruitment of methyl binding proteins that occupy the transcription factor binding site (104). Therefore, depending on whether these transcription factors are activating or repressive, methylation can activate or supress gene transcription. However, there is now some evidence to suggest that DNA methylation can enhance the binding of some transcription factors (105)(106). Hu et al. screened the entire family of human transcription factors and cofactors to determine which ones were able to bind to 150 different pieces of methylated DNA. They identified 47 transcription factors that could bind to sites containing a methylated cytosine, with some transcription factors and cofactors only able to bind to specific methylated sequences demonstrating that the surrounding sequence is important (106). Hu et al. suggest that methylated cytosine acts as the 5^{th} base, and these data show that methylation of a particular sequence is important in regulating gene expression (106).

CpG -783 is located within a putative transcription factor binding site for HNF3β, HNF1α, HOXB9 and PBX1. Binding was reduced by the presence of an excess of unlabelled consensus sequence for the HOXB9-PBX1 heterodimer. HOXB9 is a transcription factor involved in cell proliferation and differentiation, and is known to cooperatively bind with PBX1; a TALE (three amino acid loop extension) class of homeodomain transcription factor which regulates many developmental processes (143)(186). Specific antibodies for HOXB9 and PBX1 both blocked binding to the methylated sequence, whereas only the PBX1 antibody reduced protein binding to the unmethylated

sequence, with the HOXB9 antibody having no effect on binding. Because the antibodies were added before the addition of biotin-labelled probe, this suggests that the failure of the HOXB9 antibody to block binding to the unmethylated probe is not caused by a different conformation of the HOXB9-PBX1 heterodimer. Rather, these data suggest that the HOXB9-PBX1 heterodimer bound to the methylated sequence, but when unmethylated, PBX1 binds with an unidentified partner. Therefore, the binding partner of PBX1 to unmethylated CpG -783 needs to be identified.

There are 39 HOX homeobox genes that are divided into 4 groups (A, B, C and D), which are further subdivided into paralog groups (1 through to 13) (143). A pentapeptide sequence present in HOX proteins has been shown to be important for binding to the PBX1 homeodomain (144). It is possible there may be a second pentapeptide sequence within HOXB9 that specifically binds to the methylated cytosine residue of CpG -783 in the PGC1α promoter; however, when the cytosine residue is unmethylated this disrupts the binding surface for HOXB9. PBX1 has been previously shown to dimerise with MEIS1 and several other members of the HOX protein family such as HOXA10, HOXB8 or HOXB7 (143)(193). Interestingly, the consensus sequence for HOXA10-PBX1 is identical to that of the HOXB9-PBX1 heterodimer, with the important exception of a T in place of the C corresponding to the C of CpG -783. Therefore, the HOXA10-PBX1 heterodimer may bind to the unmethylated sequence if the cytosine residue is not part of the homeodomain core recognition site, but when the cytosine residue is methylated the methyl group may interfere with binding (143). This could be confirmed using EMSA and unlabelled oligonucleotide for the consensus binding sequence of the HOXA10-PBX1 heterodimer to determine if binding to the unmethylated probe was reduced.

HOX-PBX1 heterodimers can from trimeric complexes with PREP1, which are known to be transcriptional activators (194). Therefore, PBX1 may be binding to the unmethylated sequence with a different HOX protein and PREP1, which could be tested by repeating the EMSA with the consensus binding sequence for these trimeric complexes.

HPIP (hematopoietic PBX-interacting protein) have been shown to interact with PBX1 in liver cells, which inhibited the ability of HOXA9 and HOXA10-PBX1 heterodimers to bind to their target sequences (194). Whilst this effect was seen in liver cells, HPIP may be able to bind to PBX1 in different cell types and inhibit the binding of HOX-PBX1 heterodimers. In the developing mouse embryo, it has been shown that ZFPIP (Zinc finger PBX1 interacting protein) binds to PBX1 which prevents HOXA9-PBX1 binding to its consensus DNA site (195). These data suggest that other transcription factors can potentially activate or inhibit the binding of HOX-PBX1 complexes to their target sequence.

Obesity occurs via the expansion of white adipose tissue either by increased adipocyte size (hypertrophy), or by increased adipocyte numbers (hyperplasia). Data from a study by Monteiro et al. suggest that PBX1 regulates adipocyte development in mice by promoting the generation of adipocyte progenitor cells from embryonic stem cells, and by the proliferation of adipocyte progenitor cells postnatally (186). $PBX1^{-/-}$ mouse embryonic stem cells are unable to generate adipocytes, demonstrating the importance of PBX1 in the generation of adipocyte progenitor cells (186). In humans, PBX1 also promotes the differentiation of adipose derived stem cells *in vitro* into mature adipocytes (186). PBX1 expression has been shown to be positively correlated with BMI when measured in subcutaneous adipose tissue from lean, overweight and obese subjects (186). These data suggest that PBX1 is involved in the development of obesity and increased expression may lead to increased proliferation and expansion of a pre-existing pool of adipocyte progenitor cells. Results from chapter 4 have shown that increased methylation of CpG -783 in peripheral blood associated with increased adiposity in childhood. Taken with the findings from these experiments, where methylation of CpG -783 enhanced the binding of PBX1, this suggests that children with increased methylation of this locus may have increased binding of PBX1, which in turn may promote the proliferation of adipocyte progenitor cells increasing adipocyte numbers.

These findings have also shown that PBX1 decreases the expression of unmethylated PGC1α, which further supports an association between methylation of this locus and adiposity because enhanced binding of PBX1 may decrease the expression of PGC1α *in vivo*. This supports a previous study where obese subjects had increased methylation of this specific locus, and whilst methylation didn't correlate with gene expression, these subjects also had a decrease in PGC1α mRNA expression in subcutaneous adipose tissue, when compared to lean subjects (79). This suggests that methylation of this locus may influence the binding of PBX1 and adipogenesis, and PBX1 in turn influences PGC1α expression and the development of obesity. However, the effect of PBX1 on methylated PGC1α expression also needs to be determined because the effect may be different when CpG -783 is methylated. In order to confirm that methylated CpG -783 is crucial for the binding of PBX1 and HOXB9, the CpG site could be mutated and co-transfected with PBX1 and HOXB9 separately in order to determine the effect on PGC1α expression.

The HOX genes are known to involved in the transcriptional regulation of adipogenesis, with the expression of individual HOX genes being detected in specific deposits of adipose tissue (191)(196). For example, HOXB4 has been shown to transcriptionally silent in subcutaneous and intra-peritoneal white adipose tissue, whereas HOXD4 is transcriptionally active in these tissues (191).

Another consideration is the stage of development as the expression of HOXA4 is known to be expressed in preadipocytes, however expression is decreased following differentiation into mature adipocytes (191). HOXA5 expression has been shown to increase with increasing BMI in visceral and subcutaneous adipose tissue from men and women, suggesting that expression is associated with obesity (196). Therefore, binding of the HOXB9-PBX1 heterodimer to this region of the PGC1α may be important in the regulation of gene expression in adipose tissue specifically, and may be important in the development of obesity.

5.5 Summary

These data support the hypothesis that differential methylation of CpG -783 in the PGC1α promoter alters transcription factor binding, with methylation of CpG -783 specifically resulting in the increased binding of the pro-adipogenic HOXB9-PBX1 heterodimer. When CpG -783 was unmethylated, PBX1 bound with an unidentified partner. These findings demonstrate for the first time that methylation can alter transcription factor composition, which increases the complexity of gene regulation. PBX1 resulted in a decrease in PGC1α expression, suggesting that methylation of this locus may alter PGC1α transcription and have an effect on adipocyte development. Therefore, differential methylation of CpG -783 measured in peripheral blood that predicts future adiposity could reflect variation in the epigenetic regulation of PCG1α in adipose tissue.

Chapter 6- Parental DNA Methylation Does Not Predict the Methylation of Biomarkers of Adiposity in the Child

6.1 Introduction

6.1.1 Transgenerational epigenetic inheritance

In humans, the epigenome undergoes demethylation during gametogenesis and between fertilisation and the 2-cell stage. Methylation has been shown to decrease from 54% in sperm and 48% in oocytes to 41% in the zygote, which decreases further to 32% by the 2-cell stage (108)(109). This is to ensure totipotency for cellular differentiation (109). Following implantation, genome wide remethylation occurs, where CpG dense regions tend to be hypomethylated and regions with lower CpG density are hypermethylated (108). However, there is now evidence to suggest that this epigenetic reprogramming may be incomplete because environmentally induced alterations to the epigenome can be transmitted to future generations, despite the initial environmental cue no longer being present (197–205). This is known as epigenetic inheritance, which is defined as the transmission of epigenetic variations that do not occur from variations in the DNA sequence to subsequent generations of cells or organisms, whereas transgenerational epigenetic inheritance is the transmission of these epigenetic variations across multiple generations (206).

Evidence from animal studies has shown that transgenerational epigenetic inheritance occurs. For example, Hoile et al. have shown that the offspring of F0 pregnant dams fed a protein restricted diet had altered methylation and expression of phosphoenolpyruvate carboxykinase (PEPCK), which persisted up to the F3 generation, despite the offspring receiving a normal chow diet (197). Burdge et al. have shown that feeding pregnant rats a protein restricted diet during pregnancy results in decreased methylation of the PPARα and GR promoter in the livers of male mice in the F1 and F2 generation, compared to control mice, despite these generations receiving the normal control diet (198). These data suggest that a dietary challenge during pregnancy can influence the methylation of specific CpG loci and gene expression in future generations.

There is some data in humans to suggest that transgenerational epigenetic inheritance may occur. For example data from the Dutch Hunger Winter famine has shown that pregnant women who were exposed to the famine during late gestation had smaller babies who went onto have increased adiposity later in life, and these F1 females also went on to have smaller babies themselves, despite a normal diet during pregnancy (199)(200). These data suggest that the effects of the famine persist into future generations. However, the F1 germ cells that produced the F2 generation were also exposed to the famine, and therefore in order to be truly transgenerational the effects must be present in the F3 generation. Skinner states that in order to be transgenerational inheritance the generation in which the phenotypic variation is observed must not have been directly exposed to the

environmental challenge at the level of either the germline or the organism (206)(207). If a male or non-pregnant female is exposed to the environmental challenge then only 2 generations of transmission are required to be termed 'transgenerational' (206)(207).

6.1.2 Sex-specific epigenetic inheritance

There is some evidence in humans to suggest that epigenetic inheritance can occur in a sex-specific manner. For example, data from the Dutch Hunger Winter famine has shown that women exposed to the famine during early gestation have an increased BMI and waist circumference at age 50 years compared to non-exposed women, whereas in 50 year old men, BMI nor waist circumference were significantly altered by exposure to the famine (201). Kaati et al. have shown that paternal grandfather overfeeding during childhood before puberty increases the risk of cardiovascular disease and diabetes in grandsons, but not granddaughters, suggesting that the effects of over nutrition are transmitted via the male line (202).

Environmental challenges that induce variation in the epigenome can also include factors such as smoking, along with altered nutrition. For example, Pembrey et al. have shown that paternal smoking before 11 years is associated with increased BMI in the sons at 9 years, but not the daughters (203). This suggests that this male-line generational effect could be transmitted via the Y chromosome and that environmental challenges could affect the spermatozoal epigenome. However, this effect may be explained by societal factors.

Parental obesity is known to increase the risk of obesity in children, and evidence suggests this may occur in a sex-specific manner (208). A study by Soubry et al. has shown that paternal obesity, defined as a BMI above 30kg/m^2, associates with hypomethylation of the IGF2 promoter in umbilical cord blood leukocytes of the offspring, suggesting that paternal obesity can influence the epigenetic regulation of specific loci (205). Previous data from the EarlyBird cohort has shown that girls from overweight mothers had a higher BMI than boys from overweight mothers, and boys from overweight fathers had a higher BMI than girls from overweight fathers, suggesting a same-sex paring effect (87). These data suggest that maternal and paternal BMI may influence the body composition of the child, and provides a potential mechanism for the prevention of childhood obesity.

6.1.3 Aims

Pyrosequencing was used to test the hypothesis that the methylation of specific CpG loci in SIRT1 and PGC1α in children is predicted by the methylation of these loci in the parents in peripheral blood. Whilst there is some transgenerational and multigenerational epigenetic inheritance data in humans, there is a lack of evidence to determine if the methylation of a specific CpG locus is maintained between generations, and whether methylation of the previous generation can predict the methylation of the next generation. There is also a lack of evidence on whether sex-specific associations occur between parental and child methylation levels. The results from these experiments will provide some evidence on whether previously identified biomarkers of adiposity from chapter 4 are present in the previous generation, which would allow for earlier prediction and intervention.

6.2 Methods

The methylation of specific CpG loci in the SIRT1 and PGC1α promoter were measured in the peripheral blood from the parents of the EarlyBird subjects. Parental blood samples were taken at the start of the EarlyBird study when the children were aged 5 years. Due to missing peripheral blood samples for some of the parents, DNA was not available for the mother and the father for all subjects. However, DNA was available for either the mother or father for all subjects except subject 201. The methylation of the mothers (n=37) and the fathers (n=34) was plotted along with the mean methylation of the children aged 5-7 years (n=40). Whilst the results from chapter 3 have shown the methylation of each CpG loci in SIRT1 and PGC1α was temporally stable between 5-7 and 14 years, parental methylation was also plotted against the methylation of the children at 14 years, as this was a single methylation value rather than a mean.

A two-tailed paired t-test was used to determine if there was a significant difference between the methylation of the mother and the children at 5-7 and 14 years, and also between the methylation of the father and the children at 5-7 and 14 years. A linear regression was used to determine if the methylation of the mother and father was significantly associated with the methylation of the child at 5-7 and 14 years. A linear regression was also used to determine if the methylation of the mother and father was significantly associated with the methylation of just the girls, or just the boys at 5-7 and 14 years.

6.3 Results

6.3.1 Is the methylation of specific CpG loci in the child predicted by the methylation of the same loci in the parents?

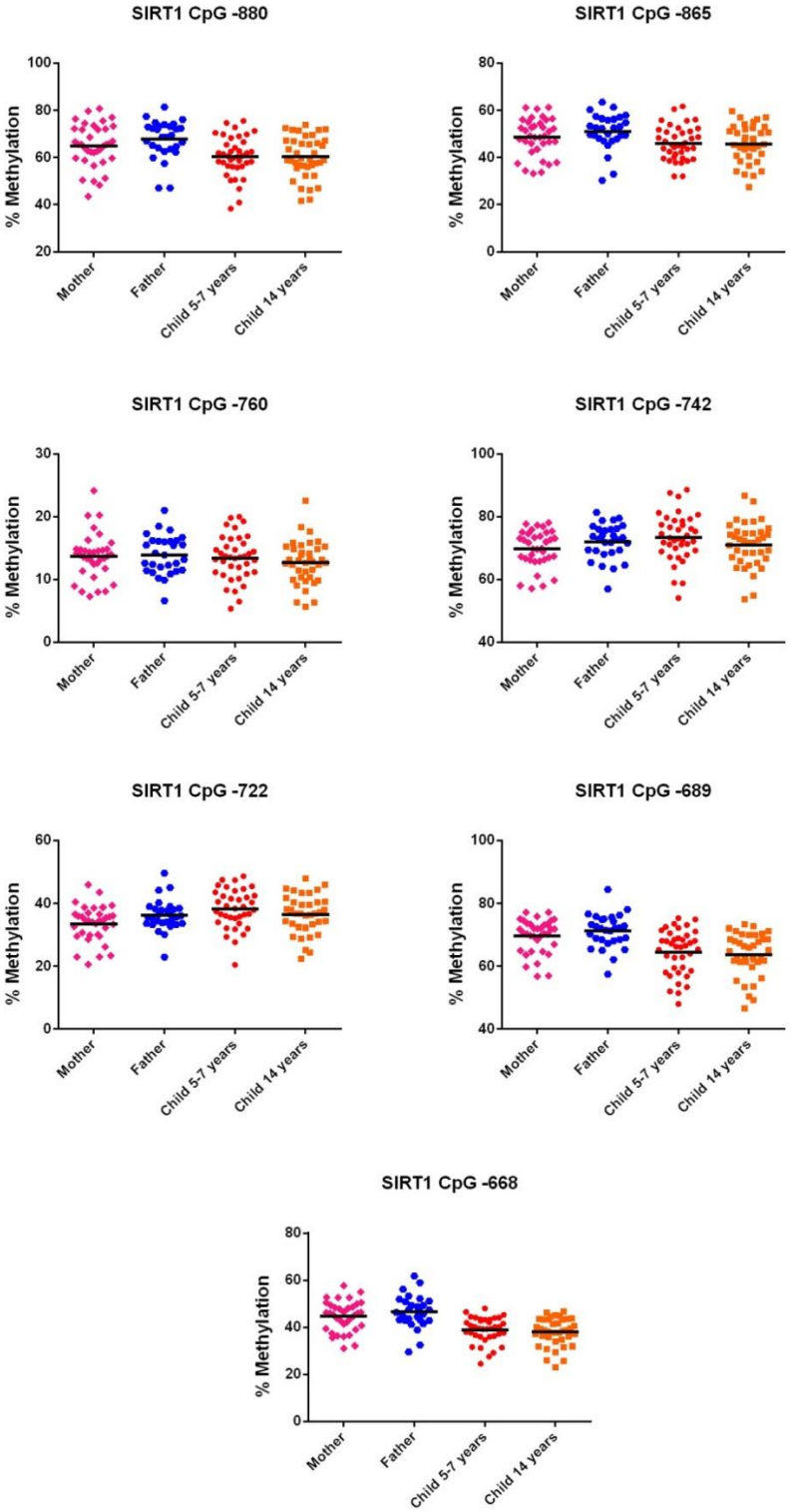


Figure 6.1- Parental DNA methylation compared to the child's methylation at 5-7 and 14 years in the SIRT1 promoter. Each panel represents the methylation of every mother, father and child at 5-7 and 14 years, for each CpG loci measured in the SIRT1 promoter. CpG loci are numbered according to their distance from the transcription start site.

For CpGs -880, -865 and -760 the mean methylation of the mothers does not significantly differ from the mean methylation of the children at 5-7 and 14 years, determined using a two-tailed paired t-test ($p>0.050$). For CpGs -742, -722, -689 and -668 the mean methylation of the mothers is significantly different from the mean methylation of the children at 5-7 and 14 years ($p\leq0.014$).

For CpGs -865, -760, -742 and -722 the mean methylation of the fathers does not significantly differ from the mean methylation of the children at 5-7 years and 14 ($p>0.050$). For CpGs -880, -689 and -668 the mean methylation of the fathers is significantly different from the mean methylation of the children at 5-7 and 14 years ($p\leq0.016$).

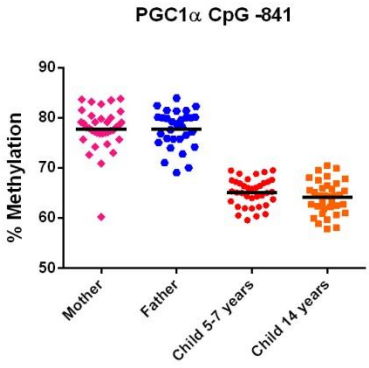
Table 6.1- The association between parental methylation and the methylation in the child at 5-7 years in the SIRT1 promoter

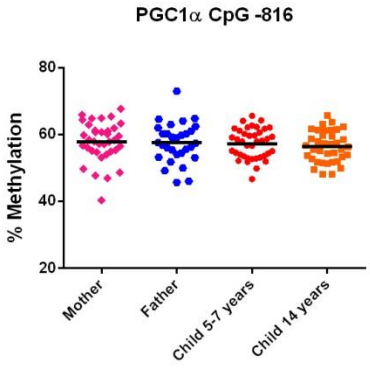
	Does mothers methylation predict the child's methylation at 5-7 years?			Does fathers methylation predict the child's methylation at 5-7 years?			Does mothers methylation predict the girls methylation at 5-7 years?			Does fathers methylation predict the girls methylation at 5-7 years?			Does mothers methylation predict the boys methylation at 5-7 years?			Does fathers methylation predict the boys methylation at 5-7 years?		
	n	b	p-value	n	b	p-value	n	b	p-value	n	b	p-value	n	b	p-value	n	b	p-value
SIRT1 CpG -880	34	-0.032	0.849	26	-0.088	0.690	16	0.147	0.414	13	0.145	0.528	17	-0.301	0.340	12	-0.250	0.528
SIRT1 CpG -865	33	-0.138	0.390	25	-0.101	0.607	15	0.132	0.509	12	-0.028	0.920	17	-0.401	0.115	12	-0.117	0.698
SIRT1 CpG -760	32	0.068	0.702	26	-0.311	0.181	14	0.274	0.223	13	-0.152	0.737	17	-0.093	0.732	12	-0.379	0.245
SIRT1 CpG -742	32	0.307	0.171	26	-0.166	0.548	14	0.522	0.055	13	-0.340	0.472	17	0.083	0.822	12	-0.116	0.765
SIRT1 CpG -722	31	0.079	0.698	25	-0.140	0.599	13	0.201	0.408	12	-0.646	0.253	17	-0.050	0.881	12	-0.025	0.946
SIRT1 CpG -689	33	-0.125	0.575	27	-0.526	0.051	15	-0.023	0.942	14	-0.834	0.060	17	-0.320	0.368	12	-0.363	0.345
SIRT1 CpG -668	32	0.017	0.915	25	-0.074	0.648	14	0.189	0.443	12	-0.399	0.293	17	-0.134	0.535	12	0.012	0.951

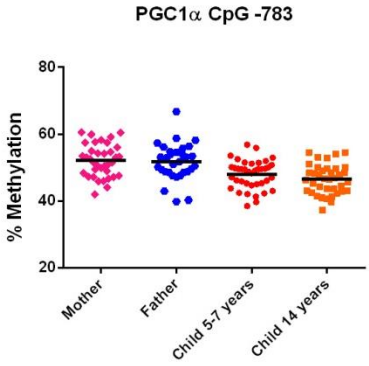
Table 6.2- The association between parental methylation and the methylation in the child at 14 years in the SIRT1 promoter

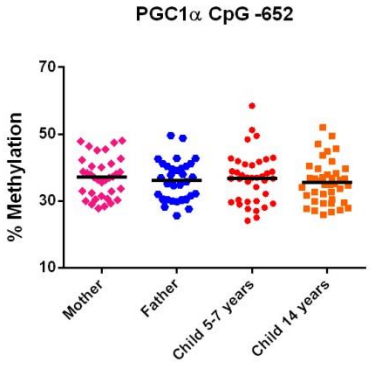
	Does mothers methylation predict the child's methylation at 14 years?			Does fathers methylation predict the child's methylation at 14 years?			Does mothers methylation predict the girls methylation at 14 years?			Does fathers methylation predict the girls methylation at 14 years?			Does mothers methylation predict the boys methylation at 14 years?			Does fathers methylation predict the boys methylation at 14 years?		
	n	b	p-value	n	b	p-value	n	b	p-value	n	b	p-value	n	b	p-value	n	b	p-value
SIRT1 CpG -880	34	-0.007	0.966	26	-0.120	0.574	15	0.149	0.493	12	0.190	0.425	18	-0.233	0.434	13	-0.370	0.309
SIRT1 CpG -865	32	-0.212	0.292	24	-0.050	0.814	14	0.017	0.952	11	0.231	0.419	17	-0.432	0.144	12	-0.142	0.664
SIRT1 CpG -760	32	-0.234	0.152	26	-0.443	0.079	15	-0.045	0.810	14	-0.252	0.610	16	-0.618	0.052	11	-0.550	0.085
SIRT1 CpG -742	32	0.086	0.656	26	-0.252	0.367	15	0.320	0.223	14	-0.306	0.495	16	-0.184	0.532	11	-0.217	0.602
SIRT1 CpG -722	30	0.058	0.765	25	-0.368	0.175	13	-0.102	0.669	13	-0.725	0.199	16	0.348	0.320	11	-0.270	0.456
SIRT1 CpG -689	33	-0.258	0.242	27	-0.275	0.376	15	-0.120	0.694	14	-0.729	0.143	17	-0.520	0.142	12	0.003	0.994
SIRT1 CpG -668	33	0.030	0.845	27	-0.240	0.173	15	0.086	0.698	14	-0.327	0.353	17	-0.037	0.875	12	-0.213	0.350

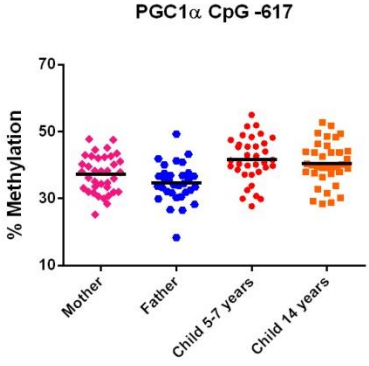
Table 6.1 and 6.2 show the output from the linear regression where ‘n’ is the number of subjects and the ‘b’ (beta) value shows the direction of the association. The p-value is also given. There were no significant associations between the methylation of any of the loci measured in the SIRT1 promoter in the mothers and the methylation of these loci in the children at 5-7 years and 14 years, nor with the methylation of just the girls or just the boys at 5-7 and 14 years ($p>0.050$). There were no significant associations between the methylation of any of the loci measured in the SIRT1 promoter in the fathers and the methylation of these loci in the children at 5-7 years and 14, when analysed together, or separated by sex. Therefore, the methylation of these CpG loci in these children is not predicted by the methylation of these loci in the mother nor the father, measured in peripheral blood.

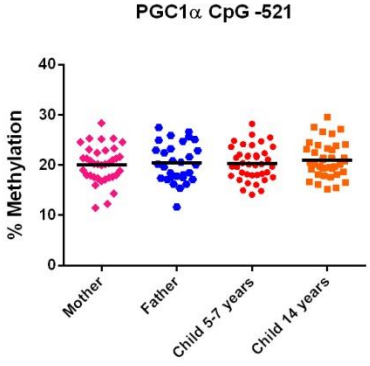

PGC1α CpG -841
% Methylation
90
80
70
60
50
Mother
Father
Child 5-7 years
Child 14 years


PGC1α CpG -816
% Methylation
80
60
40
20
Mother
Father
Child 5-7 years
Child 14 years


PGC1α CpG -783
% Methylation
80
60
40
20
Mother
Father
Child 5-7 years
Child 14 years


PGC1α CpG -652
% Methylation
70
50
30
10
Mother
Father
Child 5-7 years
Child 14 years


PGC1α CpG -617
% Methylation
70
50
30
10
Mother
Father
Child 5-7 years
Child 14 years


PGC1α CpG -521
% Methylation
40
30
20
10
0
Mother
Father
Child 5-7 years
Child 14 years

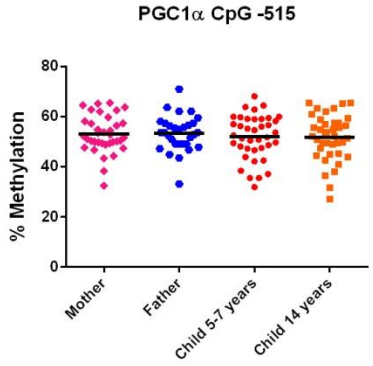

PGC1α CpG -515
% Methylation
80
60
40
20
0
Mother
Father
Child 5-7 years
Child 14 years

Figure 6.2- Parental DNA methylation compared to the child's methylation at 5-7 and years in the PGC1α promoter. Each panel represents the methylation of every mother, father and child at 5-7 and 14 years, for each CpG loci measured in the PGC1α promoter. CpG loci are numbered according to their distance from the transcription start site.

For CpGs -816, -652, -521 and -515 the mean methylation of the mothers does not significantly differ from the mean methylation of the children at 5-7 and 14 years, determined using a two-tailed paired t-test ($p>0.050$). For CpGs -841, -783 and -617 the mean methylation of the mothers is significantly different from the mean methylation of the children at 5-7 and 14 years ($p\leq0.008$).

For CpGs -816, -652, -521 and -515 the mean methylation of the fathers does not significantly differ from the mean methylation of the children at 5-7 and 14 years ($p>0.050$). For CpGs -841, -783 and -617 the mean methylation of the fathers is significantly different from the mean methylation of the children at 5-7 and 14 years ($p\leq0.004$).

Table 6.3- The association between parental methylation and the methylation in the child at 5-7 years in the PGC1α promoter

	Does mothers methylation predict the child's methylation at 5-7 years?			Does fathers methylation predict the child's methylation at 5-7 years?			Does mothers methylation predict the girls methylation at 5-7 years?			Does fathers methylation predict the girls methylation at 5-7 years?			Does mothers methylation predict the boys methylation at 5-7 years?			Does fathers methylation predict the boys methylation at 5-7 years?		
	n	b	p-value	n	b	p-value	n	b	p-value	n	b	p-value	n	b	p-value	n	b	p-value
PGC1α CpG -841	33	0.120	0.285	27	-0.094	0.481	15	0.068	0.660	13	-0.224	0.258	17	0.208	0.269	13	0.023	0.895
PGC1α CpG -816	34	0.193	0.129	28	-0.176	0.194	16	0.164	0.373	14	-0.310	0.150	17	0.221	0.293	13	-0.131	0.422
PGC1α CpG -783	34	0.093	0.521	30	-0.046	0.630	16	0.064	0.757	16	-0.080	0.543	17	0.134	0.550	13	-0.018	0.910
PGC1α CpG -652	32	0.210	0.291	29	0.158	0.452	14	0.090	0.806	14	0.251	0.432	17	0.362	0.093	14	-0.011	0.971
PGC1α CpG -617	30	0.299	0.164	27	-0.139	0.378	14	0.148	0.613	14	-0.132	0.438	15	0.509	0.098	12	-0.201	0.722
PGC1α CpG -521	34	0.151	0.262	28	0.064	0.659	16	0.213	0.079	14	-0.057	0.746	17	-0.138	0.711	13	0.272	0.299
PGC1α CpG -515	34	0.173	0.362	28	-0.148	0.456	16	0.217	0.350	14	-0.212	0.475	17	0.172	0.601	13	-0.043	0.886

Table 6.4- The association between parental methylation and the methylation in the child at 14 years in the PGC1α promoter

	Does mothers methylation predict the child's methylation at 14 years?			Does fathers methylation predict the child's methylation at 14 years?			Does mothers methylation predict the girls methylation at 14 years?			Does fathers methylation predict the girls methylation at 14 years?			Does mothers methylation predict the boys methylation at 14 years?			Does fathers methylation predict the boys methylation at 14 years?		
	n	b	p-value	n	b	p-value	n	b	p-value	n	b	p-value	n	b	p-value	n	b	p-value
PGC1α CpG -841	31	0.196	0.157	25	-0.051	0.766	15	0.106	0.568	13	-0.232	0.319	15	0.300	0.193	11	0.075	0.747
PGC1α CpG -816	32	0.122	0.376	27	-0.112	0.437	15	0.053	0.794	14	-0.145	0.537	16	0.185	0.409	12	-0.141	0.419
PGC1α CpG -783	33	-0.062	0.680	30	-0.059	0.543	15	-0.162	0.440	16	-0.087	0.504	17	0.080	0.732	13	-0.043	0.798
PGC1α CpG -652	34	0.254	0.135	31	0.214	0.265	15	0.189	0.488	15	0.212	0.446	18	0.339	0.134	15	0.238	0.431
PGC1α CpG -617	31	0.299	0.120	27	0.026	0.912	16	0.292	0.353	15	0.089	0.772	14	0.388	0.079	11	-0.487	0.270
PGC1α CpG -521	34	0.098	0.462	29	0.100	0.531	16	0.125	0.383	14	0.080	0.730	17	-0.068	0.848	14	0.132	0.586
PGC1α CpG -515	34	0.051	0.791	29	-0.184	0.383	16	0.078	0.773	14	-0.222	0.537	17	0.046	0.881	14	-0.122	0.598

Tables 6.3 and 6.4 show the output from the linear regression. There were no significant associations between the methylation of any of the loci measured in the PGC1α promoter in the mothers and the methylation of these loci in the children at 5-7 years and 14 years, nor with the methylation of just the girls or just the boys at 5-7 and 14 years ($p>0.050$). There were no significant associations between the methylation of any of the loci measured in the PGC1α promoter in the fathers and the methylation of these loci in the children at 5-7 years and 14, when analysed together, or separated by sex. Therefore, the methylation of these CpG loci in these children is not predicted by the methylation of these loci in the mother nor the father, measured in peripheral blood.

6.4 Discussion

6.4.1 Parental DNA methylation does not predict the methylation of biomarkers of adiposity in childhood

There is evidence to suggest that the human methylome is reset in the zygote to ensure totipotency for cellular differentiation, which is followed by genome wide remethylation after implantation (108)(109). These findings support this idea of reprogramming of the epigenome at fertilisation as the methylation of these loci in the parents did not predict the methylation of any CpG loci measured in SIRT1 or PGC1α in the children. This is true for those CpG loci where the mean methylation of the mothers and fathers was significantly different or the same as the children. This suggests that these regions of the epigenome did not escape demethylation since methylation levels were not transmitted to the offspring. However, the sample size was small and may be underpowered to measure any significant associations between parental methylation and that of the child, particularly when just the boys or girls were used in the linear regression ($n \leq 20$).

Whilst the methylation of all CpG loci in parental peripheral blood did not predict the methylation of the same loci in the peripheral blood of the children at 5-7 years or 14 years, there is no information available on the phenotype of the parents. Therefore, any parental factors that may influence methylation could not be adjusted for in the linear regression. Paternal factors that may influence methylation and potentially the phenotype of the offspring include maternal BMI, paternal BMI and smoking. For example, it has been shown that the methylation of 3 CpG loci in the PGC1α promoter in umbilical cord was associated with maternal BMI (171). Paternal obesity has been shown to be associated with hypomethylation of IGF2 in umbilical cord blood leukocytes of the offspring (205). These data suggest that parental body composition can influence the epigenome of the offspring. Pembrey et al. have shown that paternal smoking before 11 years of age increases the risk of adiposity in sons, but not daughters, suggesting that male-line generational effects occur and can be transmitted via the Y chromosome (203). Therefore, the linear regression should be adjusted for parental BMI and smoking status.

This study also lacks information on whether the children live at home with both parents, or with just one parent. The environment is thought to influence the epigenome throughout the life course, and it has been shown that twins who spend most of their lifetime together have methylation levels more similar than twins who spend time apart (133). Therefore, the methylation levels of the children may be influenced by their home environment including factors such as stress, which may be

dependent on whether both parents live at home or not. Therefore, this could be adjusted for in the linear regression.

Peripheral blood is a heterogeneous mix of cell populations and whilst white blood cell counts were available for these children, white blood cell counts were not available for the parental peripheral blood samples. The results in chapter 3 have shown that the methylation of some of these loci is affected by the proportion of neutrophils and lymphocytes. This suggests that parental methylation levels may also be affected by the proportion of leukocytes. This should be determined before stating that the methylation of these loci in the children is not predicted by the methylation of the corresponding loci in the parents.

The results from chapter 4 have shown that the methylation of CpG -880 in SIRT1 and CpGs -841, -816, -783 and -521 in PGC1α in peripheral blood at 5-7 years was predictive of future adiposity. These findings suggest that the methylation of these loci cannot be predicted by the methylation of the same loci in the previous generation. The methylation of these loci was also shown to be temporally stable between 5-7 and 14 years in chapter 3, which suggests that the methylation of these loci is determined somewhere between fertilisation and 5-7 years of age. Therefore, it is possible the methylation of these loci is determined *in utero* which may be influenced by maternal and paternal factors such as body composition and smoking status.

6.5 Summary

These findings do not support the hypothesis that methylation of specific CpG loci in SIRT1 and PGC1α in children is predicted by the methylation of these loci in the parents, measured in peripheral blood. These findings, along with those from chapters 3 and 4 suggest that the methylation of these potential biomarkers of adiposity is determined between fertilisation and 5-7 years of age. Therefore, it would be beneficial to measure the methylation of these loci in fetal tissues to determine if the association between methylation and future adiposity was present. This may provide information about which stage in development is important for determining the levels of methylation which are associated with future adiposity.

Chapter 7

Predictive Biomarkers of Adiposity can be measured in Multiple Tissues between Birth and Adulthood

7.1 Introduction

7.1.1 Tissue specific DNA methylation

Peripheral blood is an easily accessible tissue that has been used in epidemiological studies to measure epigenetic variation in relation to a particular phenotype. The results from chapter 4 have shown that the methylation of specific CpG loci in the SIRT1 and PGC1α promoters at 5-7 years measured in peripheral blood was associated with later childhood adiposity. However, because DNA methylation is tissue specific, it is unknown if these associations between methylation in peripheral blood and adiposity would be present in different tissues, such as adipose tissue. There is conflicting evidence from human studies as to whether the methylation of particular loci varies between different tissues. For example, Armstrong et al. have shown that the methylation of 7 gene promoters and global methylation varied across the placenta, umbilical cord blood and saliva at 3 and 6 months of age, suggesting that methylation is tissue specific and each tissue may have its own epigenetic signature, which may be related to the unique function of each tissue (126). Ollikainen et al. have shown that the methylation of the imprinted IGF2/H19 locus varies between umbilical cord blood mononuclear cells and granulocytes, human umbilical vein endothelial cells, buccal epithelial cells and placental tissue (127). However, Talens et al. have shown that the methylation of several non-imprinted genes is comparable in peripheral blood and buccal cells (119). This suggests that for some CpG loci methylation is detectable across different tissues from different germline layers. Therefore, an environmental challenge *in utero* could affect the germline layers equally and the associations between methylation in peripheral blood and adiposity could also be present in different tissues.

The results from chapter 3 have shown that the methylation of these potential markers of adiposity in SIRT1 and PGC1α were stable in peripheral blood between 5 and 14 years. Measuring the methylation of these loci in fetal tissues would determine if the association between methylation and future adiposity were present from birth. This would allow for the identification of individuals with an increased risk of obesity even earlier in development. There is some evidence to suggest that maternal BMI is positively associated with the methylation of 3 CpG loci in the PGC1α promoter in umbilical cord (171). Since maternal BMI is known to be a strong predictor of birth weight and is associated with childhood obesity, measuring DNA methylation in fetal tissues may be beneficial for predicting those individuals that may be at an increased risk of obesity later in life (209).

Evidence from previous studies suggests that the expression of SIRT1 and PGC1α is altered in the subcutaneous adipose tissue of obese subjects. For example, SIRT1 mRNA expression in subcutaneous adipose tissue has been shown to be inversely correlated with BMI (72). The

methylation of 3 CpGs in the PGC1α promoter were shown to be increased in abdominal subcutaneous adipose tissue from obese subjects, compared to lean subjects (79). Whilst PGC1α mRNA expression was decreased in the subcutaneous adipose tissue from these obese subjects, compared to lean subjects, expression was not associated with the mean methylation of the 5 CpG loci measured (79). Very little is known about the epigenetic regulation of SIRT1 in humans, and no previous studies have measured the methylation of the SIRT1 promoter in adipose tissue from lean and obese subjects. In order to determine if the methylation of these loci in SIRT1 and PGC1α is altered in obesity when the diseased state is present, the methylation was measured by pyrosequencing in subcutaneous abdominal adipose tissue from lean and obese subjects.

The purpose of the experiments in this chapter was to determine if the methylation of these loci is associated with different measures of adiposity when measured in a range of tissues from different germline layers, and also at different time points in development from birth through to adulthood. These experiments will also determine if the same association between methylation of these loci and adiposity occurs when obesity is present, or whether these associations are lost when the diseased state is present

7.1.2 Different techniques for measuring adiposity

There are several techniques that are widely used to evaluate obesity risk, which vary in complexity, ease of use, accuracy and reliability. Some of these techniques are used in the statistical analysis in this chapter to determine if the methylation of specific loci in SIRT1 and PGC1α is associated with adiposity, all of which have advantages and disadvantages.

BMI is the most widely used technique for determining if a subject is overweight or obese; however it is just a measure of weight (kg) divided by the square of height (m) and does not directly measure adiposity. BMI is dependent on both fat and lean mass and provides no information on the location of the fat depots, and is also unsuitable for use in children (21)(16). Waist circumference and waist to hip ratio both measure central abdominal obesity, however neither measurement distinguishes between subcutaneous and visceral fat (20). Waist to hip ratio is inaccurate as it relies on two measurements making it more susceptible to error and ratios are less sensitive to smaller weight changes (20). Skinfold thickness measures the size of specific subcutaneous fat depots, including subscapular and triceps, and is suitable for all age groups including children and provides evidence on regional adiposity (20). However, skinfold thickness does not measure visceral fat, nor lean or bone mass (20). DXA scans can measure total and regional fat mass, lean mass, and bone

mineral mass, and provides a percentage of each measurement also. DXA scans are advantageous as they accurately measure adiposity in children, however there is conflicting evidence on whether DXA scans are able to distinguish between subcutaneous and visceral fat (24)(25)(23). Bioelectrical impedance determines the amount of fat mass based on the rate at which an electrical current travels through the body and the amount of resistance, or bioelectrical impedance. Previous studies have shown that bioelectrical impedance is as accurate as DXA scans for measuring percentage body fat (24)(25)(23)(210). However, bioelectrical impedance is sensitive to hydration status and varies for different ethnicities (16). The gold standard for measuring adiposity is magnetic resonance imaging (MRI) as this is the only technique that can accurately distinguish between subcutaneous and visceral fat (20). However, MRI is costly and has limited availability, and therefore was not used to measure the adiposity of subjects in the experiments in this chapter.

Because each technique has advantages and disadvantages, it is unlikely that a single one is suitable to measure obesity risk in all populations, as the distribution and accumulation of fat depots are known to differ with age, gender and ethnicity (26). Those techniques which are reliable and accurate in children may be advantageous in order to better intervene during early life. Techniques which measure specific fat depots, rather than total body fat, may be more beneficial for predicting those individuals at greatest risk of obesity.

7.1.3 Aims

In order to test the hypothesis that the methylation of specific CpG loci in SIRT1 and PGC1α measured in fetal tissues is associated with adiposity in childhood, pyrosequencing was used. In order to test the hypothesis that the methylation and mRNA expression of SIRT1 and PGC1α differs in the abdominal subcutaneous adipose tissue of lean and obese people, pyrosequencing and real time PCR were used. This will determine if these potential biomarkers of adiposity are present in different tissues from different germline layers, at different stages of development from birth through to adulthood. It would be advantageous if altered methylation of these loci could be measured in fetal tissues because they are easily accessible and would allow for even earlier identification of individuals at an increased risk of obesity. This will also determine if the methylation of these loci is altered when the diseased state is present.

7.2 Methods

The methylation of specific CpG loci in the promoter of SIRT1 and PGC1α was measured in umbilical cord (n=440) and umbilical cord blood (n=300) from the Southampton Women's Survey (SWS) cohort, using pyrosequencing. The mean methylation ±SD for each CpG loci in umbilical cord and umbilical cord blood are shown in table 7.1. Subjects were not matched for the umbilical cord and umbilical cord blood samples.

Table 7.1- The mean methylation of SIRT1 and PGC1α in umbilical cord and umbilical cord blood

CpG loci	Mean methylation (%)	Standard deviation
SIRT1 Umbilical Cord		
-880	62	5.25
-865	58	4.86
SIRT1 Umbilical Cord Blood		
-880	61	10.51
-865	51	10.32
-760	14	3.93
-742	75	6.60
-722	27	6.24
-689	60	6.15
-668	36	5.40
PGC1α Umbilical Cord		
-841	20	7.78
-816	17	5.27
-783	16	4.84
-521	5	2.66
-515	12	6.31
PGC1α Umbilical Cord Blood		
-841	77	7.20
-816	62	5.70
-783	51	7.01
-652	34	5.28
-617	39	5.58
-521	19	4.10
-515	52	6.68

DXA scans were available for all subjects at birth, 4 years and 6 years. Skinfold thickness measurements were also available for these subjects at birth, 6 months, 12 months, 2 years, 3 years and 6 years. To determine if the methylation of specific CpG loci in SIRT1 and PGC1α at birth was associated with various measurements of adiposity, a continuous linear regression was used. Where applicable the data was adjusted for gestational age, sex and age when the measurement was taken.

The output from the linear regression are shown in tables 7.1 to 7.20 where significant p values (≤0.050) are highlighted in yellow and p values showing a trend toward significance (0.051-0.099) are highlighted in green. The beta value (b) shows the direction of the association.

The methylation of specific CpG loci in the promoter of SIRT1 and PGC1α was measured in the abdominal subcutaneous adipose tissue from 23 lean and 42 obese subjects, grouped according to BMI, using pyrosequencing. Subcutaneous abdominal adipose tissue biopsies were taken from men and women enrolled on the BIOCLAIMS (Biomarkers of robustness of metabolic homeostasis for nutrigenomics-derived health claims made on food) study, set up to discover biomarkers allowing for the assessment of health benefits from certain food components (subject characteristics summarized in table 2.13). A power calculation was used to determine that 40 subjects were required for each group at the start of the BIOCLAIMS study. Because the number of subjects in the lean group is less than 40, data may be underpowered to detect any significant differences between these groups.

To determine if there was a significant difference between the methylation of specific CpG loci in the SIRT1 and PGC1α promoter in the adipose tissue of lean and obese subjects, a Mann Whitney U test was used. Subjects were split into lean or obese groups according to BMI, % fat, waist circumference and waist to hip ratio. A binary logistic regression was used to determine if there was a significant difference between the methylation of each CpG analysed in the lean and obese groups, adjusted for sex and age.

SIRT1 and PGC1α mRNA expression was measured in the adipose tissue of lean and obese subjects, using real-time PCR. To determine if there was a significant difference between SIRT1 and PGC1α mRNA expression in the adipose tissue of lean and obese subjects, an unpaired t-test was used. A binary logistic regression was used to determine if there was a significant difference between the mRNA expression of SIRT1 and PGC1α in the lean and obese groups, adjusted for sex and age.

7.3 Results

7.3.1 Is the methylation of SIRT1 and PGC1α measured in fetal tissues associated with childhood adiposity?

7.3.1.1 SIRT1 methylation in umbilical cord

Table7.2- The association between methylation of SIRT1 in SWS umbilical cord DNA and birth weight

	Birthweight (g), adjusted for sex and gestational age		
	n	**b**	**p-value**
SIRT1 CpG -880	336	-8.704	0.064
SIRT1 CpG -865	334	-12.106	0.019

Table 7.2 shows that the methylation of both CpG loci measured in the SIRT1 promoter in umbilical cord shows either a trend or was significantly associated with birth weight after adjusting for sex and gestational age, where the methylation was negatively associated with birth weight.

Table7.3- The association between methylation of SIRT1 in SWS umbilical cord DNA and fat mass in childhood

	Baby DXA: Total fat (kg), adjusted for gestational age, sex and age (log transformed)			Baby DXA: Percentage fat, adjusted for gestational age, sex and age (log transformed)			4 yr DXA: Total fat (kg), without heads, adjusted for sex (log transformed)			4 yr DXA: Percentage fat, without heads, adjusted for sex			6 yr DXA: Total fat (kg), without heads, adjusted for sex and age (log transformed)			6 yr DXA: Percentage fat, without heads, adjusted for sex and age (log transformed)		
	n	b	p-value	n	b	p-value	n	b	p-value	n	b	p-value	n	b	p-value	n	b	p-value
SIRT1 CpG -880	163	-0.003	0.273	163	-0.002	0.365	149	-0.001	0.744	149	-0.084	0.271	201	-0.005	0.041	200	-0.003	0.048
SIRT1 CpG -865	162	-0.002	0.414	162	-0.001	0.649	148	-0.005	0.011	148	-0.191	0.041	199	-0.006	0.021	198	-0.003	0.053

Table 7.3 shows that the methylation of CpG -880 and -865 in SIRT1 in umbilical cord was negatively associated with total and percentage fat at 6 years and also at 4 years for CpG -865. There was no association between the methylation of either CpG loci in SIRT1 and fat mass at birth (p>0.050).

Table7.4- The association between methylation of SIRT1 in SWS umbilical cord DNA and lean mass in childhood

	Baby DXA: Total lean (kg), adjusted for gestational age, sex and age			Baby DXA: Percentage lean, adjusted for gestational age, sex and age			4 yr DXA: Total lean (kg), without heads, adjusted for sex			4 yr DXA: Percentage lean, without heads, adjusted for sex			6 yr DXA: Total lean (kg), without heads, adjusted for sex and age (log transformed)			6 yr DXA: Percentage lean, without heads, adjusted for sex and age		
	n	b	p-value	n	b	p-value	n	b	p-value	n	b	p-value	n	b	p-value	n	b	p-value
SIRT1 CpG -880	163	-0.009	0.050	163	0.059	0.365	149	0.025	0.214	149	0.082	0.278	200	-0.001	0.289	200	0.136	0.083
SIRT1 CpG -865	162	-0.010	0.025	162	0.016	0.819	148	-0.034	0.166	148	0.183	0.047	198	-0.002	0.103	198	0.163	0.046

Table 7.4 shows that the methylation of CpG -880 and -865 in SIRT1 in umbilical cord was negatively associated with total lean mass at birth. The methylation of CpG -865 was positively associated with percentage lean mass at 4 and 6 years.

Table7.5- The association between methylation of SIRT1 in SWS umbilical cord DNA and bone mass in childhood

	Baby DXA: Total Prentice BMD (kg), adjusted for gestational age, sex and age			Baby DXA: Total BMC (kg), adjusted for gestational age, sex and age			Baby DXA: Percentage BMC, adjusted for gestational age, sex and age			4 yr DXA: Total Prentice BMD (kg), without heads, adjusted for sex			4 yr DXA: Total BMC (kg), without heads, adjusted for sex			4 yr DXA: Percentage BMC, without heads, adjusted for sex		
	n	b	p-value	n	b	p-value	n	b	p-value	n	b	p-value	n	b	p-value	n	b	p-value
SIRT1 CpG -880	162	8.00E-06	0.833	163	-1.82E-04	0.398	163	0.002	0.656	175	3.46E-04	0.170	175	0.001	0.105	149	0.003	0.379
SIRT1 CpG -865	161	-5.00E-06	0.892	162	-2.53E-04	0.258	162	-0.003	0.532	174	-1.58E-04	0.589	174	-0.001	0.385	148	0.008	0.033

	6 yr DXA: Total Prentice BMD (kg), without heads, adjusted for sex and age			6 yr DXA: Total BMC (kg), without heads, adjusted for sex and age			6 yr DXA: Percentage BMC, without heads, adjusted for sex and age		
	n	b	p-value	n	b	p-value	n	b	p-value
SIRT1 CpG -880	198	8.00E-05	0.827	202	-0.001	0.366	200	0.006	0.127
SIRT1 CpG -865	196	-3.14E-04	0.430	200	-0.002	0.054	198	0.006	0.105

Table 7.5 shows that the methylation of CpG -865 in SIRT1 in umbilical cord was positively associated with percentage bone mineral content (BMC) at 4 years, and showed a trend toward significance with total BMC at 6 years, however the association was negative. The methylation of CpG -880 was not associated with any measures of bone mass at any age (p>0.050).

Table7.6- The association between methylation of SIRT1 in SWS umbilical cord DNA and skinfold thickness in childhood

	Subscapular skinfold (mm) at birth, adjusted for gestational age, sex and age (ln transformed)			Triceps skinfold (mm) at birth, adjusted for gestational age, sex and age (ln transformed)			Subscapular skinfold (mm) at 6 months, adjusted for sex and age (ln transformed)			Triceps skinfold (mm) at 6 months, adjsuted for sex and age			Subscapular skinfold (mm) at 12 months, adjusted for sex and age (ln transformed)			Triceps skinfold (mm) at 12 months, adjusted for sex and age		
	n	b	p-value	n	b	p-value	n	b	p-value	n	b	p-value	n	b	p-value	n	b	p-value
SIRT1 CpG -880	333	-0.005	0.031	334	-0.002	0.350	291	0.003	0.325	292	0.037	0.140	289	-4.45E-04	0.867	286	-0.012	0.652
SIRT1 CpG -865	331	-0.007	0.004	332	-0.003	0.188	290	-0.003	0.389	291	0.005	0.858	287	-0.004	0.156	285	-0.064	0.028

	Subscapular skinfold (mm) at 2 years, adjusted for sex and age (inverse transformed)			Triceps skinfold (mm) at 2 years, adjusted for sex and age			Subscapular skinfold (mm) at 3 years, adjusted for sex and age (inverse transformed)			Triceps skinfold (mm) at 3 years, adjusted for sex and age (ln transformed)			Subscapular skinfold (mm) at 6 years, adjusted for sex and age (inverse transformed)			Triceps skinfold (mm) at 6 years, adjusted for sex and age (inverse transformed)		
	n	b	p-value	n	b	p-value	n	b	p-value	n	b	p-value	n	b	p-value	n	b	p-value
SIRT1 CpG -880	295	8.80E-05	0.836	291	0.040	0.089	154	1.37E-04	0.827	155	-0.003	0.459	44	0.003	0.113	44	0.002	0.284
SIRT1 CpG -865	293	0.001	0.026	289	-0.038	0.156	153	2.78E-04	0.696	154	-0.006	0.127	43	-1.35E-04	0.942	43	-0.001	0.515

Table 7.6 shows that the methylation of CpG -880 and -865 in SIRT1 in umbilical cord was negatively associated with subscapular skinfold thickness at birth and also at 2 years for CpG -865, however the association was positive. The methylation of CpG -865 negatively associated with tricep skinfold thickness at 12 months. The methylation of CpG -880 and -865 was not associated with any other measures of skinfold thickness at any other age (p>0.050).

In order to visually demonstrate some of the significant associations shown in tables 7.2-7.5, % methylation of CpG -880 in SIRT1 in umbilical cord was split into quartiles and plotted against various DXA and skinfold thickness measurements at several ages. A single CpG locus was chosen to demonstrate the relationship between methylation and the different measures of adiposity. Where applicable, DXA measurements are pre-adjusted for gestational age, sex, and age when the measurement was taken. P-values and N were calculated in the continuous linear regression model. These graphs do not represent categorical or grouped analysis.

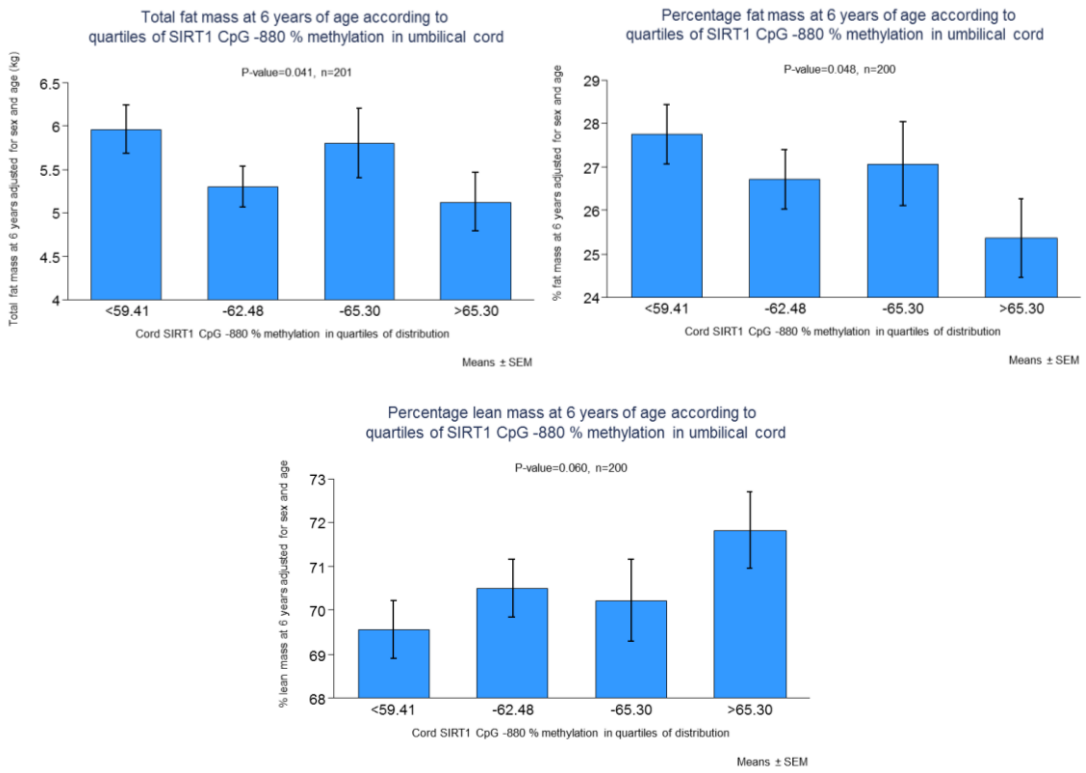


Figure 7.1- Various measures of adiposity during childhood according to quartiles of SIRT1 CpG -880 methylation measured in SWS umbilical cord. The methylation of CpG -880 in SIRT1 is shown split into quartiles and plotted against total fat mass, % fat mass and % lean mass at 6 years. As the methylation increases, total fat mass and percentage fat mass decrease at 6 years of age, whereas percentage lean mass increases ($p<0.060$). Despite the middle quartile of methylation (65.30%) not fitting this pattern, the overall trend is an increase in methylation is associated with a decrease in total fat mass and percentage fat mass, and an increase in percentage lean mass. This is also shown by a negative beta value for total fat mass and percentage fat mass at 6 years, and a positive beta value for percentage lean mass at 6 years in tables 7.3-7.5.

7.3.1.2 SIRT1 methylation in umbilical cord blood

Table7.7- The association between methylation of SIRT1 in SWS umbilical cord blood DNA and birth weight

	Birthweight (g), adjusted for sex and gestational age		
	n	**b**	**p-value**
SIRT1 CpG -880	287	3.031	0.162
SIRT1 CpG -865	285	2.380	0.275
SIRT1 CpG -760	256	4.077	0.515
SIRT1 CpG -742	248	7.312	0.049
SIRT1 CpG -722	234	5.341	0.189
SIRT1 CpG -689	287	6.767	0.071
SIRT1 CpG -668	279	2.376	0.588

Table 7.7 shows that the methylation of CpG -742 in the SIRT1 promoter in umbilical cord blood was significantly associated with birth weight (p=0.049), and the methylation of CpG -689 showed a trend toward significance (p=0.071), after adjusting for sex and gestational age, where the methylation was positively associated with birth weight. There was no significant association between the methylation of any of the remaining CpG loci measured in SIRT1 and birth weight in umbilical cord blood (p>0.050).

Table7.8- The association between methylation of SIRT1 in SWS umbilical cord blood DNA and fat mass in childhood

	Baby DXA: Total fat (kg), adjusted for gestational age, sex and age (log transformed)			Baby DXA: Percentage fat, adjusted for gestational age, sex and age (log transformed)			4 yr DXA: Total fat (kg), without heads, adjusted for sex (log transformed)			4 yr DXA: Percentage fat, without heads, adjusted for sex (log transformed)			6 yr DXA: Total fat (kg), without heads, adjusted for sex and age (log transformed)			6 yr DXA: Percentage fat, without heads, adjusted for sex and age (log transformed)		
	n	b	p-value	n	b	p-value	n	b	p-value	n	b	p-value	n	b	p-value	n	b	p-value
SIRT1 CpG -880	121	0.001	0.520	121	3.91E-04	0.691	234	0.002	0.030	234	0.001	0.205	221	0.001	0.192	221	3.07E-04	0.605
SIRT1 CpG -865	120	0.001	0.694	120	1.51E-04	0.881	232	0.002	0.029	232	0.001	0.132	219	0.001	0.121	219	0.001	0.375
SIRT1 CpG -760	111	3.66E-04	0.922	111	-0.001	0.822	210	0.004	0.035	210	0.002	0.271	192	0.003	0.209	192	0.001	0.723
SIRT1 CpG -742	107	0.003	0.253	107	0.002	0.350	202	0.003	0.024	202	0.001	0.236	187	0.002	0.267	187	3.96E-04	0.695
SIRT1 CpG -722	102	0.001	0.638	102	6.22E-05	0.973	192	0.002	0.105	192	0.001	0.458	177	0.001	0.701	177	4.37E-04	0.681
SIRT1 CpG -689	124	0.003	0.230	124	0.001	0.386	235	0.002	0.059	235	0.001	0.209	222	0.001	0.364	222	7.38E-05	0.939
SIRT1 CpG -668	121	0.002	0.349	121	0.002	0.414	230	0.003	0.048	230	0.001	0.229	218	0.001	0.473	218	1.32E-04	0.907

Table 7.8 shows that the methylation of all CpG loci in SIRT1 in umbilical cord blood was positively associated with total fat at 4 years, apart from CpG -722 ($p>0.050$) and a weaker association with CpG -689 ($p=0.059$). The methylation of each CpG loci in SIRT1 was not associated with any other measures of fat mass at any other age ($p>0.050$).

Table7.9- The association between methylation of SIRT1 in SWS umbilical cord blood DNA and lean mass in childhood

	Baby DXA: Total lean (kg), adjusted for gestational age, sex and age			Baby DXA: Percentage lean, adjusted for gestational age, sex and age			4 yr DXA: Total lean (kg), without heads, adjusted for sex			4 yr DXA: Percentage lean, without heads, adjusted for sex			6 yr DXA: Total lean (kg), without heads, adjusted for sex and age (log transformed)			6 yr DXA: Percentage lean, without heads, adjusted for sex and age		
	n	b	p-value	n	b	p-value	n	b	p-value	n	b	p-value	n	b	p-value	n	b	p-value
SIRT1 CpG -880	121	0.003	0.172	121	-0.017	0.642	234	0.014	0.086	234	-0.034	0.299	221	0.001	0.021	221	-0.011	0.757
SIRT1 CpG -865	120	0.003	0.259	120	-0.005	0.884	232	0.011	0.194	232	-0.042	0.205	219	0.001	0.041	219	-0.022	0.534
SIRT1 CpG -760	111	0.007	0.236	111	0.016	0.873	210	0.043	0.061	210	-0.094	0.295	192	0.003	0.010	192	-0.027	0.781
SIRT1 CpG -742	107	0.006	0.154	107	-0.052	0.395	202	0.026	0.080	202	-0.066	0.265	187	0.001	0.054	187	-0.016	0.783
SIRT1 CpG -722	102	0.008	0.053	102	-0.007	0.919	192	0.027	0.082	192	-0.034	0.567	177	0.002	0.032	177	0.032	0.614
SIRT1 CpG -689	124	0.006	0.118	124	-0.057	0.338	235	0.016	0.256	235	-0.060	0.282	222	0.001	0.330	222	-0.004	0.949
SIRT1 CpG -668	121	0.004	0.393	121	-0.059	0.393	230	0.026	0.109	230	-0.066	0.297	218	0.001	0.078	218	-0.009	0.889

Table 7.9 shows that the methylation of CpG -880, -760, -742 and -722 in SIRT1 in umbilical cord blood showed a trend toward significance with total lean mass at 4 years, and also with total lean at birth for CpG -722, where the association was positive. The methylation of each CpG loci in SIRT1 in umbilical cord blood showed a trend toward significance or was positively associated with total lean mass at 6 years, apart from CpG -689. The methylation of each CpG loci in SIRT1 was not associated with any other measures of lean mass at any other age ($p>0.050$).

Table7.10- The association between methylation of SIRT1 in SWS umbilical cord blood DNA and bone mass in childhood

	Baby DXA: Total Prentice BMD (kg), adjusted for gestational age, sex and age			Baby DXA: Total BMC (kg), adjusted for gestational age, sex and age (log transformed)			Baby DXA: Percentage BMC, adjusted for gestational age, sex and age (log transformed)			4 yr DXA: Total Prentice BMD (kg), without heads, adjusted for sex			4 yr DXA: Total BMC (kg), without heads, adjusted for sex (log transformed)			4 yr DXA: Percentage BMC, without heads, adjusted for sex		
	n	b	p-value	n	b	p-value	n	b	p-value	n	b	p-value	n	b	p-value	n	b	p-value
SIRT1 CpG -880	121	2.80E-05	0.254	121	0.001	0.146	121	0.001	0.184	277	7.50E-05	0.452	279	0.000	0.137	234	-0.003	0.041
SIRT1 CpG -865	120	2.30E-05	0.372	120	0.001	0.278	120	0.001	0.315	275	5.50E-05	0.595	277	0.00031	0.311	232	-0.004	0.012
SIRT1 CpG -760	111	-1.50E-05	0.811	111	0.003	0.194	111	0.002	0.159	246	2.33E-04	0.405	248	0.002	0.038	210	-0.006	0.126
SIRT1 CpG -742	107	-2.20E-05	0.577	107	0.002	0.079	107	0.001	0.159	238	9.50E-05	0.595	240	0.001	0.026	202	-0.004	0.174
SIRT1 CpG -722	102	1.70E-05	0.677	102	0.003	0.043	102	0.002	0.068	226	2.38E-04	0.200	228	0.001	0.044	192	-0.002	0.468
SIRT1 CpG -689	124	3.00E-06	0.948	124	0.002	0.064	124	0.001	0.149	277	1.26E-04	0.449	279	0.001	0.043	235	-0.001	0.639
SIRT1 CpG -668	121	-1.60E-05	0.737	121	0.003	0.071	121	0.002	0.047	270	2.79E-04	0.142	272	0.001	0.119	230	-0.003	0.281

	6 yr DXA: Total Prentice BMD (kg), without heads, adjusted for sex and age			6 yr DXA: Total BMC (kg), without heads, adjusted for sex and age (log transformed)			6 yr DXA: Percentage BMC, without heads, adjusted for sex and age		
	n	b	p-value	n	b	p-value	n	b	p-value
SIRT1 CpG -880	226	8.00E-05	0.607	229	0.001	0.041	221	-0.001	0.536
SIRT1 CpG -865	224	2.60E-05	0.868	227	0.001	0.091	219	-0.002	0.317
SIRT1 CpG -760	196	1.77E-04	0.676	199	0.002	0.045	192	-0.006	0.256
SIRT1 CpG -742	192	-5.70E-05	0.826	194	0.001	0.069	187	-0.002	0.512
SIRT1 CpG -722	182	1.29E-04	0.643	184	0.001	0.026	177	0.001	0.766
SIRT1 CpG -689	227	4.60E-05	0.855	230	0.001	0.037	222	-0.001	0.825
SIRT1 CpG -668	223	4.66E-04	0.107	226	0.001	0.082	218	-0.001	0.875

Table 7.10 shows that the methylation of CpG -880 and -865 in SIRT1 in umbilical cord was negatively associated with percentage bone mineral content (BMC) at 4 years and also at birth for CpG -668. The methylation of each CpG loci in SIRT1 was not associated with percentage BMC at 6 years ($p>0.050$).

The methylation of CpGs -742, -722, -689 and -668 in SIRT1 weakly associated with total BMC at birth, where the association was positive. The methylation of CpGs -760, -742, -722 and -689 positively associated with total BMC at 4 years. By 6 years of age, all CpG loci in SIRT1 positively associated with or showed a trend toward significance with total BMC ($p<0.091$). The methylation of each CpG loci in SIRT1 was not associated with total bone mineral density (BMD) at any age ($p>0.050$).

Table7.11- The association between methylation of SIRT1 in SWS umbilical cord blood DNA and skinfold thickness in childhood

	Subscapular skinfold (mm) at birth, adjusted for gestational age, sex and age (log transformed)			Triceps skinfold (mm) at birth, adjusted for gestational age, sex and age			Subscapular skinfold (mm) at 6 months, adjusted for age and sex (log transformed)			Triceps skinfold (mm) at 6 months, adjsuted for age and sex			Subscapular skinfold (mm) at 12 months, adjusted for sex and age (log transformed)			Triceps skinfold (mm) at 12 months, adjusted for sex and age		
	n	b	p-value	n	b	p-value	n	b	p-value	n	b	p-value	n	b	p-value	n	b	p-value
SIRT1 CpG -880	262	3.12E-04	0.544	263	0.009	0.067	263	0.001	0.053	263	0.002	0.902	261	0.001	0.027	260	0.032	0.027
SIRT1 CpG -865	260	2.56E-04	0.615	261	0.008	0.104	261	0.001	0.149	261	-0.009	0.492	259	0.001	0.135	258	0.024	0.102
SIRT1 CpG -760	231	1.10E-05	0.994	232	0.015	0.270	233	0.002	0.189	233	-0.024	0.502	230	0.003	0.051	228	0.062	0.133
SIRT1 CpG -742	224	4.33E-04	0.617	225	0.015	0.059	226	0.001	0.134	226	-0.015	0.495	223	0.002	0.063	221	0.040	0.114
SIRT1 CpG -722	210	3.83E-04	0.676	211	0.009	0.303	212	0.001	0.196	212	-0.031	0.188	209	0.001	0.183	207	0.041	0.139
SIRT1 CpG -689	262	0.001	0.511	263	0.017	0.044	263	0.001	0.123	263	0.002	0.936	261	0.001	0.184	259	0.042	0.090
SIRT1 CpG -668	255	-1.31E-04	0.896	256	0.008	0.392	256	0.002	0.044	256	-0.023	0.345	254	0.002	0.079	252	0.027	0.345

	Subscapular skinfold (mm) at 2 years, adjusted for sex and age (log transformed)			Triceps skinfold (mm) at 2 years, adjusted for sex and age (log transformed)			Subscapular skinfold (mm) at 3 years, adjusted for sex and age (log transformed)			Triceps skinfold (mm) at 3 years, adjusted for sex and age (log transformed)			Subscapular skinfold (mm) at 6 years, adjusted for sex and age (log transformed)			Triceps skinfold (mm) at 6 years, adjusted for sex and age (log transformed)		
	n	b	p-value	n	b	p-value	n	b	p-value	n	b	p-value	n	b	p-value	n	b	p-value
SIRT1 CpG -880	243	6.10E-05	0.916	243	0.001	0.076	251	0.001	0.044	255	0.001	0.060	247	0.001	0.282	251	0.001	0.126
SIRT1 CpG -865	241	-8.10E-05	0.894	241	0.001	0.055	249	0.001	0.069	253	0.001	0.024	245	0.001	0.193	249	0.001	0.049
SIRT1 CpG -760	213	0.001	0.553	213	0.001	0.589	221	0.003	0.126	225	0.003	0.097	218	0.001	0.598	221	0.002	0.338
SIRT1 CpG -742	207	2.35E-04	0.824	207	0.001	0.338	215	0.001	0.451	219	4.01E-04	0.686	212	0.002	0.194	215	0.002	0.227
SIRT1 CpG -722	193	-0.001	0.474	194	-1.71E-04	0.879	201	0.001	0.421	205	0.001	0.448	198	0.001	0.497	201	-1.09E-04	0.937
SIRT1 CpG -689	244	-3.29E-04	0.740	243	0.001	0.273	252	0.001	0.256	256	0.001	0.289	247	2.75E-04	0.849	251	2.59E-04	0.836
SIRT1 CpG -668	237	-3.60E-04	0.747	236	2.22E-04	0.848	246	0.001	0.217	249	0.001	0.379	240	4.39E-04	0.788	244	0.001	0.721

Table 7.11 shows that the methylation of CpG -880 in SIRT1 in umbilical cord blood showed a trend toward significance or was positively associated with tricep skinfold thickness at birth, 12 months, 2 years and 3 years. The methylation of CpG -880 in SIRT1 also showed a trend toward significance or was positively associated with subscapular skinfold thickness at 6 months, 12 months and 3 years. The methylation of CpG -865 in SIRT1 was positively associated with tricep skinfold thickness at 3 and 6 years. The methylation of CpG -689 was positively associated with tricep skinfold thickness at birth and a weak association at 12 months. The methylation of CpG -668 was positively associated with subscapular skinfold thickness at 6 months and a weak association at 12 months. The methylation of CpG -722 was not associated with any measures of skinfold thickness at any age ($p>0.050$), whilst CpGs -760 and -742 weakly associated with some measures of skinfold thickness ($p<0.100$).

In order to visually demonstrate some of the significant associations shown in tables 7.8-7.11, % methylation of CpG -880 in SIRT1 in umbilical cord blood was split into quartiles and plotted against various DXA and skinfold thickness measurements at several ages. Where applicable, DXA measurements are pre-adjusted for gestational age, sex, and age when the measurement was taken. Where applicable, p-values for skinfold thickness measurements have been adjusted for gestational age, sex, and age when the measurement was taken, however the actual measurements were not pre-adjusted. P-values and N were calculated in the continuous linear regression model. These graphs do not represent categorical or grouped analysis.

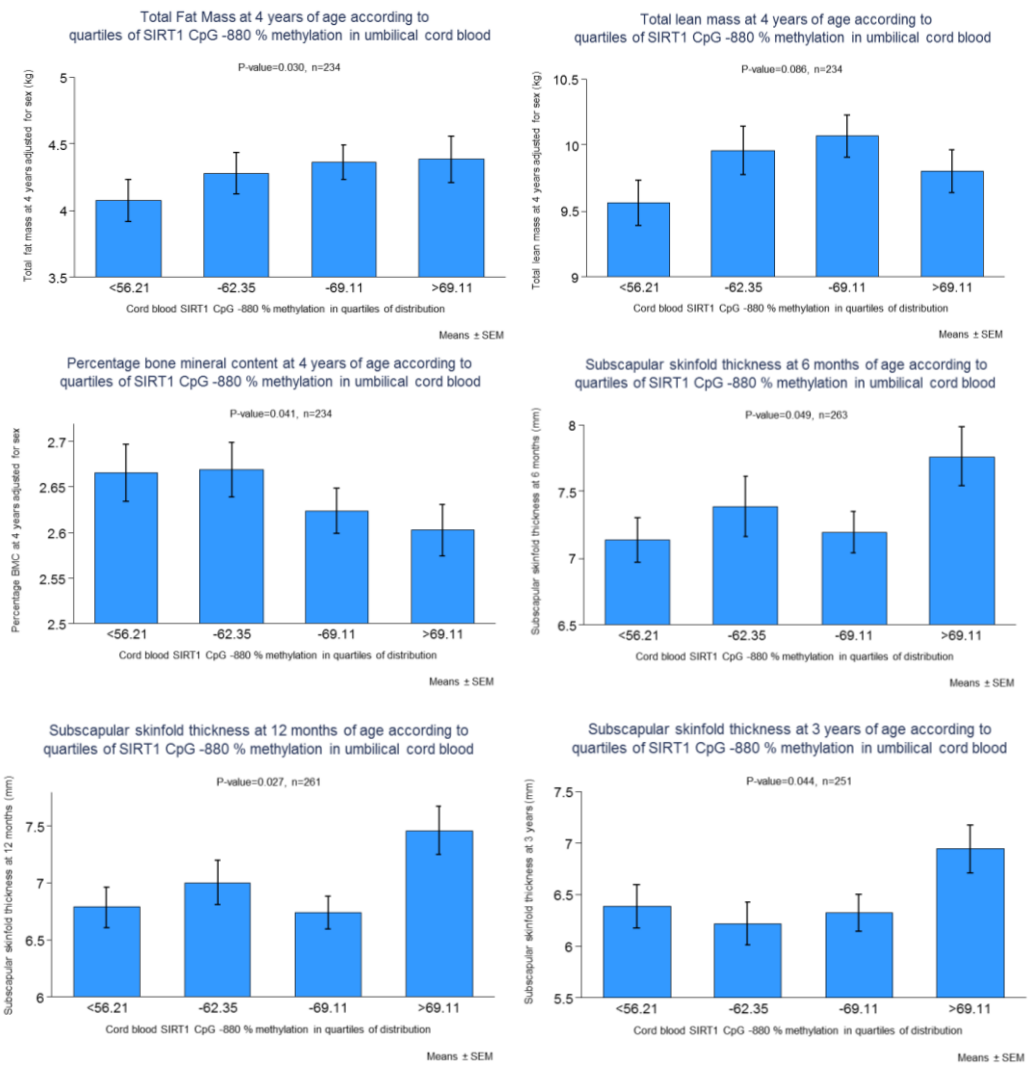


Figure 7.2- Various measures of adiposity during childhood according to quartiles of SIRT1 CpG -880 methylation measured in SWS umbilical cord blood. The methylation of CpG -880 in SIRT1 is shown split into quartiles and plotted against various DXA and skinfold thickness measurements at several ages. As the methylation increases, total fat mass and total lean mass increase at 4 years, whereas percentage bone mineral content decreases ($p<0.090$). Despite the upper quartile of methylation (>69.11%) being plotted against a lower total lean mass at 4 years than the previous quartile of methylation, the overall trend is an increase in methylation is associated with an increase in lean mass. As the methylation of CpG -880 increases, subscapular skinfold thickness at 6 months, 12 months and 3 years also increases ($p<0.05$). Despite the middle quartiles of methylation (62.35% and 69.11%) not always fitting this pattern, the overall trend is an increase in methylation is associated with an increase in subscapular skinfold thickness at these ages. This is also shown by the beta values in tables 7.8-7.11.

7.3.1.3 PGC1α methylation in umbilical cord

Table7.12- The association between methylation of PGC1α in SWS umbilical cord DNA and birth weight

	Birthweight (g), adjusted for sex and gestational age		
	n	**b**	**p-value**
PGC1α CpG -841 Log Transformed	417	92.695	0.559
PGC1α CpG -816 Log Transformed	415	265.009	0.164
PGC1α CpG -783 Log Transformed	420	18.210	0.921
PGC1α CpG -521 Log Transformed	209	222.908	0.190
PGC1α CpG -515 Log Transformed	210	401.988	0.012

Table 7.12 shows that the methylation of CpG -515 in the PGC1α promoter in umbilical cord significantly associated with birth weight after adjusting for sex and gestational age, where an increase in methylation was associated with an increase in birth weight. There was no significant association between the methylation of the remaining CpG loci measured in PGC1α and birth weight in umbilical cord ($p>0.050$).

Table7.13- The association between methylation of PGC1α in SWS umbilical cord DNA and fat mass in childhood

	Baby DXA: Total fat (kg), adjusted for gestational age, sex and age (log transformed)			Baby DXA: Percentage fat, adjusted for gestational age, sex and age (log transformed)			4 yr DXA: Total fat (kg), without heads, adjusted for sex (log transformed)			4 yr DXA: Percentage fat, without heads, adjusted for sex			6 yr DXA: Total fat (kg), without heads, adjusted for sex and age (log transformed)			6 yr DXA: Percentage fat, without heads, adjusted for sex and age (log transformed)		
	n	b	p-value	n	b	p-value	n	b	p-value	n	b	p-value	n	b	p-value	n	b	p-value
PGC1α CpG -841 Log Transformed	208	0.077	0.344	208	0.043	0.472	190	0.166	0.005	190	6.161	0.026	256	0.125	0.105	255	0.034	0.452
PGC1α CpG -816 Log Transformed	210	0.107	0.272	210	0.057	0.431	193	0.176	0.011	193	6.081	0.054	255	0.092	0.315	254	0.009	0.869
PGC1α CpG -783 Log Transformed	212	0.005	0.962	212	-0.019	0.801	195	0.155	0.028	195	6.176	0.056	260	-0.013	0.887	259	-0.048	0.371
PGC1α CpG -521 Log Transformed	96	0.110	0.301	96	0.078	0.316	77	0.121	0.097	77	4.451	0.182	123	0.234	0.003	123	0.134	0.004
PGC1α CpG -515 Log Transformed	100	0.167	0.067	100	0.126	0.062	80	0.110	0.092	80	4.205	0.169	126	0.126	0.093	125	0.078	0.089

Table 7.13 shows that the methylation of each CpG loci in PGC1α in umbilical cord showed a trend toward significance or positively associated with total fat at 4 years, and with fat mass at 6 years for CpGs -521 and -515. The methylation of CpGs -841, -816 and -783 showed a trend toward significance or positively associated with percentage fat at 4 years and with percentage mass at 6 years for CpGs -521 and -515. The methylation of CpG -515 in PGC1α also showed a trend toward significance with total fat and percentage fat at birth, which persisted at 4 and 6 years of age.

Table7.14- The association between methylation of PGC1α in SWS umbilical cord DNA and lean mass in childhood

	Baby DXA: Total lean (kg), adjusted for gestational age, sex and age			Baby DXA: Percentage lean, adjusted for gestational age, sex and age			4 yr DXA: Total lean (kg), without heads, adjusted for sex			4 yr DXA: Percentage lean, without heads, adjusted for sex			6 yr DXA: Total lean (kg), without heads, adjusted for sex and age (log transformed)			6 yr DXA: Percentage lean, without heads, adjusted for sex and age		
	n	b	p-value	n	b	p-value	n	b	p-value	n	b	p-value	n	b	p-value	n	b	p-value
PGC1α CpG -841 Log Transformed	208	0.218	0.150	208	-1.670	0.418	190	0.932	0.192	190	-6.088	0.025	255	0.046	0.119	255	-1.758	0.514
PGC1α CpG -816 Log Transformed	210	0.415	0.023	210	-2.194	0.375	193	1.200	0.130	193	-5.917	0.056	254	0.045	0.182	254	-0.507	0.876
PGC1α CpG -783 Log Transformed	212	0.351	0.062	212	0.332	0.897	195	0.697	0.392	195	-6.107	0.054	259	0.025	0.462	259	3.134	0.320
PGC1α CpG -521 Log Transformed	96	0.192	0.339	96	-2.380	0.379	77	0.758	0.348	77	-4.218	0.198	123	0.041	0.135	123	-7.285	0.008
PGC1α CpG -515 Log Transformed	100	0.209	0.222	100	-4.683	0.049	80	0.549	0.452	80	-3.922	0.192	125	0.021	0.466	125	-4.505	0.094

Table 7.14 shows that the methylation of CpG -816 and -783 in PGC1α in umbilical cord showed a trend toward significance or negatively associated with total lean mass at birth. The methylation of CpG -841, -521 and -515 in PGC1α was not associated with total lean mass at any age (p>0.050). The methylation of CpG -515 negatively associated with percentage lean at birth. The methylation of CpGs -841, -816 and -783 showed a trend toward significance or negatively associated with percentage lean mass at 4 years and with percentage lean mass at 6 years for CpGs -521 and -515.

Table7.15- The association between methylation of PGC1α in SWS umbilical cord DNA and bone mass in childhood

	Baby DXA: Total Prentice BMD (kg), adjusted for gestational age, sex and age			Baby DXA: Total BMC (kg), adjusted for gestational age, sex and age			Baby DXA: Percentage BMC, adjusted for gestational age, sex and age			4 yr DXA: Total Prentice BMD (kg), without heads, adjusted for sex			4 yr DXA: Total BMC (kg), without heads, adjusted for sex			4 yr DXA: Percentage BMC, without heads, adjusted for sex		
	n	b	p-value	n	b	p-value	n	b	p-value	n	b	p-value	n	b	p-value	n	b	p-value
PGC1α CpG -841 Log Transformed	207	-4.83E-04	0.718	208	0.007	0.303	208	0.074	0.554	221	0.006	0.505	221	0.047	0.041	190	-0.072	0.495
PGC1α CpG -816 Log Transformed	209	0.001	0.742	210	0.015	0.071	210	0.175	0.253	224	0.005	0.607	224	0.042	0.101	193	-0.164	0.172
PGC1α CpG -783 Log Transformed	211	1.73E-04	0.917	212	-2.00E-05	0.999	212	-0.132	0.407	226	0.005	0.607	226	0.044	0.092	195	-0.069	0.575
PGC1α CpG -521 Log Transformed	95	-0.002	0.309	96	0.008	0.365	96	0.105	0.466	93	0.006	0.546	93	0.005	0.837	77	-0.233	0.050
PGC1α CpG -515 Log Transformed	99	-1.32E-04	0.918	100	0.012	0.091	100	0.100	0.417	98	-0.002	0.861	98	-0.013	0.566	80	-0.284	0.006

	6 yr DXA: Total Prentice BMD (kg), without heads, adjusted for sex and age			6 yr DXA: Total BMC (kg), without heads, adjusted for sex and age			6 yr DXA: Percentage BMC, without heads, adjusted for sex and age		
	n	b	p-value	n	b	p-value	n	b	p-value
PGC1α CpG -841 Log Transformed	254	-0.004	0.768	258	0.028	0.433	255	-0.247	0.054
PGC1α CpG -816 Log Transformed	253	0.005	0.744	257	0.024	0.563	254	-0.245	0.108
PGC1α CpG -783 Log Transformed	258	0.016	0.270	262	0.015	0.719	259	-0.005	0.975
PGC1α CpG -521 Log Transformed	123	-0.013	0.328	123	0.047	0.192	123	-0.294	0.035
PGC1α CpG -515 Log Transformed	125	-0.016	0.235	126	-0.004	0.902	125	-0.342	0.013

Table 7.15 shows that the methylation of CpG -841 in PGC1α in umbilical cord positively associated with total BMC at 4 years, and CpG -783 showed a trend toward significance. The methylation of CpGs -816 and -515 weakly associated with total BMC at birth. There were no significant associations between the methylation of any CpG loci and total BMC at 6 years.

The methylation of CpG -521 and -515 in PGC1α negatively associated with percentage BMC at 4 and 6 years. The methylation of each CpG loci in PGC1α was not associated with total prentice BMD at any age ($p>0.050$).

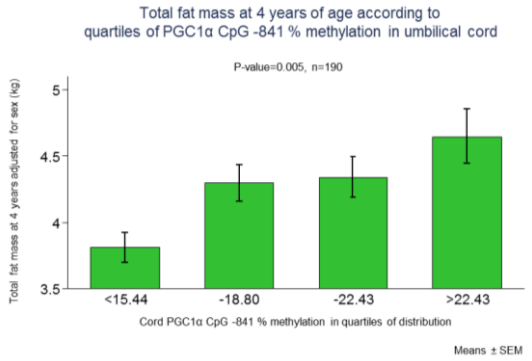
Table7.16- The association between methylation of PGC1α in SWS umbilical cord DNA and skinfold thickness in childhood

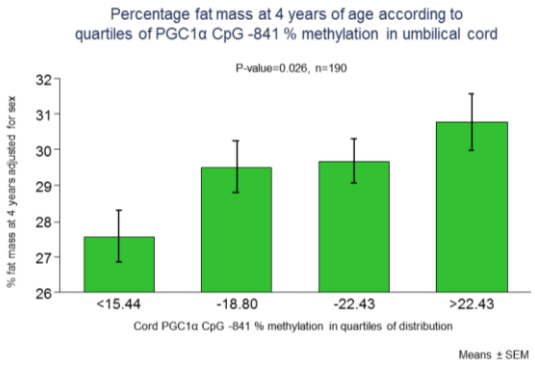
	Subscapular skinfold (mm) at birth, adjusted for gestational age, sex and age (ln transformed)			Triceps skinfold (mm) at birth, adjusted for gestational age, sex and age (ln transformed)			Subscapular skinfold (mm) at 6 months, adjusted for sex and age (ln transformed)			Triceps skinfold (mm) at 6 months, adjsuted for sex and age			Subscapular skinfold (mm) at 12 months, adjusted for sex and age (ln transformed)			Triceps skinfold (mm) at 12 months, adjusted for sex and age		
	n	b	p-value	n	b	p-value	n	b	p-value	n	b	p-value	n	b	p-value	n	b	p-value
PGC1α CpG -841 Log Transformed	414	0.079	0.291	415	0.164	0.022	369	-0.061	0.483	370	2.190	0.010	365	-0.013	0.887	362	0.773	0.388
PGC1α CpG -816 Log Transformed	412	0.108	0.225	413	0.128	0.131	367	-0.060	0.552	368	2.179	0.028	362	0.033	0.754	359	1.232	0.235
PGC1α CpG -783 Log Transformed	417	0.077	0.370	418	0.105	0.198	372	-0.053	0.594	373	1.217	0.213	367	-0.118	0.251	364	0.213	0.834
PGC1α CpG -521 Log Transformed	207	0.109	0.186	208	0.151	0.059	172	0.013	0.886	172	0.528	0.567	171	0.104	0.278	170	-0.059	0.952
PGC1α CpG -515 Log Transformed	208	0.145	0.055	209	0.200	0.008	173	-0.090	0.296	173	0.558	0.515	175	0.102	0.280	174	0.685	0.486

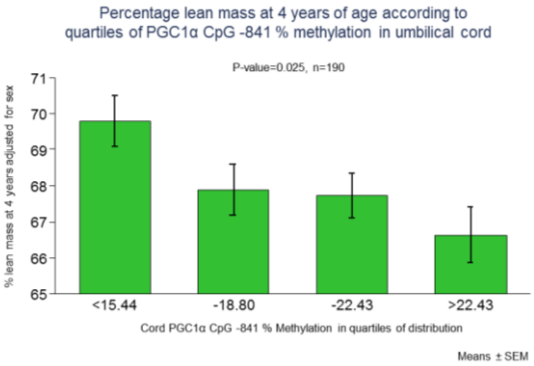
	Subscapular skinfold (mm) at 2 years, adjusted for sex and age (inverse transformed)			Triceps skinfold (mm) at 2 years, adjusted for sex and age			Subscapular skinfold (mm) at 3 years, adjusted for sex and age (inverse transformed)			Triceps skinfold (mm) at 3 years, adjusted for sex and age (ln transformed)			Subscapular skinfold (mm) at 6 years, adjusted for sex and age (inverse transformed)			Triceps skinfold (mm) at 6 years, adjusted for sex and age (inverse transformed)		
	n	b	p-value	n	b	p-value	n	b	p-value	n	b	p-value	n	b	p-value	n	b	p-value
PGC1α CpG -841 Log Transformed	363	-0.019	0.164	360	1.401	0.074	199	-0.040	0.049	203	0.425	0.0003	66	-0.021	0.692	66	-0.059	0.234
PGC1α CpG -816 Log Transformed	361	-0.027	0.096	358	2.665	0.004	201	-0.038	0.102	204	0.407	0.003	64	-0.038	0.484	64	-0.068	0.185
PGC1α CpG -783 Log Transformed	366	-0.008	0.630	363	1.106	0.231	204	-0.030	0.175	207	0.250	0.055	65	0.033	0.591	65	0.042	0.465
PGC1α CpG -521 Log Transformed	176	-0.013	0.358	175	1.252	0.126	70	-0.043	0.044	72	0.225	0.070	36	-0.094	0.006	36	-0.065	0.034
PGC1α CpG -515 Log Transformed	180	-0.013	0.349	180	2.049	0.011	74	-0.051	0.017	75	0.358	0.005	39	-0.067	0.064	39	-0.065	0.043

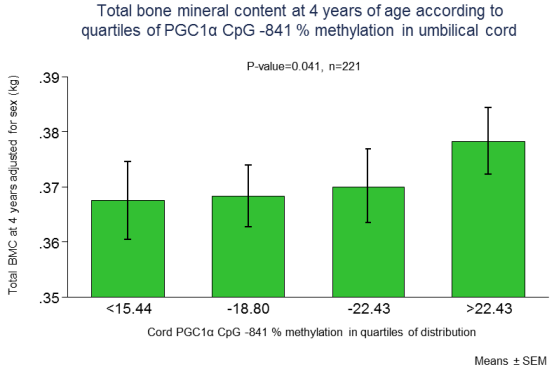
Table 7.16 shows that the methylation of CpG -841 in PGC1α in umbilical cord showed a trend toward significance or positively associated with tricep skinfold thickness at birth, 6 months, 2 years and 3 years, and also negatively associated with subscapular skinfold thickness at 3 years. The methylation of CpG -816 positively associated with tricep skinfold thickness at birth, 2 years and 3 years. The methylation of CpG -783 in PGC1α showed a weak association with tricep skinfold thickness at 3 years only. The methylation of CpGs -521 and -515 showed a trend toward significance or positively associated with tricep skinfold thickness at birth, 2, 3 and 6 years, however the association at 6 years was negative. The methylation of CpG -521 and -515 negatively associated with subscapular skinfold thickness at 3 and 6 years.

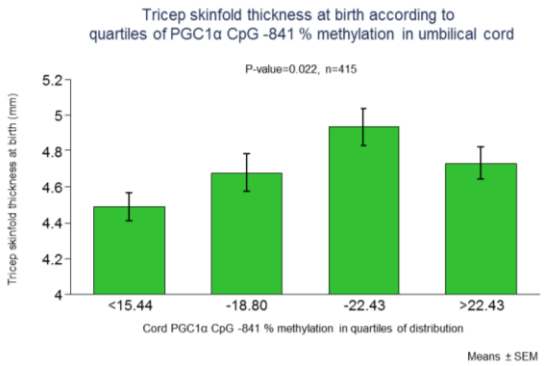
In order to visually demonstrate some of the significant associations shown in tables 7.13-7.16, % methylation of CpG -841 in PGC1α in umbilical cord was split into quartiles and plotted against various DXA and skinfold thickness measurements at several ages. Each measure of adiposity is pre-adjusted as described previously.

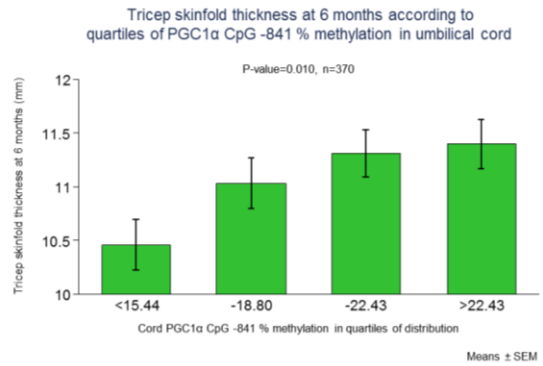

Total fat mass at 4 years of age according to
quartiles of PGC1α CpG -841 % methylation in umbilical cord
P-value=0.005, n=190
Total fat mass at 4 years adjusted for sex (kg)
5
4.5
4
3.5
<15.44
-18.80
-22.43
>22.43
Cord PGC1α CpG -841 % methylation in quartiles of distribution
Means ± SEM


Percentage fat mass at 4 years of age according to
quartiles of PGC1α CpG -841 % methylation in umbilical cord
P-value=0.026, n=190
% fat mass at 4 years adjusted for sex
32
31
30
29
28
27
26
<15.44
-18.80
-22.43
>22.43
Cord PGC1α CpG -841 % methylation in quartiles of distribution
Means ± SEM


Percentage lean mass at 4 years of age according to
quartiles of PGC1α CpG -841 % methylation in umbilical cord
P-value=0.025, n=190
% lean mass at 4 years adjusted for sex
71
70
69
68
67
66
65
<15.44
-18.80
-22.43
>22.43
Cord PGC1α CpG -841 % Methylation in quartiles of distribution
Means ± SEM


Total bone mineral content at 4 years of age according to
quartiles of PGC1α CpG -841 % methylation in umbilical cord
P-value=0.041, n=221
Total BMC at 4 years adjusted for sex (kg)
.39
.38
.37
.36
.35
<15.44
-18.80
-22.43
>22.43
Cord PGC1α CpG -841 % methylation in quartiles of distribution
Means ± SEM


Tricep skinfold thickness at birth according to
quartiles of PGC1α CpG -841 % methylation in umbilical cord
P-value=0.022, n=415
Tricep skinfold thickness at birth (mm)
5.2
5
4.8
4.6
4.4
4.2
4
<15.44
-18.80
-22.43
>22.43
Cord PGC1α CpG -841 % methylation in quartiles of distribution
Means ± SEM


Tricep skinfold thickness at 6 months according to
quartiles of PGC1α CpG -841 % methylation in umbilical cord
P-value=0.010, n=370
Tricep skinfold thickness at 6 months (mm)
12
11.5
11
10.5
10
<15.44
-18.80
-22.43
>22.43
Cord PGC1α CpG -841 % methylation in quartiles of distribution
Means ± SEM

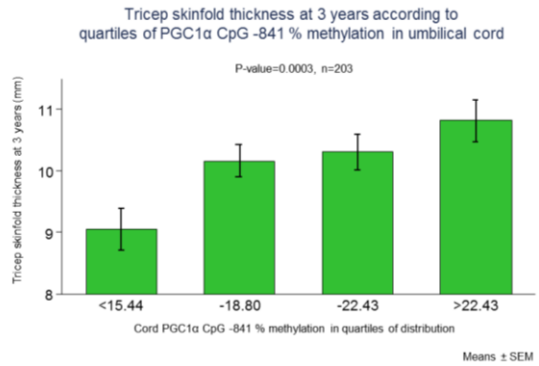

Tricep skinfold thickness at 3 years according to
quartiles of PGC1α CpG -841 % methylation in umbilical cord
P-value=0.0003, n=203
Tricep skinfold thickness at 3 years (mm)
11
10
9
8
<15.44
-18.80
-22.43
>22.43
Cord PGC1α CpG -841 % methylation in quartiles of distribution
Means ± SEM

Figure 7.3- Various measures of adiposity during childhood according to quartiles of PGC1α CpG -841 methylation measured in SWS umbilical cord. The methylation of CpG -841 in PGC1α is shown split into quartiles and plotted against various DXA and skinfold thickness measurements at several ages. As the methylation increases, total fat mass, percentage fat mass and total bone mineral content at 4 years of age all increase, as does tricep skinfold thickness at birth, 6 months and 3 years, whereas % lean mass at 4 years of age decreases ($p<0.050$). Despite the upper quartile of methylation (>22.43%) not fitting this pattern for tricep thickness at birth, the overall trend is an increase in methylation is associated with an increase in tricep thickness. This is also shown by a positive beta value for total fat mass, percentage fat mass and total bone mineral content at 4 years and tricep skinfold thickness at birth, 6 months and 3 years, and a negative beta value for % lean mass at 4 years in tables 7.13-7.16.

7.3.1.4 PGC1α methylation in umbilical cord blood

Table7.17- The association between methylation of PGC1α in SWS umbilical cord blood DNA and birth weight

	Birthweight (g), adjusted for sex and gestational age		
	n	**b**	**p-value**
PGC1α CpG -841	285	-2.247	0.479
PGC1α CpG -816	263	-0.617	0.883
PGC1α CpG -783	269	-2.071	0.531
PGC1α CpG -652	289	-0.034	0.994
PGC1α CpG -617	285	-3.594	0.380
PGC1α CpG -521	272	-2.104	0.716
PGC1α CpG -515	268	0.271	0.939

Table 7.17 shows that the methylation of each CpG loci in the PGC1α promoter in umbilical cord blood was not significantly associated with birth weight ($p>0.050$).

Table7.18- The association between methylation of PGC1α in SWS umbilical cord blood DNA and fat mass in childhood

	Baby DXA: Total fat (kg), adjusted for gestational age, sex and age (log transformed)			Baby DXA: Percentage fat, adjusted for gestational age, sex and age (log transformed)			4 yr DXA: Total fat (kg), without heads, adjusted for sex (log transformed)			4 yr DXA: Percentage fat, without heads, adjusted for sex (log transformed)			6 yr DXA: Total fat (kg), without heads, adjusted for sex and age (log transformed)			6 yr DXA: Percentage fat, without heads, adjusted for sex and age (log transformed)		
	n	b	p-value	n	b	p-value	n	b	p-value	n	b	p-value	n	b	p-value	n	b	p-value
PGC1α CpG -841	122	-0.001	0.635	122	-0.001	0.652	232	4.43E-04	0.658	232	0.001	0.464	219	-1.05E-04	0.938	219	1.06E-04	0.907
PGC1α CpG -816	110	4.11E-04	0.869	110	0.001	0.662	212	0.001	0.319	212	0.002	0.084	202	-2.60E-05	0.988	202	4.74E-04	0.679
PGC1α CpG -783	112	0.001	0.707	112	0.001	0.591	217	-2.50E-04	0.818	217	1.61E-04	0.832	207	-1.00E-04	0.943	207	4.54E-04	0.623
PGC1α CpG -652	122	0.001	0.635	122	0.001	0.541	238	4.57E-04	0.737	238	0.001	0.362	223	-0.001	0.653	223	6.04E-05	0.959
PGC1α CpG -617	119	-3.90E-04	0.875	119	1.72E-04	0.925	234	-2.04E-04	0.875	234	0.001	0.503	219	-0.002	0.222	219	-0.001	0.546
PGC1α CpG -521	113	-0.001	0.805	113	-2.66E-04	0.915	226	4.10E-05	0.982	226	0.001	0.407	208	-0.001	0.546	208	1.03E-04	0.947
PGC1α CpG -515	112	0.002	0.351	112	0.002	0.222	223	-4.57E-05	0.967	223	0.001	0.257	206	-0.001	0.424	206	1.98E-04	0.830

Table 7.18 shows that the methylation of each CpG loci in PGC1α in umbilical cord blood was not significantly associated with any measures of fat mass at any age (p>0.050), apart from a weak positive association between the methylation of CpG -816 and percentage fat at 4 years (p=0.084).

Table7.19- The association between methylation of PGC1α in SWS umbilical cord blood DNA and lean mass in childhood

	Baby DXA: Total lean (kg), adjusted for gestational age, sex and age			Baby DXA: Percentage lean, adjusted for gestational age, sex and age			4 yr DXA: Total lean (kg), without heads, adjusted for sex			4 yr DXA: Percentage lean, without heads, adjusted for sex			6 yr DXA: Total lean (kg), without heads, adjusted for sex and age (log transformed)			6 yr DXA: Percentage lean, without heads, adjusted for sex and age		
	n	b	p-value	n	b	p-value	n	b	p-value	n	b	p-value	n	b	p-value	n	b	p-value
PGC1α CpG -841	122	-0.003	0.421	122	0.054	0.372	232	-0.006	0.595	232	-0.030	0.505	219	-2.86E-04	0.623	219	0.013	0.801
PGC1α CpG -816	110	-0.005	0.259	110	4.74E-04	0.994	212	-0.017	0.256	212	-0.095	0.111	202	-0.001	0.268	202	-0.022	0.741
PGC1α CpG -783	112	-0.004	0.305	112	-0.017	0.763	217	-0.009	0.452	217	-0.008	0.876	207	-0.001	0.319	207	-0.017	0.750
PGC1α CpG -652	122	-0.002	0.707	122	-0.020	0.777	238	-0.014	0.368	238	-0.046	0.456	223	-0.001	0.283	223	0.005	0.939
PGC1α CpG -617	119	-0.004	0.321	119	0.009	0.896	234	-0.021	0.174	234	-0.028	0.634	219	-0.001	0.082	219	0.050	0.453
PGC1α CpG -521	113	-0.001	0.821	113	0.030	0.736	226	-0.031	0.136	226	-0.044	0.595	208	-0.001	0.140	208	0.025	0.782
PGC1α CpG -515	112	-0.003	0.467	112	-0.057	0.325	223	-0.026	0.040	223	-0.046	0.350	206	-0.001	0.111	206	0.032	0.562

Table 7.19 shows that the methylation of CpG -515 in PGC1α in umbilical cord blood negatively associated with total lean mass at 4 years. The methylation of CpG -617 weakly associated with total lean mass at 6 years, where the association was negative. The methylation of the remaining CpG loci in PGC1α was not associated with any other measures of lean mass at any age (p>0.050).

Table7.20- The association between methylation of PGC1α in SWS umbilical cord blood DNA and bone mass in childhood

	Baby DXA: Total Prentice BMD (kg), adjusted for gestational age, sex and age			Baby DXA: Total BMC (kg), adjusted for gestational age, sex and age (log transformed)			Baby DXA: Percentage BMC, adjusted for gestational age, sex and age (log transformed)			4 yr DXA: Total Prentice BMD (kg), without heads, adjusted for sex			4 yr DXA: Total BMC (kg), without heads, adjusted for sex (log transformed)			4 yr DXA: Percentage BMC, without heads, adjusted for sex		
	n	b	p-value	n	b	p-value	n	b	p-value	n	b	p-value	n	b	p-value	n	b	p-value
PGC1α CpG -841	122	4.30E-05	0.295	122	-0.002	0.058	122	-0.002	0.027	274	-1.50E-04	0.297	276	-2.96E-04	0.495	232	3.87E-04	0.846
PGC1α CpG -816	110	-2.90E-05	0.517	110	-0.001	0.571	110	-2.04E-04	0.841	251	-2.49E-04	0.190	253	-1.79E-04	0.752	212	0.001	0.745
PGC1α CpG -783	112	3.70E-05	0.356	112	-0.001	0.681	112	-2.38E-04	0.786	257	-5.10E-05	0.741	259	-1.12E-04	0.808	217	0.003	0.154
PGC1α CpG -652	122	-1.70E-05	0.721	122	1.08E-04	0.942	122	2.19E-04	0.832	278	3.70E-05	0.852	280	-2.40E-05	0.968	238	0.002	0.358
PGC1α CpG -617	119	4.00E-06	0.925	119	1.62E-04	0.908	119	0.001	0.352	274	1.11E-04	0.560	276	-3.23E-04	0.569	234	0.004	0.136
PGC1α CpG -521	113	-4.00E-05	0.497	113	-0.001	0.575	113	-4.71E-04	0.721	263	-3.62E-04	0.164	265	-0.001	0.331	226	-0.001	0.739
PGC1α CpG -515	112	2.00E-06	0.961	112	-0.001	0.540	112	-0.001	0.378	259	-1.65E-04	0.307	261	-0.001	0.101	223	-2.78E-04	0.899

	6 yr DXA: Total Prentice BMD (kg), without heads, adjusted for sex and age			6 yr DXA: Total BMC (kg), without heads, adjusted for sex and age (log transformed)			6 yr DXA: Percentage BMC, without heads, adjusted for sex and age		
	n	b	p-value	n	b	p-value	n	b	p-value
PGC1α CpG -841	225	-3.65E-04	0.119	227	-0.001	0.297	219	-2.44E-04	0.925
PGC1α CpG -816	207	-4.29E-04	0.158	209	-0.001	0.311	202	0.002	0.591
PGC1α CpG -783	212	-1.34E-04	0.581	214	-3.70E-04	0.515	207	0.002	0.356
PGC1α CpG -652	228	-1.45E-04	0.633	231	-0.001	0.412	223	0.004	0.235
PGC1α CpG -617	224	-4.10E-05	0.888	227	-0.001	0.198	219	0.007	0.025
PGC1α CpG -521	214	-3.37E-04	0.413	216	-0.001	0.175	208	0.003	0.546
PGC1α CpG -515	212	-3.45E-04	0.160	214	-0.001	0.042	206	0.002	0.579

Table 7.20 shows that the methylation of CpG -841 in PGC1α in umbilical cord blood negatively associated with percentage BMC at birth, and showed a trend toward significance with total BMC at birth. The methylation of CpG -617 positively associated with percentage BMC at 6 years. The methylation of CpG -515 negatively associated with total BMC at 6 years. The methylation of the remaining CpG loci in PGC1α was not associated with any other measures of bone mass at any other age ($p>0.050$).

Table7.21- The association between methylation of PGC1α in SWS umbilical cord blood DNA and skinfold thickness in childhood

	Subscapular skinfold (mm) at birth, adjusted for gestational age, sex and age (log			Triceps skinfold (mm) at birth, adjusted for gestational age, sex and age			Subscapular skinfold (mm) at 6 months, adjusted for age and sex (log transformed)			Triceps skinfold (mm) at 6 months, adjsuted for age and sex			Subscapular skinfold (mm) at 12 months, adjusted for sex and age (log transformed)			Triceps skinfold (mm) at 12 months, adjusted for sex and age		
	n	b	p-value	n	b	p-value	n	b	p-value	n	b	p-value	n	b	p-value	n	b	p-value
PGC1α CpG -841	259	-0.001	0.188	260	-0.014	0.051	260	-0.001	0.504	260	0.005	0.798	258	-3.43E-04	0.665	257	-0.014	0.502
PGC1α CpG -816	240	-0.001	0.159	240	-0.014	0.160	241	-2.47E-04	0.817	241	-0.008	0.745	238	0.001	0.536	236	0.017	0.546
PGC1α CpG -783	245	-0.001	0.070	245	-0.007	0.372	246	-3.92E-07	1.000	246	-5.90E-05	0.998	243	1.39E-04	0.872	241	-0.008	0.725
PGC1α CpG -652	263	-0.001	0.249	264	-0.014	0.160	264	-0.001	0.232	264	-0.023	0.377	262	-0.001	0.498	260	-0.012	0.676
PGC1α CpG -617	259	-0.002	0.054	260	-0.017	0.090	260	-0.001	0.276	260	-0.034	0.176	258	-0.001	0.268	256	-0.033	0.249
PGC1α CpG -521	249	-0.001	0.664	250	-0.017	0.206	250	-0.001	0.711	250	-0.027	0.427	248	-0.001	0.327	246	-0.016	0.675
PGC1α CpG -515	245	2.20E-05	0.978	246	-0.013	0.109	246	-5.48E-05	0.995	246	-0.014	0.493	244	-1.22E-04	0.889	242	0.004	0.857

	Subscapular skinfold (mm) at 2 years, adjusted for sex and age (log transformed)			Triceps skinfold (mm) at 2 years, adjusted for sex and age (log transformed)			Subscapular skinfold (mm) at 3 years, adjusted for sex and age (log transformed)			Triceps skinfold (mm) at 3 years, adjusted for sex and age (log transformed)			Subscapular skinfold (mm) at 6 years, adjusted for sex and age (log transformed)			Triceps skinfold (mm) at 6 years, adjusted for sex and age (log transformed)		
	n	b	p-value	n	b	p-value	n	b	p-value	n	b	p-value	n	b	p-value	n	b	p-value
PGC1α CpG -841	241	-2.20E-05	0.980	241	-0.001	0.202	247	-0.001	0.488	251	-0.00049	0.570	244	-0.001	0.377	248	-0.001	0.341
PGC1α CpG -816	223	-1.10E-04	0.923	223	-0.001	0.605	228	1.01E-04	0.928	232	-0.001	0.260	226	2.00E-04	0.906	230	-0.002	0.190
PGC1α CpG -783	228	-0.001	0.533	228	-3.94E-04	0.674	233	-3.36E-04	0.717	237	-0.001	0.119	231	-1.11E-04	0.936	235	-0.001	0.340
PGC1α CpG -652	243	-0.001	0.546	243	-2.37E-04	0.848	251	-2.23E-04	0.854	255	-0.001	0.235	247	-1.87E-04	0.913	251	-0.002	0.217
PGC1α CpG -617	239	-0.001	0.390	239	-0.002	0.211	247	-0.001	0.270	251	-0.002	0.193	243	-0.003	0.110	247	-0.003	0.021
PGC1α CpG -521	230	-0.002	0.276	230	-0.001	0.711	239	4.63E-04	0.770	243	-0.001	0.669	234	-0.001	0.769	238	-0.002	0.356
PGC1α CpG -515	226	-4.68E-04	0.617	226	-1.37E-04	0.888	235	-0.001	0.508	239	-1.20E-05	0.990	230	-3.59E-04	0.792	234	9.87E-05	0.934

Table 7.21 shows that the methylation of each CpG loci in PGC1α in umbilical cord blood was not associated with any measures of skinfold thickness at any age ($p>0.050$), apart from a negative association between the methylation of CpG -617 and tricep skinfold thickness at 6 years, and a weak negative association with subscapular and tricep skinfold thickness at birth for CpG -841, -783 and -617.

Because there were very few significant associations between the methylation of PGC1α in umbilical cord blood with DXA and skinfold thickness measurements, there were no suitable plots to visually demonstrate methylation plotted against different measures of adiposity.

7.3.2 Does DNA methylation of SIRT1 and PGC1α differ in the adipose tissue of lean subjects versus obese subjects?

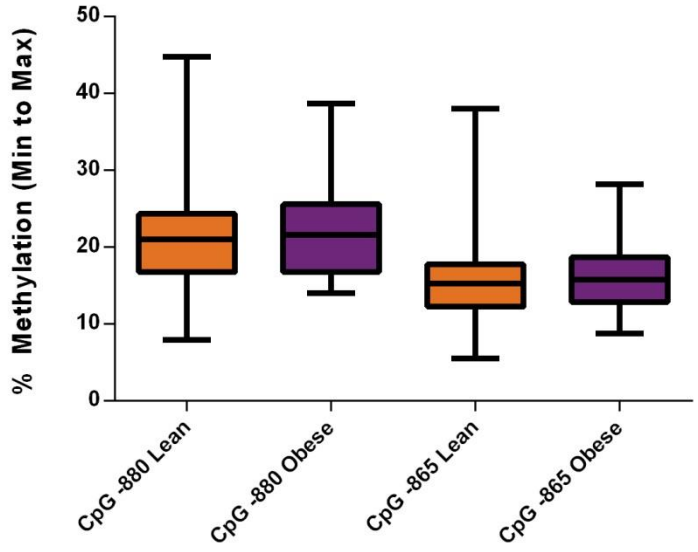


Figure 7.4- The methylation of SIRT1 in the subcutaneous adipose tissue of lean and obese subjects. The methylation of CpG -880 and CpG -865 was not significantly different in the subcutaneous adipose tissue form lean and obese subjects, determined by a Mann Whitney U test ($p>0.050$).

Table 7.22- The ability of SIRT1 methylation in subcutaneous adipose tissue to predict measures of adiposity

BMI (kg/m^2)	Does methylation predict BMI (kg/m^2)? Adjusted for sex and age						
	n	R2 (Naglekerke)	B	Exp (B)	Lower 95% CI	Upper 95% CI	p-value
SIRT1 CpG -880	50	0.182	-0.013	0.987	0.904	1.078	**0.774**
SIRT1 CpG -865	49	0.186	-0.044	0.957	0.862	1.063	**0.410**
% fat	**Does methylation predict % fat? Adjusted for sex and age**						
	n	R2 (Naglekerke)	B	Exp (B)	Lower 95% CI	Upper 95% CI	p-value
SIRT1 CpG -880	48	0.415	0.054	1.056	0.946	1.178	**0.330**
SIRT1 CpG -865	47	0.393	0.027	1.027	0.910	1.160	**0.662**
Waist measurement (cm)	**Does methylation predict waist measurement (cm)? Adjusted for sex and age**						
	n	R2 (Naglekerke)	B	Exp (B)	Lower 95% CI	Upper 95% CI	p-value
SIRT1 CpG -880	50	0.350	0.036	1.036	0.937	1.146	**0.488**
SIRT1 CpG -865	49	0.332	0.017	1.017	0.907	1.140	**0.776**
Waist:hip ratio	**Does methylation predict waist:hip ratio? Adjusted for sex and age**						
	n	R2 (Naglekerke)	B	Exp (B)	Lower 95% CI	Upper 95% CI	p-value
SIRT1 CpG -880	50	0.231	-0.016	0.984	0.900	1.076	**0.724**
SIRT1 CpG -865	49	0.224	-0.034	0.966	0.869	1.074	**0.523**

The criteria used to determine if a subject was lean or obese for each measure of adiposity in adults are as follows. A subject with a BMI of less than 25.0 kg/m^2 was lean and a BMI of more than 30.0 kg/m^2 was obese (211). A male with % fat ≥24% and a female with % fat ≥30% were obese according to the 1995 World Health Organisation (WHO) (211). A male with a waist circumference of ≥94cm and a female with a waist circumference of ≥80cm were obese according to the International Diabetes Federation (212)(213). A male with a waist to hip ratio of ≥0.90 and a female with a WHR of ≥0.85 were obese according to the WHO (213).

Table 7.22 shows the output from the binary logistic regression, where 'n' shows the number of subjects. R^2 (Naglekerke) describes the amount of variance in the phenotypic outcome that is explained by methylation. The beta value (B) shows the direction of the association, whereas the odds ratio (Exp B) is the odds of being obese for each increase in the predictor (% methylation). The lower and upper 95% confidence intervals are also shown, which determine the certainty of the Exp B value. When the lower and upper 95% confidence intervals span 1.0 the association is not significant

at the 5% level, whereas a value of less than 1.0 shows a significant negative association, and a value of more than 1.0 shows a significant positive association. Significant p-values less than 0.050 are highlighted in yellow, and those that show a trend toward significance with a p-value between 0.051 and 0.100 are highlighted in green.

The methylation of each CpG loci analysed in SIRT1 was not significantly associated with BMI, % body fat, waist circumference or waist to hip ratio ($p>0.050$). Therefore, the methylation of these CpGs is not suitable as a biomarker of different measurements of obesity in subcutaneous adipose tissue.

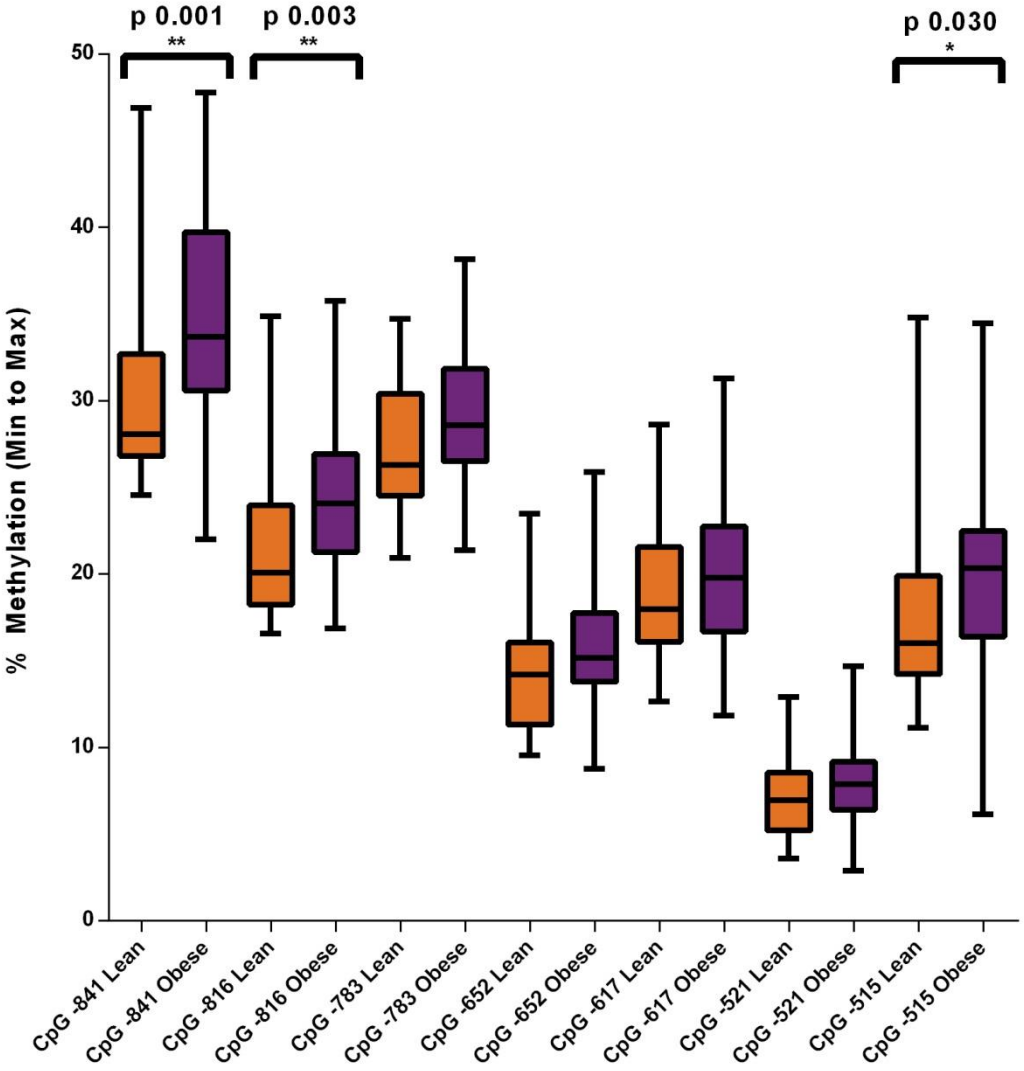


Figure 7.5- The methylation of PGC1α in the subcutaneous adipose tissue of lean and obese subjects. The methylation of CpG -841 (p=0.001), -816 (p=0.003) and -515 (p=0.029) was significantly higher in the adipose tissue from obese subjects, compared to lean subjects, determined by a Mann Whitney U test. There was no significant difference between the methylation of any of the other CpGs measured in the adipose tissue from obese subjects, compared to lean subjects, determined by a Mann Whitney U test (p>0.050). However, the methylation of CpG -783 showed a trend toward significance (p=0.058), where the methylation was higher in the obese subjects, compared to the lean subjects.

Table 7.23- The ability of PGC1α methylation in subcutaneous adipose tissue to predict measures of adiposity

BMI (kg/m^2)	Does methylation predict BMI (kg/m2)? Adjusted for sex and age						
	n	R2 (Naglekerke)	B	Exp (B)	Lower 95% CI	Upper 95% CI	p-value
PGC1α CpG -841	51	0.261	0.099	1.104	0.996	1.224	**0.059**
PGC1α CpG -816	50	0.255	0.121	1.129	0.988	1.291	**0.075**
PGC1α CpG -783	51	0.234	0.129	1.138	0.970	1.334	**0.113**
PGC1α CpG -652	50	0.191	0.036	1.036	0.870	1.234	**0.688**
PGC1α CpG -617	48	0.175	0.039	1.039	0.897	1.204	**0.608**
PGC1α CpG -521	51	0.189	0.111	1.117	0.843	1.479	**0.441**
PGC1α CpG -515	51	0.185	0.035	1.036	0.927	1.158	**0.533**
% fat	**Does methylation predict %fat? Adjusted for sex and age**						
	n	R2 (Naglekerke)	B	Exp (B)	Lower 95% CI	Upper 95% CI	p-value
PGC1α CpG -841	49	0.497	0.138	1.148	1.017	1.297	**0.026**
PGC1α CpG -816	48	0.468	0.151	1.163	0.994	1.360	**0.059**
PGC1α CpG -783	49	0.464	0.182	1.199	0.991	1.452	**0.062**
PGC1α CpG -652	48	0.422	0.099	1.104	0.893	1.364	**0.361**
PGC1α CpG -617	46	0.392	0.033	1.033	0.871	1.226	**0.706**
PGC1α CpG -521	49	0.410	0.155	1.167	0.832	1.638	**0.370**
PGC1α CpG -515	49	0.421	0.080	1.083	0.949	1.237	**0.238**
Waist measurement (cm)	**Does methylation predict waist measurement (cm)? Adjusted for sex and age**						
	n	R2 (Naglekerke)	B	Exp (B)	Lower 95% CI	Upper 95% CI	p-value
PGC1α CpG -841	51	0.425	0.121	1.128	1.006	1.266	**0.040**
PGC1α CpG -816	50	0.395	0.122	1.130	0.977	1.307	**0.100**
PGC1α CpG -783	51	0.375	0.125	1.134	0.953	1.349	**0.158**
PGC1α CpG -652	50	0.361	0.075	1.078	0.885	1.312	**0.456**
PGC1α CpG -617	48	0.332	0.006	1.006	0.856	1.181	**0.945**
PGC1α CpG -521	51	0.353	0.149	1.161	0.847	1.591	**0.354**
PGC1α CpG -515	51	0.363	0.075	1.077	0.951	1.220	**0.241**
Waist:hip ratio	**Does methylation predict waist:hip ratio? Adjusted for sex and age**						
	n	R2 (Naglekerke)	B	Exp (B)	Lower 95% CI	Upper 95% CI	p-value
PGC1α CpG -841	51	0.226	0.023	1.023	0.928	1.127	**0.649**
PGC1α CpG -816	50	0.229	0.021	1.021	0.900	1.159	**0.744**
PGC1α CpG -783	51	0.222	0.007	1.007	0.860	1.179	**0.927**
PGC1α CpG -652	50	0.236	0.086	1.090	0.909	1.307	**0.086**
PGC1α CpG -617	48	0.230	-0.039	0.962	0.826	1.120	**0.616**
PGC1α CpG -521	51	0.224	0.046	1.048	0.792	1.385	**0.744**
PGC1α CpG -515	51	0.222	0.001	1.001	0.896	1.119	**0.984**

Table 7.23 shows the output from the binary logistic regression as described previously. The methylation of CpG -841 and -816 in PGC1α was not significantly associated with BMI, however there was a trend toward significance (p=0.059 and 0.075 respectively). The methylation of CpG -841 in PGC1α significantly associated with % body fat and waist circumference ($p<0.05$). The methylation of CpG -816 in PGC1α was not significantly associated with % body fat or waist circumference, however there was a trend toward significance (p=0.059 and 0.100 respectively). The methylation of CpG -783 showed a trend toward significance with % body fat only (p=0.062), however there were no associations with BMI, waist circumference or waist to hip ratio. The methylation of CpGs -652, -617, -521 and -515 in PGC1α was not significantly associated with BMI, % body fat, waist circumference or waist to hip ratio ($p>0.05$). Therefore, the methylation of CpGs -841, -816 and -783 are suitable as biomarkers of several measures of obesity in subcutaneous adipose tissue.

7.3.3 Does SIRT1 and PGC1α mRNA expression differ in the adipose tissue of lean subjects versus obese subjects?

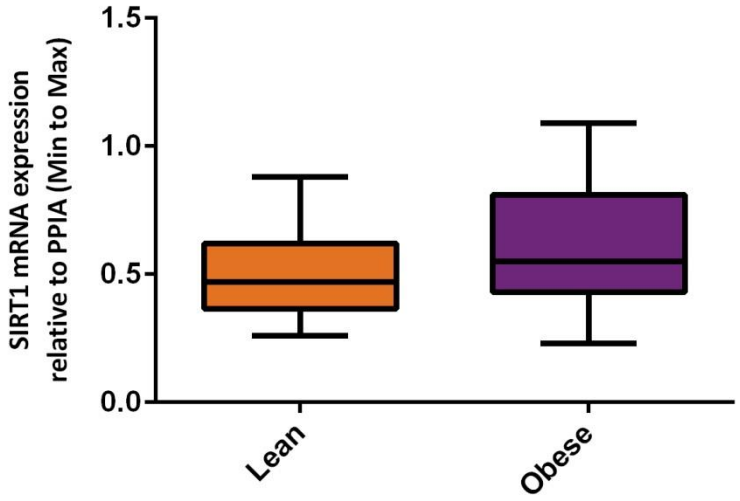


Figure 7.6- SIRT1 mRNA expression in the subcutaneous adipose tissue of lean and obese subjects. SIRT1 mRNA expression was not significantly different in the subcutaneous adipose tissue form lean and obese subjects, determined by an unpaired t-test ($p>0.050$). However, there was a trend toward significance with mRNA expression being higher in the obese subjects, compared to the lean subjects ($p=0.081$).

Table 7.24- The ability of SIRT1 mRNA expression in subcutaneous adipose tissue to predict measures of adiposity

BMI (kg/m^2)	Does mRNA expression predict BMI (kg/m^2)? Adjusted for sex and age						
	n	R2 (Naglekerke)	B	Exp (B)	Lower 95% CI	Upper 95% CI	p-value
SIRT1 mRNA expression relative to PPIA	46	0.293	0.149	1.161	0.113	11.897	**0.900**
% fat	**Does mRNA expression predict % fat? Adjusted for sex and age**						
	n	R2 (Naglekerke)	B	Exp (B)	Lower 95% CI	Upper 95% CI	p-value
SIRT1 mRNA expression relative to PPIA	45	0.517	1.133	3.105	0.127	76.019	**0.487**
Waist measurement (cm)	**Does mRNA expression predict waist measurement (cm)? Adjusted for sex and age**						
	n	R2 (Naglekerke)	B	Exp (B)	Lower 95% CI	Upper 95% CI	p-value
SIRT1 mRNA expression relative to PPIA	46	0.494	0.717	2.049	0.119	35.186	**0.621**
Waist:hip ratio	**Does mRNA expression predict waist:hip ratio? Adjusted for sex and age**						
	n	R2 (Naglekerke)	B	Exp (B)	Lower 95% CI	Upper 95% CI	p-value
SIRT1 mRNA expression relative to PPIA	46	0.307	0.801	2.227	0.178	27.861	**0.535**

The criteria used to determine if a subject was lean or obese for each measure of adiposity in adults has been described previously in section 7.2.2.

Table 7.24 shows the output from the binary logistic regression. R^2 (Naglekerke) describes the amount of variance in the phenotypic outcome that is explained by mRNA expression. The odds ratio (Exp B) is the odds of being obese for each increase in the predictor (mRNA expression).

SIRT1 mRNA expression was not significantly associated with BMI, % body fat, waist circumference or waist to hip ratio ($p>0.050$). Therefore, the expression of SIRT1 is not a suitable biomarker of different measurements of obesity in subcutaneous adipose tissue.

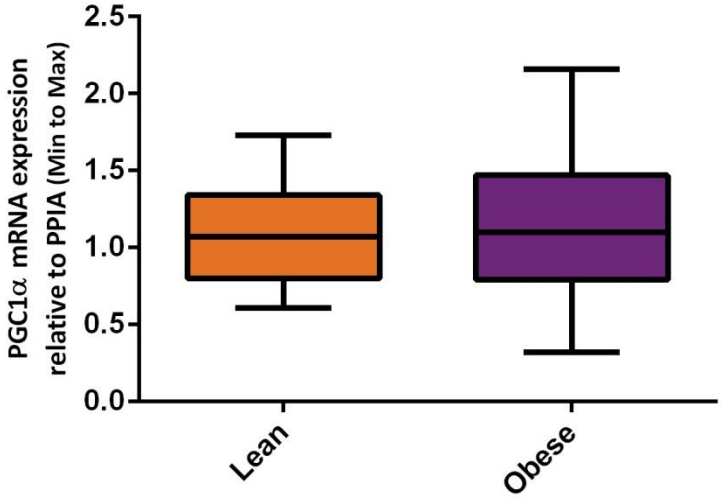


Figure 7.7- PGC1α mRNA expression in the subcutaneous adipose tissue of lean and obese subjects. PGC1α mRNA expression was not significantly different in the subcutaneous adipose tissue form lean and obese subjects, determined by an unpaired t-test (p>0.050).

Table 7.25- The ability of PGC1α mRNA expression in subcutaneous adipose tissue to predict measures of adiposity

BMI (kg/m^2)	**Does mRNA expression predict BMI (kg/m^2)? Adjusted for sex**						
	n	**R2 (Naglekerke)**	**B**	**Exp (B)**	**Lower 95% CI**	**Upper 95% CI**	**p-value**
PGC1α mRNA expression relative to PPIA	47	0.274	0.285	1.330	0.341	5.193	**0.682**
% fat	**Does mRNA expression predict % fat? Adjusted for sex and age**						
	n	**R2 (Naglekerke)**	**B**	**Exp (B)**	**Lower 95% CI**	**Upper 95% CI**	**p-value**
PGC1α mRNA expression relative to PPIA	46	0.470	0.456	1.577	0.308	8.079	**0.585**
Waist measurement (cm)	**Does mRNA expression predict waist measurement (cm)?**						
	n	**R2 (Naglekerke)**	**B**	**Exp (B)**	**Lower 95% CI**	**Upper 95% CI**	**p-value**
PGC1α mRNA expression relative to PPIA	47	0.471	0.327	1.387	0.284	6.777	**0.686**
Waist:hip ratio	**Does mRNA expression predict waist:hip ratio? Adjusted for sex**						
	n	**R2 (Naglekerke)**	**B**	**Exp (B)**	**Lower 95% CI**	**Upper 95% CI**	**p-value**
PGC1α mRNA expression relative to PPIA	47	0.300	0.548	1.730	0.418	7.149	**0.449**

Table 7.25 shows the output from the binary logistic regression. PGC1α mRNA expression was not significantly associated with BMI, % body fat, waist circumference or waist to hip ratio (p>0.050). Therefore, the expression of PGC1α is not a suitable biomarker of different measurements of obesity in subcutaneous adipose tissue.

7.4 Discussion

7.4.1 The methylation of SIRT1 and PGC1α in fetal tissues was associated with later childhood adiposity

7.4.1.1 SIRT1 methylation and adiposity in umbilical cord

The results in chapter 4 have shown that the methylation of specific CpG loci in the SIRT1 and PGC1α promoter at 5-7 years in peripheral blood was associated with later childhood adiposity. The methylation of these loci has also been shown to be temporally stable between 5 and 14 years in peripheral blood. Fetal tissue was not available for these subjects and therefore the association between methylation of these loci at birth and future adiposity could not be determined. In order to investigate when the methylation of these loci might be determined, fetal tissue from the SWS cohort was used. The results in this chapter have shown that the methylation of these loci at birth predicted future childhood adiposity, suggesting that methylation might be set up *in utero*.

The methylation of CpG -880 and -865 in SIRT1 in umbilical cord was negatively associated with subscapular skinfold thickness at birth and 2 years and with fat mass at 4 and 6 years of age. The difference in age at which these associations occurred could be explained by the differences in these two techniques. DXA scans are known to be accurate when measuring fat mass in babies and children, whereas measuring adiposity by skinfold thickness is less accurate and reproducible (20). Another consideration is that these techniques measure different types of fat depots. Skinfold thickness is a measurement of specific subcutaneous fat depots, whereas DXA scans are able to measure both subcutaneous and visceral fat and, therefore, is a combination of both types of fat (20)(23). Visceral adipose tissue undergoes lipolysis more readily than subcutaneous adipose tissue and is positively associated with insulin resistance (9). These technicalities could explain why an association with skinfold thickness is present at birth whilst the association with fat mass doesn't occur until 4 years of age. Whilst both these measurements are useful for determining risk of childhood adiposity, DXA scans may be more accurate for determining an increased risk of visceral fat which may be a better predictor of increased risk of obesity related disease, such as T2D and heart disease.

The methylation of CpGs -880 and -865 in SIRT1 in umbilical cord was negatively associated with total lean mass at birth, which is the same direction as the association with total fat mass. A positive relationship between fat mass, lean mass, bone size and bone density is known to occur in childhood and adulthood, which is thought to be a compensatory mechanism whereby there is an increase in muscle mass and increased size and density of the skeleton in order to support the

increased fat mass (214). The methylation of CpG -865 in SIRT1 in umbilical cord was positively associated with percentage lean mass at 4 and 6 years and percentage bone mineral content (BMC) at 4 years. The direction of these associations is the opposite to those seen with total measures of adiposity, including total fat mass and total lean mass. This supports previous data where the percentage measurements of adiposity were shown to be in the opposite direction to total measurements of adiposity (215).

7.4.1.2 SIRT1 methylation and adiposity in umbilical cord blood

The methylation of 5 out of the 7 CpG loci (CpGs -880, -865, -760, -742 and -668) measured in SIRT1 in umbilical cord blood was positively associated with total fat mass at 4 years. The methylation of CpG -689 and -668 was positively associated with tricep and subscapular skinfold thickness at birth and 6 months, respectively. The methylation of CpGs -880 and -865 was positively associated with subscapular and tricep thickness at 12 months, and also at 3 years, and 6 years of age for CpG -865. Therefore, the association between the methylation of these loci and skinfold thickness generally occurs at a younger age than the association between methylation and total fat mass, consistent with the findings in umbilical cord. As discussed previously, DXA scans are more accurate than skinfold thickness for determining adiposity, and these techniques measure different types of adipose tissue, which may explain the difference in age at which these associations occur (20)(23).

The methylation of CpGs -880 and -865 in SIRT1 was negatively associated with subscapular skinfold thickness and fat mass in umbilical cord, whereas the association in umbilical cord blood was positive. Whilst these significant associations occur at a similar age, the direction of the association is opposite across the two tissue types. Both of these fetal tissues are a heterogeneous mix of cellular populations. Therefore, cellular heterogeneity may explain the difference in the direction of these associations for these tissues. Because cell counts were not available for these samples, this could not be corrected for in the statistical analysis. Umbilical cord and umbilical cord blood contain mesenchymal stem cells which are multipotent and have the ability to differentiate into osteogenic, chondrogenic and adipogenic lineages (123)(124). Umbilical cord blood also contains nucleated hematopoietic stem cells, which may be responsible for the difference in the direction of the associations between methylation and measures of adiposity in these different fetal tissues. Because subjects from which the umbilical cord and umbilical cord blood samples were taken are not the same, it was not possible to determine if there was an inverse correlation between the methylation of these loci in these tissues.

Because umbilical cord blood contains hematopoietic stem cells, it might be expected that the direction of the associations in umbilical cord blood would be the same as in peripheral blood (124). However, the results from Chapter 4 showed that the methylation of CpG -880 in SIRT1 was negatively associated with future adiposity in peripheral blood. Therefore, the direction of the association in umbilical cord blood is in the opposite direction to peripheral blood. This might be explained by the fact that umbilical cord blood contains nucleated red blood cells, which are immature cells found in umbilical cord blood and fetal peripheral blood up to the 5th day of life (125). Nucleated red blood cells are not normally found in the peripheral blood of healthy children or adults as their presence is associated with severe diseases (125). Therefore, the presence of nucleated red blood cells in umbilical cord blood could explain the difference in the direction of the association between methylation and measures of adiposity in peripheral blood.

The methylation of 4 out of the 7 CpG loci (CpGs -880, -865, -760 and -722) in SIRT1 in umbilical cord blood was positively associated with total lean mass at 6 years and total BMC at 4 and 6 years. This supports evidence from studies that have shown a positive relationship between fat mass, lean mass and bone size and density in childhood and adulthood, and studies specifically in children have shown a positive relationship between total fat mass and BMC (215)(214). As discussed previously with measures of fat mass and skinfold thickness, the direction of these associations is opposite to those in umbilical cord. This further suggests that differences in methylation occur in a tissue specific manner, which is likely explained by the difference in cell populations between these fetal tissues.

7.4.1.3 PGC1α methylation and adiposity in umbilical cord

The methylation of each CpG loci in PGC1α, apart from CpG -515, in umbilical cord was positively associated with total fat mass at either 4 or 6 years. The methylation of several CpG loci positively associated with tricep skinfold thickness at birth (CpG -841 and -515), 6 months (CpG -841 and -816), 2 years (CpG -816 and -515) and 3 years (CpG -841, -816 and -515). Therefore, the age at which these associations occur is at a younger age for skinfold thickness, compared to total fat mass determined by DXA. This is consistent with findings discussed previously with SIRT1 methylation in umbilical cord and umbilical cord blood. These findings are also consistent with those shown in chapter 4, where the methylation of CpGs -841, -816, -783 and -515 at 5-7 years in peripheral blood was positively associated with childhood adiposity. Despite the actual level of methylation being different in umbilical cord and peripheral blood, the same association between methylation and

future adiposity remains, suggesting that measuring the methylation of these loci at birth may be beneficial for predicating future risk of adiposity at an even earlier age. This may also suggest that an environmental challenge that occurs *in utero* could affect different tissues equally by influencing the methylation of the multipotent mesenchymal stem cells, which go on to differentiate into the different lineages (123)(124).

The methylation of CpG -816 in PGC1α in umbilical cord was positively associated with total lean mass at birth, whereas the methylation of specific CpG loci negatively associated with percentage lean mass at birth (CpG -515), percentage lean mass at 4 years (CpG -841), percentage lean mass at 6 years (CpG -521) and percentage BMC at 4 and 6 years (CpGs -521 and -515). This further supports an inverse relationship between total and percentage measures of adiposity, when using DXA scans. Whilst these associations between methylation and total lean mass, percentage lean mass and percentage BMC do not consistently occur at the same ages, or at the same loci, measuring this region of the PGC1α promoter may be useful for predicating future lean and mass.

7.4.1.4 PGC1α methylation and adiposity in umbilical cord blood

The methylation of PGC1α in umbilical cord blood was not significantly associated with any measures of DXA fat mass at any age. Only the methylation of CpG -617 was negatively associated with tricep skinfold thickness at 6 years, which is in the opposite direction to the associations between PGC1α methylation and skinfold thickness in umbilical cord, and the association between PGC1α methylation and future adiposity in peripheral blood. This further suggests that methylation patterns in umbilical cord blood are tissue specific, likely explained by the presence of nucleated red blood cells. Therefore, umbilical cord blood appears to have a tissue specific epigenetic signature, whereas the methylation patterns in umbilical cord and peripheral blood appear to be consistent, despite these tissues being from different germline layers. Therefore, measuring DNA methylation in umbilical cord blood may not be indicative of the methylation levels seen in other tissues from different germline layers, or of the association between methylation and a phenotypic measurement. This is in agreement with other studies that have shown that DNA methylation varies between fetal tissues (127)(126).

The methylation of CpG -515 was negatively associated with total lean mass at 4 years and total BMC at 6 years. The methylation of CpG -617 was positively associated with percentage BMC at 6 years. This is consistent with the other results from this chapter discussed previously, where there is an inverse relationship between total and percentage measures of adiposity.

7.4.1.5 SIRT1 and PGC1α methylation and birth weight

The methylation of CpGs -880 and -865 in SIRT1 in umbilical cord was negatively associated with birth weight, whereas there was no association in umbilical cord blood. The methylation of CpGs -742 and -689 in SIRT1 in umbilical cord blood was positively associated with birth weight. The methylation of CpGs -742 and -689 were not measured in umbilical cord due to limited DNA, and therefore it is not known if there would be an association between the methylation of these CpGs and birth weight. For the loci where there is an association between methylation and birth weight, the direction of the associations are inverse in these fetal tissues, further suggesting that umbilical cord blood may have a unique epigenetic signature. The direction of these associations is the same as those associations between fat mass and skinfold thickness for each tissue, suggesting that birth weight may be a proxy measure for future body composition. This is consistent with evidence that has shown that birth weight is associated with measures of adiposity during childhood and adulthood (216). However, there is now evidence to suggest that birth weight may not be a good indicator of future body composition and what may be of greater importance is the relative size and length of the baby, and small babies who undergo catch up weight gain during early childhood may be at greatest risk of later adiposity (217)(83)(86)(57). Therefore, associations between the methylation of SIRT1 in these tissues and birth weight may not be as useful and accurate at predicting future risk of obesity as DXA scans.

The methylation of CpG -515 in umbilical cord was positively associated with birth weight; however there was no association in umbilical cord blood. Gemma et al. have shown that maternal BMI was positively associated with the methylation of CpG -515 in umbilical cord, along with 3 other loci in the promoter (171). Maternal BMI is a strong predictor of birth weight and is associated with childhood obesity (209). Results from this chapter have demonstrated that the methylation of CpG -515 is positively associated with various measures of fat mass, lean mass, bone mineral mass and skinfold thickness at several different ages in umbilical cord, and also some associations in umbilical cord blood. Taken together, this may suggest that maternal body composition is what determines the level of methylation of this locus in the offspring, in umbilical cord and potentially other tissues, which in turn determines the birth weight and future adiposity. In order to test this hypothesis, maternal BMI could be corrected for in the statistical analysis along with maternal weight gain during pregnancy to determine if the association between methylation of CpG -515 and future measures of adiposity still remains after correcting for maternal body composition.

Taken together, these findings suggest that the methylation of SIRT1 and PGC1α in fetal tissues is associated with several measures of adiposity during childhood. Umbilical cord blood appears to have a unique epigenetic signature, whereas the associations in umbilical cord and peripheral blood appear to be consistent, despite these tissues having different methylation patterns and being from different germline layers. Therefore, measuring the methylation of these CpG loci at birth may be beneficial for predicting those individuals at an increased risk of childhood obesity at an even earlier age.

7.4.2 The methylation of PGC1α but not SIRT1 is altered in the subcutaneous adipose tissue of lean and obese subjects

Because previous results have shown the methylation of specific CpG loci in the SIRT1 and PGC1α promoter in fetal tissues and peripheral blood is associated with future adiposity, the methylation of these loci was measured in subcutaneous adipose tissue from lean and obese subjects to determine if the methylation was altered in obesity and if the association between methylation and adiposity remains when the disease state is present.

The methylation of each CpG analysed in SIRT1 was not significantly different in subcutaneous adipose tissue from lean and obese subjects, and the methylation was not associated with any of the measures of obesity tested. To date there are no studies that have measured the methylation of SIRT1 in adipose tissue. Because only 2 CpG loci were measured in the promoter region of SIRT1 due to limited DNA, it is unknown if the methylation of other CpG loci within the promoter or in a separate region might be different in the subcutaneous adipose tissue of lean and obese subjects. A power calculation was used to determine that 40 subjects were required for each group at the start of the BIOCLAIMS study. Therefore, because there were less than 40 subjects in the lean group the sample size may be inadequately powered to detect a significant difference in methylation of these loci between these two groups. If the methylation of these CpGs was measured in a larger population there may be a significant difference between lean and obese subjects.

The methylation of 3 CpG loci (-841, -816 and -515) measured in the PGC1α promoter was significantly higher in the subcutaneous adipose tissue of obese subjects, compared to lean, and the methylation of CpG -783 showed a trend towards higher methylation in the obese subjects, compared to the lean when subjects were classified according to their BMI. These findings support data from a previous study by Chen et al. that showed the mean methylation of CpG -841 -816, -783, -652 and -617 was higher in subcutaneous adipose tissue from obese subjects, compared to lean subjects,

classified according to BMI (79). Chen et al. also measured the methylation of these CpG loci in omental visceral adipose tissue, which showed no difference between lean and obese subjects, suggesting a subcutaneous fat specific effect (79). It is unknown if the methylation of these loci would decrease if BMI were to decrease. Further work would be required to determine if increased BMI is causal of increased methylation, or if increased methylation is causal of increased BMI because these results simply show an association between increased methylation and increased BMI and do not show that methylation if functionally involved in the expansion of subcutaneous adipose tissue.

The methylation of CpGs -841, -816 and -783 was positively associated with percentage fat, determined by bioelectrical impedance. This has been shown to an accurate technique for measuring percentage body fat, equally as accurate as DXA (24)(25)(23)(210). The methylation of CpGs 841 and -816 was positively associated with waist measurement, which is a measure of central abdominal obesity, suggesting that methylation of these loci might be beneficial as a marker of central obesity (20). The methylation of CpGs -841 and -816 also showed a trend toward significance with BMI, which has several disadvantages associated with it. Firstly, BMI is not a direct measure of adiposity. Secondly, BMI cannot distinguish between fat, lean and bone mass. Thirdly, BMI is not suitable for use in children (21)(16). The methylation of all the CpGs analysed in the PGC1α promoter were not significantly associated with waist to hip ratio. This is not an accurate measure of adiposity as it relies on two measurements making it more susceptible to error and ratios are less sensitive to smaller weight (20). Whilst the methylation of CpG -515 was significantly different in the subcutaneous adipose tissue of lean and obese subjects, the methylation was not associated with any of the measures of adiposity tested. This suggests that this may be a type I error and if the methylation of this loci were measured in a larger population there may not be a significant difference between lean and obese subjects. Therefore, measuring the methylation of CpGs -841, -816 and -783 in subcutaneous adipose tissue could potentially predict percentage fat and waist measurement, and to a lesser extent BMI, before the onset of obesity.

7.4.3 The expression of SIRT1 and PGC1α is not altered in the subcutaneous adipose tissue of lean and obese subjects

These findings have shown that SIRT1 mRNA expression showed a trend toward significance where SIRT1 mRNA expression was higher in the subcutaneous adipose tissue of obese subjects compared to lean subjects. SIRT1 mRNA expression was not associated with any of the measures of obesity tested. SIRT1 is known to inhibit adipogenesis and a previous study has shown that SIRT1

mRNA expression is inversely associated with BMI (218)(67)(72). Therefore, it would be expected that the obese subjects would have decreased SIRT1 mRNA expression, rather than showing a trend toward higher expression, and that SIRT1 mRNA expression might be associated with BMI. However, the number of subjects in the lean group is less than determined by the power calculation at the start of the study. Therefore, the number of subjects in which the SIRT1 mRNA expression was measured may be inadequately powered to detect associations with BMI, percentage fat, waist measurement and waist to hip ratio. Another consideration is that the abdominal subcutaneous adipose tissue biopsy was taken 8 hours after eating. It is possible that SIRT1 mRNA expression might have been increased in these subjects in response to fasting as caloric restriction is known to rapidly increase SIRT1 mRNA expression, which could explain why expression was not different between the two groups (73). SIRT1 mRNA expression should be measured in the same group of subjects after eating to truly determine if there is a difference between expression in the lean and obese groups.

PGC1α mRNA expression did not significantly differ in the subcutaneous adipose tissue of lean and obese subjects, classified according to BMI, and mRNA expression was not associated with any of the measures of obesity tested. This does not agree with data from a study by Chen et al. where PGC1α mRNA expression was decreased in the subcutaneous adipose tissue from obese subjects, compared to lean subjects, classified according to BMI (79). However, the number of subjects in the lean and obese groups was not matched, and the total number of subjects may be underpowered to detect a significant difference in PGC1α mRNA expression between the two groups. Therefore, if the mRNA expression was measured in a larger population the expression may be decreased in the subcutaneous adipose tissue from obese subjects, compared to lean subjects, as Chen et al. have shown.

PGC1α is a co-activator and is known to bind to many transcription factors, for example SREBP1, which is involved in adipogenesis and lipogenesis, and also PPARγ which plays a central role in adipogenesis (219)(8). The methylation of several CpG loci in PGC1α was shown to be higher in the subcutaneous adipose tissue from the obese subjects, compared to lean. It is possible that methylation could alter the binding of PGC1α to these transcription factors, which may result in altered expression of these genes in lean and obese subjects. Therefore, PGC1α may be indirectly involved in the development of obesity via altered methylation and transcription factor binding.

Taken together, these findings show that SIRT1 and PGC1α mRNA expression does not differ in the subcutaneous adipose tissue of lean and obese subjects, and is not associated with any of the measures of obesity tested. This suggests that SIRT1 and PGC1α might be associated with adiposity

via alternative mechanisms besides altered expression in adipose tissue. Because both genes are expressed in a wide range of tissues and involved in many different pathways, altered expression elsewhere in the body could result in obesity. For example altered expression in neuronal tissue which may affect the appetite, altered expression in the liver resulting in increased fatty acid storage, or altered expression in skeletal muscle resulting in decreased uptake into the muscle and lipotoxicity. Therefore, the SIRT1 and PGC1α mRNA expression could be measured in easily accessible tissues such as skeletal muscle in the same subjects to determine if the expression was altered between lean and obese subjects.

7.5 Summary

These findings support the hypothesis that the methylation of specific CpG loci in SIRT1 and PGC1α measured in fetal tissues is associated with adiposity in childhood. These findings in part support the hypothesis that the methylation and mRNA expression of SIRT1 and PGC1α differs in the abdominal subcutaneous adipose tissue of lean and obese people, with increased PGC1α methylation in obese subjects compared to lean. These results, along with those from chapter 4, have shown that the methylation of specific loci in SIRT1 and PGC1α is associated with several measures of adiposity in several different tissues from different germline layers, and these associations are present at several stages of development from birth through to adulthood. Despite the level of methylation varying across the different tissue types, the association between methylation of these loci and adiposity still remains, even when obesity has already occurred. This suggests that the methylation of these loci may be determined *in utero*. Therefore, measuring the methylation of these loci at birth may predict those individuals on a set trajectory to an increased risk of obesity later in life. Further work is required in order to determine if maternal and paternal factors may influence the methylation of these loci *in utero*.

Chapter 8

The Effect of Fatty Acid Supplementation *in vitro* on SIRT1 and PGC1α Expression and Methylation

8.1 Introduction

8.1.1 Dietary fatty acid intake can influence the risk of obesity

The results from chapter 7 have shown that the methylation of specific CpG loci in the SIRT1 and PGC1α promoter in fetal tissues is associated with future adiposity during childhood. Because these associations are present from birth, this suggests that the methylation of these loci may be determined *in utero* and the prenatal environment may influence the fetal epigenome. Environmental factors that could potentially alter the fetal epigenome include stress, pollution, exercise and maternal diet (220). Of these factors, the effect of maternal diet on the methylation of the offspring has been thoroughly investigated in animals and humans (113)(198)(221)(222). There has been an increased focus over recent years on the effect of maternal dietary fat on the epigenome of the offspring due to the rise in obesity worldwide. Both the amount and type of fat have been suggested to be important in the development of obesity (14). Because the epigenetic regulation of genes that are involved in metabolism varies between individuals, this may contribute to their nutritional requirements and influence their susceptibility to disease (223). However, because diet has been shown to modify the epigenome, dietary intervention during pregnancy and throughout the life course may be a mechanism for altering the methylation and expression of specific genes and pathways, including SIRT1 and PGC1α, which may be beneficial for the prevention of obesity later in life.

In humans, there are 2 essential polyunsaturated fatty acids (PUFAs); α-linolenic acid (ALA) and linoleic acid (LA), which are known as the 'parents' of the omega-3 (n-3) and omega-6 (n-6) subfamilies of PUFAs (28). ALA and LA undergo a series of desaturation and elongation steps in order to produce longer chain PUFAs for many cellular processes, including fetal development (27). ALA and LA cannot be synthesised *de novo* and therefore must be consumed within the diet (29). Therefore, the maternal diet is important for the intake of these PUFAs for the developing fetus

Over the past two decades there has been a reduction in the consumption of saturated fats in the western diet, which have been replaced with 'healthier' plant based alternatives rich in LA, which has resulted in a shift in the ratio of n-3 to n-6 PUFAs (28). In humans, the optimal ratio of ALA to LA in humans is thought to be between 1:3 and 1:5, whereas in some populations the ratio has been shown to be as high as 1:22 (28)(13). This change in the ratio of n-3 to n-6 PUFAs has been suggested to have contributed to the development of childhood and adult obesity (13). Data from animal models support this when the offspring of mice fed a high fat diet rich in LA during pregnancy and lactation were shown to have increased body weight and fat mass, and increased adipocyte size,

compared to the offspring of dams fed a high fat diet enriched in equal amounts of LA and ALA (30). These differences persisted into adulthood suggesting that the amount and type of PUFA intake during early life can increase the risk of obesity.

Data from animal and human studies suggests that dietary supplementation with n-3 PUFAs may be beneficial for decreasing adiposity, perhaps by increasing the ratio of n-3 to n-6 PUFAs (31)(32)(221)(224). For example, Donahue et al. have shown that higher concentrations of docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) in the maternal diet of pregnant women was associated with lower adiposity in their children at 3 years of age (221). Royal jelly, which contains the monounsaturated fatty acid 10-hydroxy-2E-decenoic acid (10-H=DA) and the saturated fatty acid 10-hydroxydecanoic acid (10-HDA), has been shown to promote weight loss and reduce inflammation in humans after only 8 weeks of supplementation (224). Guo et al. have shown that royal jelly significantly reduced total cholesterol and low density lipoprotein (LDL) in healthy weight subjects, after 4 weeks of supplementation (225). Therefore royal jelly could be beneficial for the prevention of obesity. Taken together, these data suggest that the amount and type of dietary fat during early life and throughout the life course may have long term consequences on human health.

8.1.2 Fatty acid supplementation can alter PGC1α DNA methylation and gene expression

There is some evidence from animal studies that suggests dietary fatty acid intake may be associated with altered PGC1α DNA methylation and gene expression, *in vivo*. For example, Larker et al. have shown that the offspring of mice fed a high fat diet 6 weeks before and during pregnancy had increased methylation of a specific CpG locus in the PGC1α promoter, which associated with a trend toward decreased PGC1α mRNA expression in skeletal muscle (226). These changes in methylation and expression were present from birth and persisted into adulthood, suggesting that maternal diet is a potential mechanism for altering the methylation and expression of specific genes.

Several *in vitro* studies have shown that fatty acid supplementation can induce changes in the expression of PGC1α specifically (31)(227)(228). For example, Staiger et al. have shown that the unsaturated fatty acids oleic acid (OA) and LA increased PGC1α mRNA expression in human myotubes, whereas the saturated fatty acid palmitic acid (PA) had no effect on expression, suggesting a specific effect dependent on the class of fatty acid (227). Flachs et al. have shown that adult male mice fed a diet where 44% of dietary lipids were replaced with n-3 concentrate (51% DHA) had increased PGC1α mRNA expression in their white adipose tissue, but had no effect on expression in the liver (31). These data suggest that fatty acid supplementation can induce changes in PGC1α gene

expression in a tissue specific manner which may be beneficial as PCG1α is expressed in many metabolic tissues and therefore a more targeted approach may be advantageous.

There is some evidence to suggest that *in vitro* fatty acid supplementation can alter the methylation of PGC1α. For example, Barrès et al. have shown that primary human skeletal muscle cells supplemented with OA and PA *in vitro* induces non-CpG methylation of the PGC1α promoter, whereas there was no effect on CpG methylation (189). Cells that were supplemented with PA also had a significant decrease in PGC1α mRNA expression, suggesting a fatty acid specific effect on gene expression (189).

To date there is limited data on the effect of fatty acid supplementation on the methylation and expression of SIRT1. However, there is some evidence to suggest that both SIRT1 and PGC1α are covalently modified as a result of fatty acid supplementation *in vitro* (229). For example, mouse skeletal muscle cells supplemented with OA have increased SIRT1 phosphorylation at Ser483 and PGC1α deacetylation, resulting in increased fatty acid oxidation, suggesting a potential mechanism for reducing elevated levels of NEFAs that occurs in obesity (229).

8.1.3 Aims

In order to test the hypothesis that fatty acids can alter the expression and methylation of SIRT1 and PGC1α *in vitro*, human liposarcoma cells were supplemented with fatty acids including n-3 PUFAs, an n-6 PUFA, monounsaturated fatty acids and a saturated fatty acid. Altering the amount and type of dietary fat in the maternal diet and throughout the life course provides a potential mechanism for altering the methylation and expression of genes *in vivo*. Different classes of fatty acids may have different effects on the methylation and expression of specific genes and pathways, which may occur in a tissue specific manner. More evidence is required on the effect of fatty acid supplementation on the methylation and expression of SIRT1 and PGC1α specifically, to determine if dietary supplementation before pregnancy, during pregnancy, and throughout the life course may be beneficial for the prevention of obesity later in life.

8.2 Methods

Human liposarcoma cells were grown in media supplemented with n-3 PUFAs (ALA, EPA and DHA), an n-6 PUFA (LA), monounsaturated fatty acids (10-H=DA and OA) and a saturated fatty acid (10-HDA) and at an increasing concentration (10, 50 and 100μM), for 72 hours (n=6 per concentration for each fatty acid)(section 2.2.16). Fatty acids were chosen based on previous *in vitro* studies that suggest they may alter the regulation and expression of SIRT1and PGC1α (31)(227)(228)(189)(229). Human liposarcoma cells were also grown in media without fatty acid supplementation for 72 hours, as a control group (n=6). SIRT1 and PGC1α mRNA expression was normalised to the house keeping gene cyclophilin (PPIA), which was confirmed to be stably expressed under these conditions using the geNorm Reference Gene Selection Kit. To determine if there was a significant effect of concentration on expression, one-way ANOVA and Tukey's multiple comparison tests were used.

The effect of ALA on the methylation of the PGC1α promoter was determined by pyrosequencing (n=6 per concentration). To determine if there was a significant effect of concentration on methylation, one-way ANOVA and Tukey's multiple comparison tests were used.

8.3 Results

8.3.1 Do fatty acids alter the expression of SIRT1 and PGC1α *in vitro*?

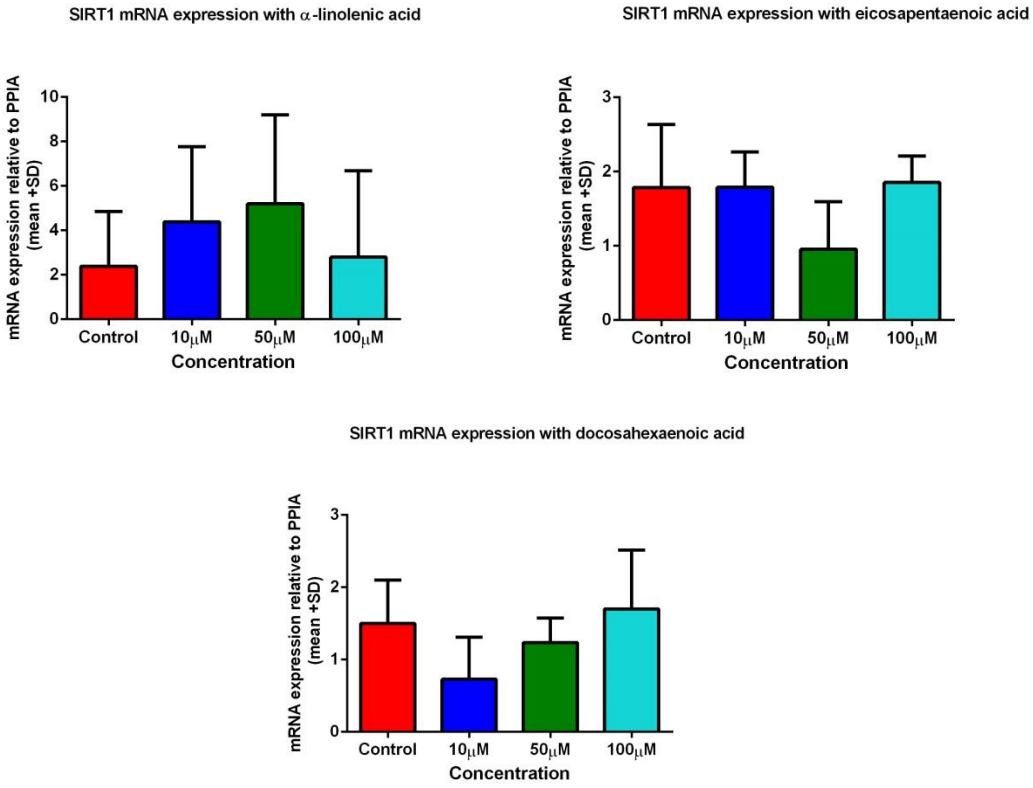


Figure 8.1- The effect of omega-3 polyunsaturated fatty acids on SIRT1 expression. There was no significant effect of ALA, EPA or DHA on SIRT1 mRNA expression at any of the concentrations tested, determined by one-way ANOVA and Tukey's multiple comparison ($p>0.05$).

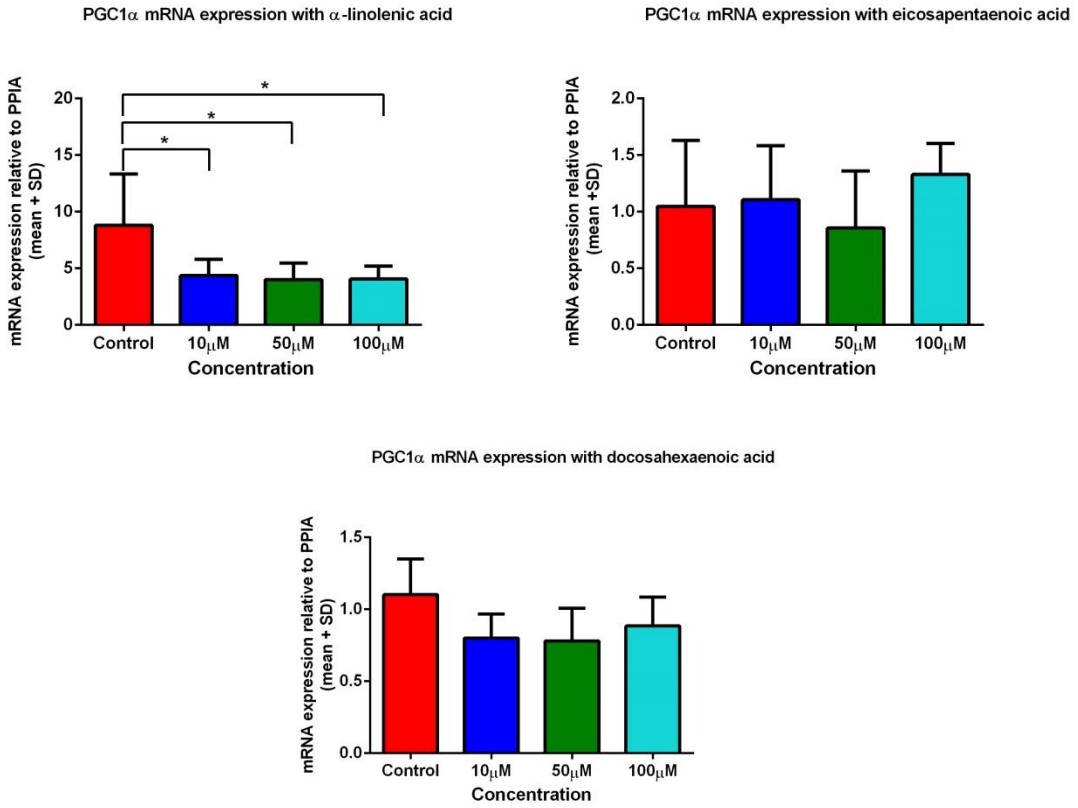


Figure 8.2- The effect of omega-3 polyunsaturated fatty acids on PGC1α expression. There was a significant effect of ALA on PGC1α mRNA expression, where expression was decreased at all concentrations tested, determined by one-way ANOVA and Tukey's multiple comparison ($p<0.03$). There was no significant effect of EPA or DHA on PGC1α mRNA expression at any of the concentrations tested, determined by one-way ANOVA and Tukey's multiple comparison ($p>0.05$).

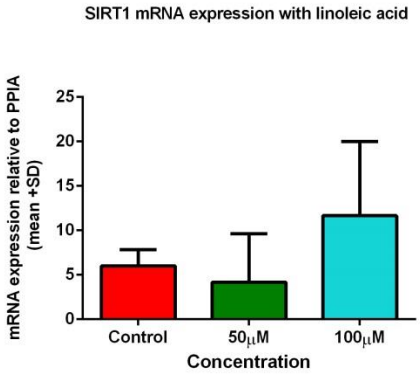


Figure 8.3- The effect of an omega-6 polyunsaturated fatty acid on SIRT1 expression. Data is unavailable for LA at 10μM because the quality of the RNA was poor (260/280 ratios less than 1.2), which was determined by Nanodrop, and the RNA was degraded when ran on an agarose gel. Therefore, samples for LA at 10μM were discarded from further analysis. There was no significant effect of LA on SIRT1 mRNA expression at either of the concentrations tested, determined by one-way ANOVA and Tukey's multiple comparison ($p>0.05$).

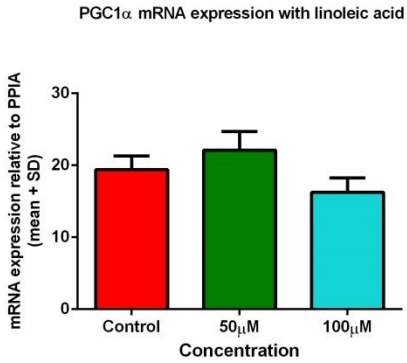


Figure 8.4- The effect of an omega-6 polyunsaturated fatty acid on PGC1α expression. Data is unavailable for LA at 10μM due to poor quality RNA, as described previously. There was no significant effect of LA on PGC1α mRNA expression at either of the concentrations tested, determined by one-way ANOVA and Tukey's multiple comparison ($p>0.05$).

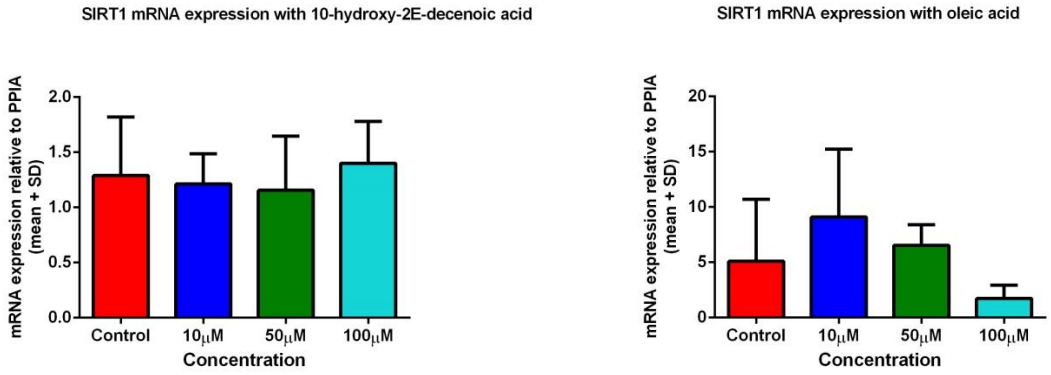


Figure 8.5- The effect of omega-9 monounsaturated fatty acids on SIRT1 expression. There was no significant effect of 10-H=DA or OA on SIRT1 mRNA expression at any of the concentrations tested, determined by one-way ANOVA and Tukey's multiple comparison ($p>0.05$).

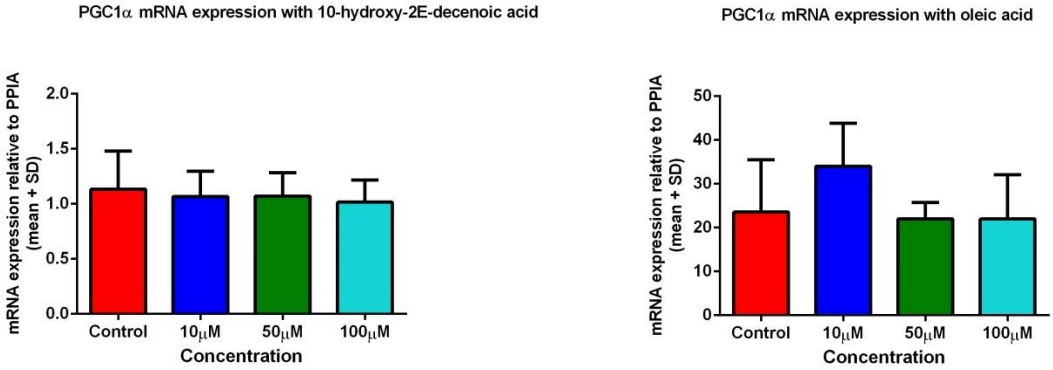


Figure 8.6- The effect of omega-9 monounsaturated fatty acids on PGC1α expression. There was no significant effect of 10-H=DA or OA on PGC1α mRNA expression at any of the concentrations tested, determined by one-way ANOVA and Tukey's multiple comparison ($p>0.05$).

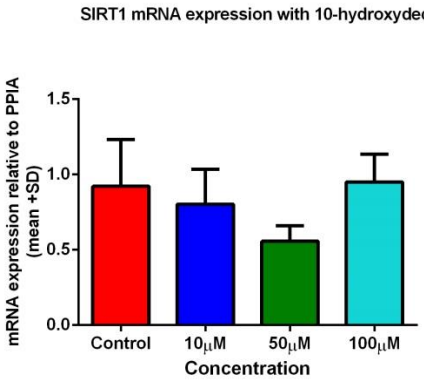


Figure 8.7- The effect of a saturated fatty acid on SIRT1 expression. There was a significant difference between 10-HDA at 50µM and 100µM, determined by Tukey's multiple comparisons test (p=0.04). However, there was no significant effect of 10-HDA on SIRT1 mRNA expression at any of the concentrations tested, when compared to the control group (p>0.05).

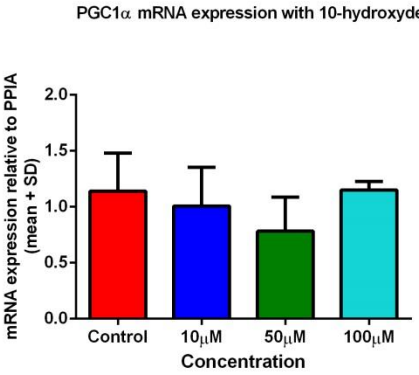
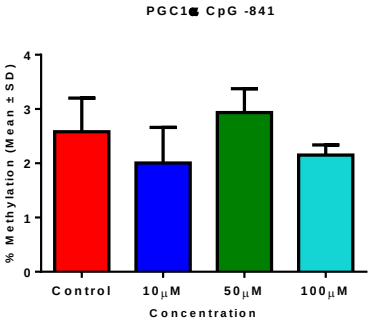
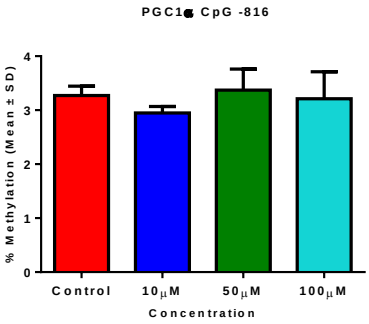


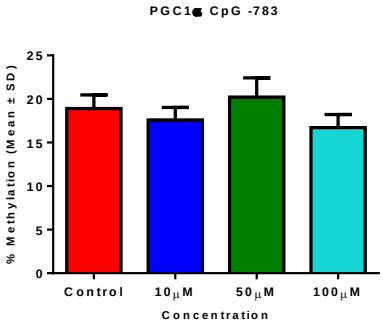
Figure 8.8- The effect of a saturated fatty acid on PGC1α expression. There was no significant effect of 10-HDA on PGC1α mRNA expression at any of the concentrations tested, determined by one-way ANOVA and Tukey's multiple comparison (p>0.05).

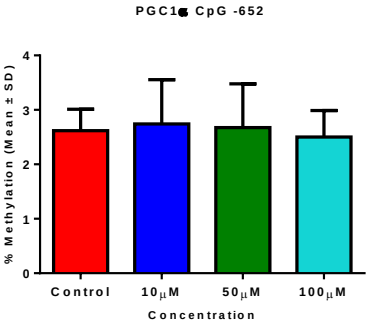
8.3.2 Does α-linolenic acid alter the methylation of PGC1α *in vitro?*

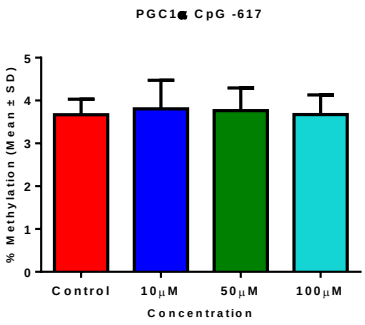
Having shown that ALA supplementation induced a decrease in PGC1α expression *in vitro*, the effect of ALA on the methylation of the PGC1α promoter was determined by pyrosequencing (n=6 per concentration).

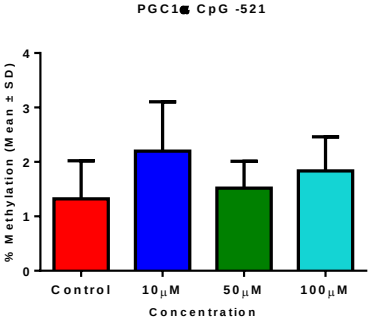












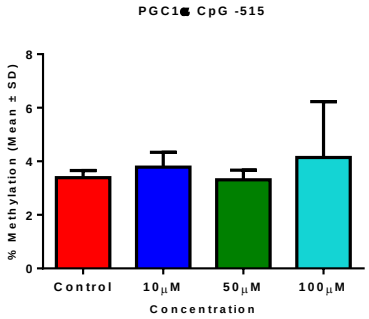


Figure 8.9- The effect of α-linolenic acid on the methylation of PGC1α. There was no significant effect of ALA on the methylation of CpG -816, -652, -617, -521 and -515 in the PGC1α promoter, determined by one-way ANOVA and Tukey's multiple comparison ($p>0.05$). There was a significant difference between the methylation of CpG -841 and -783 in the PGC1α promoter at 10μM and 100μM compared with the methylation at 50μM, determined by Tukey's multiple comparison ($p=0.04$ and 0.01 respectively). However, there was no significant effect of ALA on the methylation of CpG -841 and -783 at any of the concentrations tested, when compared to the control group ($p>0.05$). Therefore, supplementation with ALA did not significantly alter the methylation of these 7 CpG loci in the PGC1α promoter, when compared to the control group.

8.4 Discussion

8.4.1 ALA decreases the expression of PGC1α *in vitro*

These findings show that at physiological concentrations ALA significantly decreases the expression of PGC1α in human liposarcoma cells *in vitro*. There is a predicted PPAR/RXR (peroxisomal proliferator-activated receptor/retinoid X receptor) heterodimer binding site located within the PGC1α promoter (figure 3.6), and PPARs are known to be activated by a wide range of fatty acids (230). PPARs are able to bind fatty acids and serve as intracellular receptors, whilst also functioning as transcription factors by binding to specific regions of DNA to regulate gene expression (231). It is known that PPARs are able to activate and repress the expression of target genes, including adipocyte specific genes (230). Therefore, ALA supplementation may result in decreased PGC1α expression in human liposarcoma cells *in vitro* via the activation of PPAR, which in turn causes the repression of PGC1α.

ALA has been shown to increase PGC1α mRNA expression in adipocytes from mice, after 24 hour supplementation *in vitro* (31). These differences in the effect of ALA on PGC1α mRNA expression may be explained by the different transcripts found in humans and mice and the differences between using a cell line and primary cells. Another consideration is that the adipocytes were taken from male mice fed a diet supplemented with a n-3 concentrate containing DHA, EPA and ALA (31). Whilst these fatty acids are all from the same family, they have different biological properties and it is unknown if the effect of ALA supplementation on PGC1α mRNA expression was the result of one of the other fatty acids, or a combination of all three.

Other studies that have looked at the effect of *in vitro* fatty acid supplementation on the expression of PGC1α typically use one concentration (31)(227)(189). A range of concentrations were tested for each fatty acid in order to determine the effect of increasing concentration and if there was a dose response. The lowest concentration of ALA tested was 10μM, which decreased PGC1α mRNA expression to the same level as the highest concentration of 100μM, suggesting that the effect was not dependent on increasing concentration. However, the effect of ALA at a concentration less than 10μM could be tested in order to determine the exact concentration when decreased expression occurs.

ALA did not alter the expression of SIRT1, suggesting that ALA may work in a gene specific manner. Whilst there is a predicted PPARγ (peroxisomal proliferator-activated receptor gamma) binding site within the SIRT1 promoter (figure 3.3), it does not form a heterodimer with RXR as in the PGC1α promoter. Therefore, ALA may alter PGC1α gene expression via the PPAR/RXR binding site as

this is known to bind to the PPRE (peroxisomal proliferator response element) which may be located elsewhere within the promoter, and may cause the decrease in PGC1α gene expression (230). This could explain why ALA had no effect on SIRT1 expression. The other n-3 PUFAs tested were EPA and DHA, which did not alter the expression of SIRT1 or PGC1α, suggesting that specific fatty acids are important in the regulation of specific genes. There is evidence to suggest that specific fatty acids are able to bind to PPAR's whereas other cannot, which could explain why EPA and DHA had no effect on SIRT1 and PGC1α gene expression (231)(232).

LA had no effect on the expression of SIRT1 or PGC1α in human liposarcoma cells *in vitro*. Staiger et al. have shown that human myotubes supplemented with LA *in vitro* increased PGC1α mRNA expression, which suggests that LA may alter PGC1α in a tissue and cell specific manner (227). This could be explained by differing distribution and function of the PPAR subtypes within different metabolic tissues, which will affect target genes in a tissue specific manner (230). Another consideration is that six replicates were not enough to detect a significant effect on gene expression. A power calculation could be used with the results obtained in order to determine the minimum sample size required to measure a significant change, and then the experiment could be repeated.

The effect of different ratios of ALA:LA was not tested. The ratio of n-3 to n-6 PUFAs has been suggested to be important in the development of childhood and adulthood obesity (13). Therefore, it would be interesting to look at the effect of different ratios on the expression of SIRT1 and PGC1α in human liposarcoma cells *in vitro*. These findings have shown that ALA decreased PGC1α expression, whereas LA had no effect on PGC1α expression. Therefore, different ratios of ALA:LA in these cells may cause the expression of PGC1α to also change. For example, the expression of PGC1α may increase to levels more similar to the control cells if the ratio of ALA:LA were increased as lower ratios are associated with decreased adiposity as is increased PGC1α expression (13)(77)(79).

The monounsaturated fatty acid OA had no effect on the expression of SIRT1 or PGC1α in human liposarcoma cells *in vitro*. A previous study has shown that human myotubes supplemented with OA at 0.2mM and 0.4Mm *in vitro* increased PGC1α mRNA expression, in a dose dependent manner (228). However, whilst this study treated cells with different concentrations that are physiologically relevant, the cells were also treated with the saturated fatty acid PA at the same time as OA, at equal concentrations. Therefore, these effects on PGC1α expression may be the result of PA, rather than OA, or the combined effect of both fatty acids. Another study has shown that when adipocytes from mice fed a diet supplemented with a n-3 concentrate were supplemented with OA *in vitro*, there was no effect on PGC1α mRNA expression (31). Whilst these *in vitro* studies suggest that

OA may effect gene expression in a tissue specific manner, they do not specifically look at the effect of just OA on the expression of PGC1α mRNA expression because either the diet was previously supplemented or the cells were supplemented with a different class of fatty acid at the same time.

Royal jelly is comprised of 3-7% lipids, of which the monounsaturated fatty acid 10-H=DA and the saturated fatty acid 10-HDA are important (224). These fatty acids have been suggested to have health benefits in humans including anti-inflammatory, hypotensive and weight loss effects (224). It has been shown that supplementation with these fatty acids decreases body weight, BMI and total energy intake in T2D female patients, after only 8 weeks, when compared to the control group given a placebo (224). This suggests that these fatty acids may be beneficial for the prevention of obesity. However, human liposarcoma cells supplemented with 10-H=DA and 10-HDA *in vitro* did not affect SIRT1 or PGC1α mRNA expression. This could be because the cells were only supplemented for 72 hours and a longer period of time may be required in order to see an effect. The concentration of these fatty acids may not be enough in order to see an effect, or both fatty acids may be required together to cause an effect on gene expression. Another consideration is that the weight loss effects of royal jelly may occur via different genes involved in the development of obesity.

Taken together, these findings suggest that ALA decreases the expression of PGC1α in human liposarcoma cells *in vitro*, with no effect on SIRT1 expression. Other classes of fatty acids and other n-3 PUFAs had no effect on the expression of SIRT1 or PGC1α, suggesting that the effects of ALA supplementation are both fatty acid and gene specific. Therefore, because decreased expression of PGC1α *in vivo* is known to occur in obesity, decreasing the level of ALA in the diet may be a potential mechanism for decreasing the risk of obesity (79). However, more evidence is required to determine if ALA reduces PGC1α expression *in vivo* and its effects in different tissue types to determine if ALA plays a functional role in the development of obesity.

8.4.2 ALA does not alter the methylation of the PGC1α promoter *in vitro*

Because ALA decreased the expression of PGC1α in human liposarcoma cells *in vitro*, the effect of ALA on the methylation of the PGC1α promoter was tested. A range of concentrations were tested in order to determine the effect of increasing concentration on methylation and if there was a dose response.

These findings show that ALA did not alter the methylation of any of the CpG loci measured in the PGC1α promoter. However, the methylation of these loci was less than 5%, except for CpG -783 where the methylation was about 18%. Therefore, the range of methylation may be too small to

detect subtle differences in methylation between the control and ALA supplemented cells. Because only 7 CpG loci were measured, it is unknown if ALA supplementation altered the methylation of other loci within the PGC1α promoter. Another consideration is that cells were supplemented for 72 hours, which may not be long enough to induce methylation changes. However, the cells could not be supplemented for longer because they would have been too confluent and cell death would have occurred, which is a caveat to this experiment.

A previous study by Barrès et al. has shown that OA and PA increased non-CpG methylation of the PGC1α promoter in human skeletal muscle cells *in vitro*, but had no effect on CpG methylation (189). PA also significantly decreased PGC1α mRNA expression (189). Therefore, there may be changes in non-CpG methylation in the PGC1α promoter as a result of ALA supplementation, which could be tested in the future. Methylation was not measured around a PPRE site located elsewhere within the promoter, which is known to be important in the action of PPAR/RXR mediated fatty acid signalling (230). Altered methylation around a PPRE site may provide a mechanism for potential ALA-mediated methylation changes.

In the Barrès et al. study, the fatty acids that induced non-CpG methylation changes were a monounsaturated and saturated fatty acids, which could explain by ALA, an n-3 PUFA, had no effect on methylation. Another consideration is that Barrès et al. measured these effects in human skeletal muscle cells, suggesting there may be both a fatty acid and cell specific effect on PGC1α methylation. Also, the skeletal muscle cells were supplemented with fatty acids at a concentration of 0.5mM, which is greater than other *in vitro* studies which typically supplement cells at a concentration up to 0.3mM (31)(227)(228)(189)(154)(155)(156). Therefore, the changes seen in non-CpG methylation and expression of PGC1α may not be representative of the changes that may occur *in vivo* at more physiological concentrations.

8.5 Summary

These findings in part support the hypothesis that fatty acids can alter the expression and methylation of SIRT1 and PGC1α *in vitro*, where ALA supplementation decreased PGC1α mRNA expression, but had no effect on SIRT1 expression or PGC1α methylation. These findings suggest that PGC1α expression is regulated in a fatty acid specific manner, with other classes of fatty acids having no effect on expression. Further studies are required to determine how these fatty acid specific effects occur, which may involve the activation of a PPAR, and a PPRE. More information is required to determine if these changes in PGC1α are associated with changes in non-CpG methylation or potentially histone changes. This would allow for prenatal and postnatal dietary intervention with specific fatty acids as a potential mechanism to activate and repress specific genes and pathways, thereby reducing the risk of obesity later in life.

Chapter 9

Discussion

9.0 Discussion

Over the past 20 years there has been a rapid rise in the incidence of obesity worldwide, in both adults and children (4). Evidence from epidemiological studies suggests that the prenatal and early life environment can increase the risk of obesity in later life. Several studies have provided evidence that environmental factors such as diet and exercise can alter the epigenetic regulation of key genes involved in the regulation of metabolism, including PGC1α, which may contribute to the development of disease later in life (164)(82)(79). There is conflicting data regarding the stability of DNA methylation throughout the life course, particularly during childhood (132)(136). This suggests that the environment can potentially influence the epigenome throughout the life course, not just during critical periods in development.

This work was completed in order to increase our knowledge on the stability of DNA methylation of specific loci throughout childhood and puberty. Secondly, this work was completed to determine if the methylation of specific loci in key genes involved in metabolism and adipogenesis is induced during early life and associates with future adiposity, and may have utility as a potential epigenetic biomarker of obesity risk.

9.1 Summary of main findings

These results have shown that the methylation of specific CpG loci in the regulatory regions of five genes involved in metabolism is temporally stable throughout childhood and puberty when measured in peripheral blood. Despite peripheral blood being a heterogeneous mix of cellular populations, the methylation of the majority of the loci measured was unaffected by the proportion of leukocytes. The methylation of CpG -880 in SIRT1 and CpGs -841, -816, -783 and -521 in PGC1α in peripheral blood at 5-7 years predicted future childhood adiposity, up to 14 years. For each 10% difference in methylation at 5-7 years per cent body fat was decreased by 3.0% at CpG -880 in SIRT1, and increased by 12.7%, 7.5%, 6.4% and 7.4% at CpGs -841, -816, -783 and -521 in PGC1α, respectively. However, the methylation of these loci, nor any other loci measured in HNF4α, RXRα and GCK, was able to predict future insulin resistance.

Methylation of CpG -783 in the PGC1α promoter enhanced the binding of a HOXB9-PBX1 heterodimer, with loss of methylation causing PBX1 to bind with an unidentified partner. Both HOXB9 and PBX1 are known to be involved in adipogenesis, further supporting a positive link between increased methylation of PGC1α and adiposity.

The methylation of specific loci in the SIRT1 and PGC1α promoter that associated with future childhood adiposity was not predicted by the methylation of these loci in their parents, when measured in peripheral blood. However, the methylation of these loci in fetal tissues associated with several measures of adiposity during childhood, suggesting that the methylation of these loci may be determined *in utero*. Furthermore, the methylation of these loci in the PGC1α promoter was increased in the subcutaneous adipose tissue of obese subjects compared to lean, and positively associated with BMI, % fat and waist circumference. This suggests that these predictive biomarkers persist into adulthood and are present when the diseased state has occurred. However, there were no changes in the expression of SIRT1 or PGC1α in the subcutaneous adipose tissue of obese subjects when compared to lean subjects.

PGC1α mRNA expression was decreased in human liposarcoma cells supplemented with α-linolenic acid (ALA) *in vitro*, which was not associated with a change in methylation of the seven CpG loci measured within the promoter. This suggests that fatty acid supplementation may be a mechanism for influencing PGC1α gene expression, but may not alter the methylation of these predictive biomarkers.

9.2 Discussion of main findings

There is a lack of information about the stability of DNA methylation across the life course, in particular the stability of DNA methylation throughout childhood and puberty, which have been proposed to be periods of increased epigenetic change (95). The results in chapter 3 have shown that the methylation of the majority of the CpG loci in the regulatory regions of five genes involved in metabolism was stable between 5-7 and 14 years, when measured in peripheral blood. This supports data from animal and human studies that have shown the methylation of specific regions of the epigenome are stably maintained over time (132)(130). This suggests that the methylation of some loci may be stable during childhood and may not be influenced by future environmental challenges such as diet, pollution, exercise and hormonal changes that occur during puberty.

The methylation of each CpG loci measured in the PGC1α promoter in peripheral blood was shown to be temporally stable throughout childhood and puberty. This is conflicting with data from other studies that have shown that methylation of PGC1α is altered by exercise and diet (164)(82). However, these studies measure the methylation in skeletal muscle rather than peripheral blood where the methylation may be more dynamically regulated. Also, the effect of these factors on methylation was determined using different techniques besides pyrosequencing which have

disadvantages. For example, MeDIP measures the relative enrichment of DNA rather than absolute DNA methylation and therefore changes in methylation may be very small, and bisulphite sequencing is less accurate than pyrosequencing.

There is evidence to suggest that the prenatal and early life environment can potentially induce differentially methylated loci, which, if associated with a particular disease has the ability to act as a biomarker of disease risk (177). The results in chapter 4 have shown that the methylation of CpG -880 in the SIRT1 promoter and CpGs -841, -816, -783 and -521 in the PGC1α promoter at 5-7 years in peripheral blood predicted adiposity between 9 and 14 years, independent of sex, age, pubertal timing and physical activity. Furthermore, the methylation of SIRT1 was negatively associated with future adiposity with a 10% increase in methylation predicting a 3.0% decrease in percentage fat mass, whereas the methylation of PGC1α was positively associated with future adiposity with a 10% increase in methylation predicting between 6.4% and 12.7% increase in percentage fat mass. SIRT1 is known to positively regulate PGC1α activity via deacetylation of several lysine residues, thereby increasing the ability of PGC1α to activate target genes (69)(70). SIRT1 has been shown to inhibit adipogenesis by repressing PPARγ in adipocytes and increasing fat mobilisation (63). PGC1α also inhibits adipogenesis and its activation inhibits fat storage in preadipocytes and promotes increased fat mobilisation in mature adipocytes (78). The expression of SIRT1 and PGC1α are known to be decreased in the subcutaneous adipose tissue of obese subjects, compared to lean (79)(72)(73)(49). Therefore, it may be expected that the association between methylation and future adiposity would be in the same direction for these genes. However, it is unknown if the methylation of these loci affects the binding of transcription factors, or whether methylation affects gene expression in peripheral blood. For example, NKX61 is predicted to bind a region just upstream of CpG -880 in the SIRT1 promoter, which is known to be a transcriptional repressor *in vitro* (181). Whilst no studies have shown that methylation blocks the binding of this specific transcription factor, it is well known that this can occur within gene promoters (104)(182). Therefore, increased methylation of CpG -880 in SIRT1 could block the binding of this repressive transcription factor resulting in increased gene expression. This would mean that when unmethylated there would be less SIRT1 expression and would support previous data where expression is decreased in obesity (72)(73).

Whilst the methylation of these specific loci in the SIRT1 and PGC1α promoters at 5-7 years was able to predict future adiposity, when measured in peripheral blood, the methylation of these loci was unable to predict insulin resistance after correcting for sex, age, pubertal timing and physical activity. This suggests that insulin resistance is not associated with the methylation of the loci in

childhood. Obesity can occur in childhood, without insulin resistance, and these results support previous evidence where these diseases have been shown not to correlate with each other (188)(185). Insulin secretion is known to increase during puberty, to overcome the temporary insulin resistance that occurs (183)(184)(185). This suggests that in these children, the β cells were able to increase insulin secretion to overcome this temporary insulin resistance and does not indicate any sign of β cell exhaustion that often occurs as a result of insulin resistance.

The effect of differential methylation of one of these loci in PGC1α was explored further using EMSA, to determine if methylation has any functional significance on gene expression. The results in chapter 5 have shown that methylation of CpG -783 in the PGC1α promoter enhances the binding of a HOXB9-PBX1 heterodimer. Methylation is generally associated with a reduction in transcription factor binding as the methyl group physically blocks the binding site, or recruits methyl binding proteins that occupy the binding site (104). However, there is increasing evidence to suggest that DNA methylation can enhance the binding of some transcription factors (105)(106). Whilst it has not been shown that methylation enhances the binding of HOXB9 or PBX1 to their target sequence, these findings support the growing evidence that methylation does not always inhibit transcription factor binding. Findings in chapter 5 also showed for the first time that differential methylation can lead to alternative PBX1 containing heterodimers, shown in figure 9.1. When CpG -783 was methylated PBX1 bound with HOXB9, but when unmethylated bound with an unidentified partner, suggesting that methylation at this site could potentially influence transcription factor binding which may have an effect on gene expression. PBX1 is known to bind as a heterodimer with other HOX proteins, some of which are activators, whilst others are repressors (193)(144). HOX-PBX1 heterodimers are also known to form trimeric complexes with PREP1, suggesting that differential methylation of this locus is potentially important for the binding of several transcription factors and may be a involved in a complex regulatory mechanism (194).

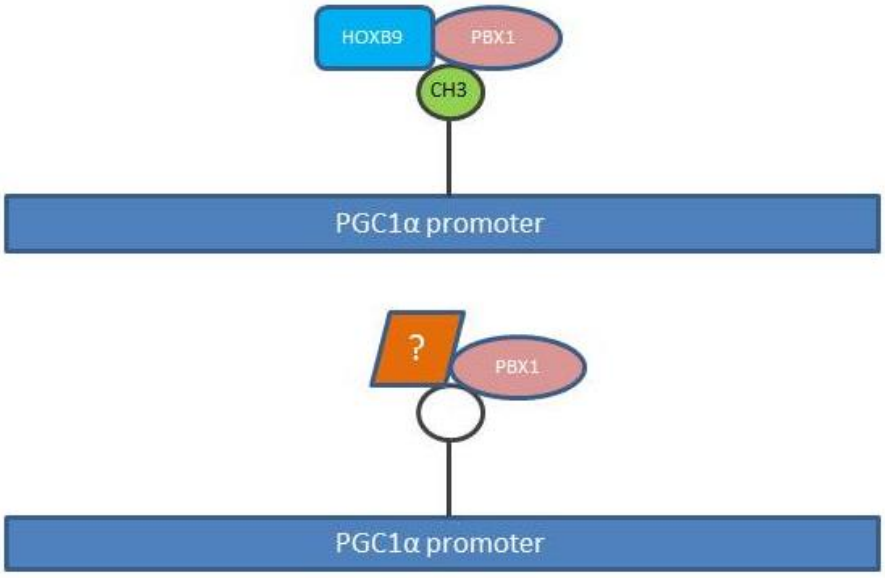


Figure 9.1- Differential methylation of CpG -783 in the PGC1α promoter alters the binding of a PBX1 heterodimer. When CpG -783 is methylated, shown by the green circle, the HOXB9-PBX1 heterodimer binds. When CpG -783 is unmethylated, shown by an empty circle, PBX1 binds with an unidentified partner.

PBX1 and the HOX genes are known to be involved in adipogenesis (186)(191)(196). In humans, PBX1 expression is increased in the subcutaneous adipose tissue of obese subjects and is known to promote adipogenesis (186). This suggests that PBX1 may be involved in the development of obesity. The results in chapter 5 have shown that PBX1 decreased the expression of unmethylated PGC1α, suggesting that methylation of CpG -783 may be important in regulation the expression of PGC1α. Furthermore, because methylation of this locus in peripheral blood was associated with increased adiposity, and because methylation of this locus enhances the binding of PBX1, this suggests that methylation may be important in determining adiposity. For example, subjects with higher methylation and adiposity may have enhanced binding of PBX1, which in turn may promote the proliferation of adipocyte progenitor cells increasing adipocyte numbers and result in decreased PGC1α expression. This would support previous evidence that has shown PGC1α expression is decreased in adiposity (79)(49). Taken together, this supports a positive relationship between methylation of this locus and adiposity and suggests that methylation may be functionally important in the development of obesity.

Because the methylation of specific loci in the SIRT1 and PGC1α promoters in peripheral blood was stable between 5-7 and 14 years and was predictive of future adiposity, the methylation of these loci was measured in peripheral blood from the parents of these children to determine if the methylation in the parents could predict the methylation of the same loci in the children. Results from chapter 6 show that the methylation of these loci was not predicted by the methylation of either the mother or father in these children. This suggests that the methylation of these loci is determined at some point in development between fertilisation and the age of 5-7 years. To explore this further, the methylation of these loci were measured in umbilical cord and umbilical cord blood from the SWS cohort. The results in chapter 7 have shown that the methylation of CpG -880 and -865 in SIRT1 in umbilical cord is negatively associated with subscapular skinfold thickness at birth and 2 years, and fat mass at 4 and 6 years of age. The methylation of several loci in the PGC1α promoter in umbilical cord positively associated with tricep skinfold thickness at several ages between birth and 3 years, and fat mass at 4 and 6 years of age. Therefore, the associations between methylation of SIRT1 and PGC1α and skinfold thickness occur at a younger age than with fat mass. This is likely explained by the different types of fat depots that these two techniques can measure. Skinfold thickness is a measurement of specific subcutaneous fat depots, whereas DXA scans are able to measure both subcutaneous and visceral fat and therefore is a combination of both types of fat (20)(23). This may suggest that associations with percentage fat are more informative for determining the risk of visceral fat, and may be a better predictor of increased risk of obesity related disease.

The methylation of several CpG loci in the SIRT1 promoter in umbilical cord blood associated with total fat mass at 4 years and with tricep and subscapular skinfold thickness at several ages between birth and 6 years of age, however the association was positive. The methylation of PGC1α in umbilical cord blood was not associated with fat mass at any age, and only the methylation of CpG -617 negatively associated with tricep skinfold thickness at 6 years. The difference in the direction of these associations with adiposity in umbilical cord blood compared to umbilical cord and peripheral blood is likely explained by differences in cell populations. Whilst umbilical cord and umbilical cord blood both contain mesenchymal stem cells, umbilical cord blood also contains nucleated red blood cells, which are not found in peripheral blood (123)(124)(125). Therefore, the presence of nucleated red blood cells in umbilical cord blood could explain the difference in the direction of the association between methylation and measures of adiposity in peripheral blood, and also umbilical cord. This suggests that umbilical cord blood has a unique epigenetic signature, whereas the methylation of

these loci in umbilical cord and peripheral blood appear to behave in a similar manner, despite these tissues being from different germline layers and having different levels of methylation.

To determine if the methylation of these potential biomarkers of adiposity was altered in obesity, and if the association between methylation and adiposity remained when the disease state was present, methylation was measured in the subcutaneous adipose tissue from lean and obese subjects. The methylation of CpG -880 and -865 in SIRT1 was not significantly different in the subcutaneous adipose tissue from lean and obese subjects, and methylation was not associated with different measures of obesity. This could be because the sample size was inadequately powered to detect a significant difference in methylation of these loci between the lean and obese groups. The methylation of CpGs -841, -816 and -783 in PGC1α was increased in the subcutaneous adipose tissue from obese subjects, compared to lean subjects, and positively associated with BMI, percentage fat and waist measurement. These findings agree with those from a study by Chen et al. where the mean methylation of CpGs -841 -816, -783, -652 and -617 in PGC1α was shown to be increased in the subcutaneous adipose tissue from obese subjects, compared to lean subjects, classified according to BMI (79). Taken together, this further supports that increased methylation of PGC1α is associated with adiposity, even when the disease state is present. The expression of SIRT1 and PGC1α was not significantly different in the subcutaneous adipose tissue from lean and obese subjects, which could be due to a small sample size as previous studies have shown the expression of these genes is decreased in adipose tissue during obesity (73)(79). Or, the expression of these genes may be altered in a different tissue and indirectly involved in the development of obesity. For example, the expression may be altered in the brain, which could influence appetite and calorie intake. Expression may be altered in the liver or skeletal muscle and may affect fatty acid storage. Alternatively, the methylation of SIRT1 and PGC1α may be biomarkers of adiposity, and methylation may not be functionally involved in the development of obesity.

Maternal diet is a potential factor that could alter the fetal epigenome and influence the methylation of these predictive biomarkers of adiposity *in utero*. The amount and type of dietary fat could be important in inducing methylation changes that are associated with future adiposity, as high fat diets have increased worldwide coinciding with the dramatic rise in obesity. The results from chapter 8 have shown that human liposarcoma cells supplemented with various fatty acids including n-3 PUFAs, n-6 PUFAs, monounsaturated fatty acids and saturated fatty acids did not alter the expression of SIRT1 or PGC1α, apart from ALA, which reduced PGC1α at all concentrations between 10 and 100μM. When cells were supplemented with the same concentrations of ALA, there was no

change in PGC1α methylation, suggesting that fatty acid intake may not be involved in determining the level of methylation of these predictive loci. It is possible that other components of the maternal diet could be influencing the level of methylation of these loci. For example, evidence from animal models suggests that protein restricted diets induce altered methylation of specific loci that are associated with obesity (113).

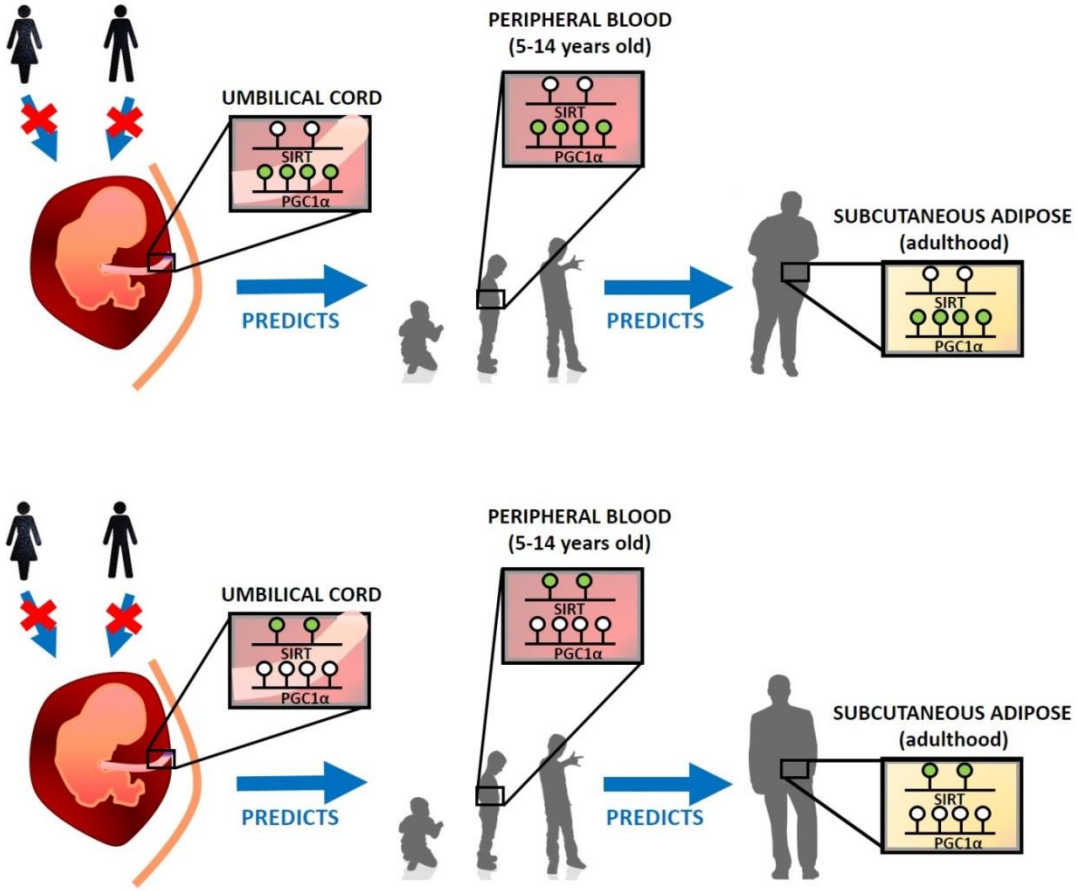


Figure 9.2- The proposed mechanism by which SIRT1 and PGC1α methylation can be epigenetic biomarkers of adiposity. The top panel shows the route by which SIRT1 and PGC1α methylation can predict adulthood obesity, whereas the bottom panel shows the route by which methylation can determine a healthy weight in adulthood. The methylation of these loci in the parents does not predict the methylation of these biomarkers of adiposity in their children, shown by the red crosses. The methylation is determined *in utero* and methylation is maintained throughout the life course. Methylation can be measured in umbilical cord, peripheral blood and subcutaneous adipose tissue to predict adiposity. The level of methylation at birth predicts which trajectory the subject is on. Decreased methylation is shown by an empty circle, whereas increased methylation is shown by a green circle.

Taken together, all the results from this thesis suggest that the methylation of CpG -880 and -865 in the SIRT1 promoter and CpGs -841, -816, -783 and -521 in the PGC1α promoter may be suitable as potential epigenetic biomarkers of adiposity. Figure 9.2 shows the proposed mechanism by which SIRT1 and PGC1α methylation can act as biomarkers of future adiposity. The methylation of these loci was associated with various measures of obesity including percentage fat, skinfold thickness, BMI and waist circumference when measured in multiple tissues from different germline layers. Because the association between methylation and adiposity was present from birth through to adulthood in these different tissue types, this suggests the methylation of these loci is determined *in utero* and then stably maintained throughout the life course. Therefore, an environmental challenge *in utero* could affect the methylation of the different germline layers equally. Furthermore, the methylation of these loci is associated with adiposity even when the disease state is present suggesting that methylation is stable during disease progression. Despite the actual levels of methylation not being the same across these tissue types, the same associations with adiposity still occur. This suggests that whilst tissues such as umbilical cord and peripheral blood are a heterogeneous mix of cell populations, they are still beneficial for measuring these potential biomarkers as they are easy to access. Therefore, measuring the methylation of these loci at birth or during early childhood may be beneficial for predicting those individuals at an increased risk of obesity in later life who may benefit from targeted intervention.

9.3 Future work

The findings in this thesis support the DOHaD theory and suggest that the methylation of specific CpG loci that are determined during early life may have utility in predicting future disease risk. However, there are some key mechanisms and questions that need further exploration which could be addressed with future work.

Differential methylation of CpG -783 in PGC1α resulted in alternative PBX1 containing heterodimers to bind to this region of the promoter. When methylated, PBX1 bound with HOXB9, however when unmethylated, PBX1 bound to the promoter region with an unidentified partner. Chromatin immunoprecipitation (ChIP) could be used to determine which transcription factor(s) bind with PBX1 to the unmethylated sequence. Sequencing would then determine the transcription factor(s) that were bound to this specific region of the PGC1α promoter. Another member of the HOX family, HOXA10, has been shown to bind to the same sequence as HOXB9 apart from the sequence contains a T in place of the C of CpG -783 (143). Therefore, HOXA10 may be able to bind to the

unmethylated sequence with PBX1, but binding to the methylated sequence may be blocked by the methyl group. It is important to determine which transcription factor(s) is bound with PBX1 to enable the effect on gene expression to be further explored.

Further studies are required to understand the mechanism by which differential methylation of CpG -783 in the PGC1α promoter may be involved in regulating gene expression. This could be determined by transfecting human liposarcoma cells with a construct containing the PGC1α promoter where CpG -783 is either unmethylated or methylated, and luciferase activity compared. Whilst this method of site specific methylation has been published recently, the ligation of methylated PCR product back into the plasmid backbone is extremely inefficient and very costly (233). Therefore, mutating the cytosine to another base and determining the effect on luciferase activity may be a more reliable method to determine if this base is important for gene expression. However, this would not determine if methylation of this specific cytosine is functionally important for the regulation of gene expression.

Results from this thesis suggest that the methylation of these loci which are associated with adiposity might be determined *in utero* and the fetus may be on a set trajectory from birth. Therefore, further statistical analyses are required to determine which factors are responsible for determining the methylation of these loci prenatally. The SWS cohort has additional information available on many maternal factors including pre-pregnancy BMI, weight gain during pregnancy, smoking status, diet throughout pregnancy, and educational attainment. This would allow further statistical models to be generated to determine which of these factors, or combination of factors, might be significantly associated with the methylation of these loci in the offspring. This may provide a mechanism by which the prenatal environment is able to determine the methylation of these loci in the offspring and increase their risk of adiposity later in life.

Further work is required to determine if altered methylation of these loci in SIRT1 and PGC1α is causally involved in the development of obesity, or if they are simply biomarkers. Whilst the methylation of PGC1α was increased in the subcutaneous adipose tissue of obese subjects, when compared to lean subjects, both PGC1α and SIRT1 expression were unaltered. Because both genes are expressed in a wide range of tissues and important in the regulation of several metabolic processes, further studies are required to determine if the methylation of these loci alters gene expression in another pathway besides adipogenesis (63)(75)(75)(66). For example, altered methylation could affect gene expression in the brain, which could affect appetite and lead to obesity as a result of increased calorie intake. SIRT1 expression in the hypothalamus is known to be important

in coordinating neuroendocrine responses that control appetite (234). PGC1α is also known to be expressed the brain of mice and therefore may be important in the control of appetite (235). Another possibility is that altered methylation may affect SIRT1 and PGC1α expression in the liver, which could lead to the development of obesity by reduced re-esterification of NEFAs to triglycerides and increased NEFAs in circulation, which could have downstream effects on fat storage (38)(8)(9). Therefore, the association between methylation and gene expression could be determined in other tissue types obtained from post mortems and surgical procedures. The mechanisms involved could also be further explored using an animal model where the maternal and offspring diet could be manipulated during various stages of development. Animal studies would determine if the methylation of these loci and the phenotype could be altered by diet. A recent study in humans has shown that the methylation of specific loci in genes involved in fatty acid biosynthesis is altered by n-3 PUFA and olive oil supplementation, in peripheral blood mononuclear cells from men and women (236). This may provide a potential mechanism to reverse the methylation of these loci and potentially the phenotype.

9.4 Implications for human health

It is well understood that the prenatal and early life environment can alter the epigenetic regulation of genes, which can increase the risk of obesity later in life. The results of this study have shown that altered methylation of temporally stable loci in SIRT1 and PGC1α associates with future adiposity in childhood. Furthermore, these associations between altered methylation and adiposity are present in several tissue types from different germline layers from birth through to adulthood, suggesting the *in utero* environment is important in determining the methylation of these loci. This suggests that peripheral blood and fetal tissues may be suitable to measure these potential biomarkers during early life which may be beneficial for predicting future risk of obesity. This would allow for the identification of individuals who may benefit from targeted and preventative mechanisms in order to decrease their risk of obesity later in life.

Chapter 10

Appendix

10.1 Appendix

Table 10.1- The stability of methylation across the SIRT1 promoter in peripheral blood each year between 5-7 and 14 years

Age (years)	CpG -880					
	8-9y	**10y**	**11y**	**12y**	**13y**	**14y**
5-7y	r= 0.93 p< 0.001	0.95 <0.001	0.95 <0.001	0.92 <0.001	0.96 <0.001	0.94 <0.001
8-9y		0.93 <0.001	0.97 0.001	0.96 <0.001	0.95 <0.001	0.90 <0.001
10y			0.93 <0.001	0.92 <0.001	0.94 <0.001	0.93 <0.001
11y				0.93 <0.001	0.92 <0.001	0.90 <0.001
12y					0.94 <0.001	0.90 <0.001
13y						0.96 <0.001
Age (years)	**CpG -865**					
	8-9y	**10y**	**11y**	**12y**	**13y**	**14y**
5-7y	r= 0.86 p<0.001	0.87 <0.001	0.85 <0.001	0.83 <0.001	0.82 <0.001	0.79 <0.001
8-9y		0.92 <0.001	0.91 <0.001	0.88 <0.001	0.79 <0.001	0.89 <0.001
10y			0.93 <0.001	0.88 <0.001	0.87 <0.001	0.82 <0.001
11y				0.90 <0.001	0.86 <0.001	0.86 <0.001
12y					0.88 <0.001	0.81 <0.001
13y						0.91 <0.001
Age (years)	**CpG -760**					
	8-9y	**10y**	**11y**	**12y**	**13y**	**14y**
5-7y	r= 0.89 p<0.001	0.81 <0.001	0.82 <0.001	0.84 <0.001	0.78 <0.001	0.85 <0.001
8-9y		0.73 <0.001	0.83 <0.001	0.85 <0.001	0.82 <0.001	0.90 <0.001
10y			0.76 <0.001	0.62 <0.001	0.64 <0.001	0.79 <0.001
11y				0.71 <0.001	0.67 <0.001	0.81 <0.001
12y					0.76 <0.001	0.81 <0.001
13y						0.85 <0.001

Age (years)	CpG -742					
	8-9y	10y	11y	12y	13y	14y
5-7y	r= 0.85 p<0.001	0.85 <0.001	0.83 <0.001	0.94 <0.001	0.91 <0.001	0.82 <0.001
8-9y		0.84 0.008	0.81 <0.001	0.85 <0.001	0.81 <0.001	0.75 <0.001
10y			0.79 <0.001	0.88 <0.001	0.80 <0.001	0.74 <0.001
11y				0.78 <0.001	0.81 <0.001	0.80 <0.001
12y					0.88 <0.001	0.86 <0.001
13y						0.91 <0.001
Age (years)	CpG -722					
	8-9y	10y	11y	12y	13y	14y
5-7y	r= 0.79 p<0.001	0.77 <0.001	0.83 <0.001	0.74 <0.001	0.84 <0.001	0.75 <0.001
8-9y		0.73 <0.001	0.84 <0.001	0.73 <0.001	0.73 <0.001	0.61 <0.001
10y			0.75 <0.001	0.58 <0.001	0.65 <0.001	0.74 <0.001
11y				0.63 <0.001	0.81 <0.001	0.75 <0.001
12y					0.62 <0.001	0.70 <0.001
13y						0.86 <0.001
Age (years)	CpG -689					
	8-9y	10y	11y	12y	13y	14y
5-7y	r= 0.86 p<0.001	0.84 <0.001	0.91 <0.001	0.73 <0.001	0.83 <0.001	0.73 <0.001
8-9y		0.85 <0.001	0.88 <0.001	0.72 <0.001	0.81 <0.001	0.80 <0.001
10y			0.88 <0.001	0.74 <0.001	0.81 <0.001	0.83 <0.001
11y				0.73 <0.001	0.90 <0.001	0.81 <0.001
12y					0.82 <0.001	0.74 <0.001
13y						0.84 <0.001

Age (years)	CpG -668					
	8-9y	**10y**	**11y**	**12y**	**13y**	**14y**
5-7y	r= 0.83 p<0.001	0.83 <0.001	0.84 <0.001	0.75 <0.001	0.74 <0.001	0.85 <0.001
8-9y		0.79 <0.001	0.84 <0.001	0.82 <0.001	0.67 <0.001	0.81 <0.001
10y			0.85 <0.001	0.68 <0.001	0.83 <0.001	0.86 <0.001
11y				0.66 <0.001	0.77 <0.001	0.81 <0.001
12y					0.58 <0.001	0.65 <0.001
13y						0.84 <0.001

Table 10.2- The stability of methylation across the PGC1α promoter in peripheral blood each year between 5-7 and 14 years

	CpG -841					
	8-9y	**10y**	**11y**	**12y**	**13y**	**14y**
5-7y	r= 0.94 p< 0.001	0.89 <0.001	0.94 <0.001	0.91 <0.001	0.82 <0.001	0.91 <0.001
8-9y		0.87 <0.001	0.94 <0.001	0.91 <0.001	0.81 <0.001	0.87 <0.001
10y			0.86 <0.001	0.90 <0.001	0.80 <0.001	0.86 <0.001
11y				0.93 <0.001	0.90 <0.001	0.90 <0.001
12y					0.86 <0.001	0.93 <0.001
13y						0.86 <0.001
	CpG -816					
	8-9y	**10y**	**11y**	**12y**	**13y**	**14y**
5-7y	r= 0.96 p< 0.001	0.97 < 0.001	0.97 < 0.001	0.95 < 0.001	0.95 < 0.001	0.94 < 0.001
8-9y		0.97 < 0.001	0.98 < 0.001	0.96 < 0.001	0.96 < 0.001	0.96 < 0.001
10y			0.97 < 0.001	0.96 < 0.001	0.97 < 0.001	0.95 < 0.001
11y				0.96 < 0.001	0.97 < 0.001	0.97 < 0.001
12y					0.96 < 0.001	0.96 < 0.001
13y						0.96 < 0.001
	CpG -783					
	8-9y	**10y**	**11y**	**12y**	**13y**	**14y**
5-7y	r= 0.94 p< 0.001	0.96 < 0.001	0.95 < 0.001	0.95 < 0.001	0.96 < 0.001	0.94 < 0.001
8-9y		0.92 < 0.001	0.94 < 0.001	0.92 < 0.001	0.94 < 0.001	0.89 < 0.001
10y			0.95 < 0.001	0.95 < 0.001	0.96 < 0.001	0.94 < 0.001
11y				0.95 < 0.001	0.97 < 0.001	0.93 < 0.001
12y					0.95 < 0.001	0.96 < 0.001
13y						0.95 < 0.001

	CpG -652					
	8-9y	**10y**	**11y**	**12y**	**13y**	**14y**
5-7y	r= 0.90 p< 0.001	0.95 < 0.001	0.93 < 0.001	0.93 < 0.001	0.96 < 0.001	0.94 < 0.001
8-9y		0.93 < 0.001	0.94 < 0.001	0.92 < 0.001	0.89 < 0.001	0.91 < 0.001
10y			0.92 < 0.001	0.97 < 0.001	0.94 < 0.001	0.97 < 0.001
11y				0.89 < 0.001	0.91 < 0.001	0.90 < 0.001
12y					0.93 < 0.001	0.98 < 0.001
13y						0.92 < 0.001
	CpG -617					
	8-9y	**10y**	**11y**	**12y**	**13y**	**14y**
5-7y	r= 0.94 p< 0.001	0.96 < 0.001	0.94 < 0.001	0.95 < 0.001	0.95 < 0.001	0.94 < 0.001
8-9y		0.98 < 0.001	0.98 < 0.001	0.97 < 0.001	0.98 < 0.001	0.97 < 0.001
10y			0.97 < 0.001	0.95 < 0.001	0.96 < 0.001	0.96 < 0.001
11y				0.97 < 0.001	0.98 < 0.001	0.97 < 0.001
12y					0.99 < 0.001	0.98 < 0.001
13y						0.99 < 0.001
	CpG -521					
	8-9y	**10y**	**11y**	**12y**	**13y**	**14y**
5-7y	r= 0.84 p< 0.001	0.65 < 0.001	0.90 < 0.001	0.90 < 0.001	0.89 < 0.001	0.88 < 0.001
8-9y		0.65 < 0.001	0.89 < 0.001	0.89 < 0.001	0.90 < 0.001	0.87 < 0.001
10y			0.62 < 0.001	0.74 < 0.001	0.72 < 0.001	0.61 < 0.001
11y				0.93 < 0.001	0.91 < 0.001	0.93 < 0.001
12y					0.93 < 0.001	0.92 < 0.001
13y						0.87 < 0.001

	CpG -515					
	8-9y	**10y**	**11y**	**12y**	**13y**	**14y**
5-7y	r= 0.90 p< 0.001	0.95 < 0.001	0.91 < 0.001	0.89 < 0.001	0.97 < 0.001	0.90 < 0.001
8-9y		0.93 < 0.001	0.92 < 0.001	0.92 < 0.001	0.93 < 0.001	0.92 < 0.001
10y			0.96 < 0.001	0.95 < 0.001	0.94 < 0.001	0.94 < 0.001
11y				0.91 < 0.001	0.88 < 0.001	0.96 < 0.001
12y					0.89 < 0.001	0.91 < 0.001
13y						0.90 < 0.001

Table 10.3- The stability of methylation across the HNF4α distal/P2 promoter in peripheral blood each year between 5-7 and 14 years

	CpG -121					
	8-9y	**10y**	**11y**	**12y**	**13y**	**14y**
5-7y	r= 0.56 p= 0.001	0.63 <0.001	0.68 <0.001	0.51 0.001	0.72 <0.001	0.52 0.001
8-9y		0.24 0.20	0.82 <0.001	0.23 0.21	0.61 <0.001	0.26 0.15
10y			0.39 0.02	0.78 <0.001	0.49 0.003	0.62 <0.001
11y				0.24 0.16	0.68 <0.001	0.25 0.13
12y					0.34 0.04	0.65 <0.001
13y						0.38 0.02
	CpG -117					
	8-9y	**10y**	**11y**	**12y**	**13y**	**14y**
5-7y	r= 0.36 p=0.04	0.25 0.16	0.26 0.13	0.17 0.32	-0.04 0.83	0.24 0.16
8-9y		0.15 0.43	0.14 0.46	-0.11 0.56	0.33 0.06	0.42 0.02
10y			-0.13 0.44	0.15 0.38	-0.14 0.43	0.13 0.46
11y				-0.10 0.57	0.25 0.15	0.36 0.03
12y					-0.11 0.52	0.06 0.74
13y						0.21 0.20
	CpG -103					
	8-9y	**10y**	**11y**	**12y**	**13y**	**14y**
5-7y	r= 0.21 p= 0.25	0.21 0.22	-0.08 0.65	0.11 0.50	0.39 0.02	0.37 0.03
8-9y		0.52 0.003	0.38 0.03	0.38 0.03	0.38 0.03	0.51 0.003
10y			0.28 0.11	0.63 <0.001	0.24 0.17	0.55 0.001
11y				0.44 0.007	0.36 0.03	0.18 0.28
12y					0.30 0.07	0.50 0.001
13y						0.30 0.07

	CpG -94					
	8-9y	**10y**	**11y**	**12y**	**13y**	**14y**
5-7y	r= 0.69 p< 0.001	0.71 <0.001	0.61 <0.001	0.64 <0.001	0.65 <0.001	0.73 <0.001
8-9y		0.65 <0.001	0.83 <0.001	0.69 <0.001	0.68 <0.001	0.72 <0.001
10y			0.66 <0.001	0.62 <0.001	0.56 <0.001	0.59 <0.001
11y				0.61 <0.001	0.83 <0.001	0.70 <0.001
12y					0.56 <0.001	0.68 <0.001
13y						0.76 <0.001
	CpG -79					
	8-9y	**10y**	**11y**	**12y**	**13y**	**14y**
5-7y	r= 0.51 p= 0.003	0.78 <0.001	0.84 <0.001	0.63 <0.001	0.76 <0.001	0.79 <0.001
8-9y		0.53 0.002	0.55 0.001	0.58 0.001	0.59 <0.001	0.58 <0.001
10y			0.73 <0.001	0.58 <0.001	0.70 <0.001	0.76 <0.001
11y				0.72 <0.001	0.76 <0.001	0.77 <0.001
12y					0.61 <0.001	0.79 <0.001
13y						0.71 <0.001

Table 10.4- The stability of methylation in the RXRα gene in peripheral blood each year between 5-7 and 14 years

	CpG -2346					
	8-9y	**10y**	**11y**	**12y**	**13y**	**14y**
5-7y	r= -0.29 p= 0.15	0.29 0.14	-0.32 0.09	0.33 0.09	-0.26 0.18	0.22 0.23
8-9y		-0.30 0.18	0.65 0.001	-0.47 0.03	0.18 0.42	-0.41 0.04
10y			-0.21 0.28	0.67 <0.001	0.04 0.83	0.29 0.15
11y				-0.33 0.09	0.14 0.48	-0.05 0.80
12y					-0.16 0.40	0.48 0.008
13y						0.04 0.85
	CpG -2335					
	8-9y	**10y**	**11y**	**12y**	**13y**	**14y**
5-7y	r= 0.44 p= 0.05	0.65 0.001	0.50 0.01	0.68 <0.001	0.47 0.02	0.32 0.12
8-9y		0.60 0.008	0.75 <0.001	0.54 0.02	0.43 0.06	0.34 0.14
10y			0.54 0.006	0.62 0.001	0.55 0.004	0.38 0.08
11y				0.65 <0.001	0.68 <0.001	0.54 0.005
12y					0.48 0.01	0.52 0.007
13y						0.59 0.001

Table 10.5- The stability of methylation across the GCK promoter in peripheral blood each year between 5-7 and 14 years

	CpG -322					
	8-9y	**10y**	**11y**	**12y**	**13y**	**14y**
5-7y	r= 0.39 p= 0.03	0.37 0.04	0.16 0.35	0.17 0.37	0.46 0.009	0.21 0.23
8-9y		0.57 0.002	0.22 0.24	0.39 0.05	0.59 0.001	0.28 0.12
10y			0.17 0.35	0.31 0.11	0.46 0.01	0.25 0.17
11y				0.09 0.64	0.27 0.15	0.16 0.38
12y					0.57 0.002	0.23 0.22
13y						0.44 0.01
	CpG -282					
	8-9y	**10y**	**11y**	**12y**	**13y**	**14y**
5-7y	r= -0.03 p= 0.90	-0.13 0.54	0.04 0.83	-0.05 0.82	0.08 0.72	0.01 0.95
8-9y		-0.08 0.74	-0.02 0.93	0.41 0.07	-0.06 0.80	0.04 0.86
10y			0.21 0.32	0.04 0.85	0.12 0.62	0.05 0.82
11y				-0.09 0.67	0.25 0.25	0.06 0.77
12y					0.01 0.97	0.30 0.14
13y						0.25 0.24
	CpG -192					
	8-9y	**10y**	**11y**	**12y**	**13y**	**14y**
5-7y	r= 0.21 p= 0.29	0.58 <0.001	0.58 <0.001	0.64 <0.001	0.33 0.06	0.31 0.06
8-9y		0.19 0.37	0.63 0.001	0.68 <0.001	-0.01 0.98	0.13 0.51
10y			0.77 <0.001	0.70 <0.001	0.53 0.002	0.73 <0.001
11y				0.47 0.007	0.33 0.08	0.42 0.01
12y					0.34 0.07	0.71 <0.001
13y						0.06 0.74

	CpG -172					
	8-9y	**10y**	**11y**	**12y**	**13y**	**14y**
5-7y	r= 0.97 p<0.001	0.94 <0.001	0.69 <0.001	0.55 <0.001	0.68 <0.001	0.91 <0.001
8-9y		0.95 <0.001	0.67 <0.001	0.11 0.63	0.55 0.01	0.94 <0.001
10y			0.66 <0.001	0.61 <0.001	0.59 0.001	0.92 <0.001
11y				0.52 0.004	0.55 0.004	0.75 <0.001
12y					0.49 0.01	0.51 0.004
13y						0.51 0.008
	CpG +30					
	8-9y	**10y**	**11y**	**12y**	**13y**	**14y**
5-7y	r= 0.52 p= 0.002	0.39 0.02	0.48 0.003	0.41 0.02	0.30 0.09	0.57 <0.001
8-9y		0.26 0.17	0.22 0.23	0.41 0.03	0.27 0.15	0.44 0.01
10y			0.48 0.003	0.32 0.07	0.17 0.34	0.20 0.24
11y				0.41 0.02	0.43 0.01	0.36 0.03
12y					0.33 0.07	0.34 0.05
13y						0.39 0.02
	CpG +34					
	8-9y	**10y**	**11y**	**12y**	**13y**	**14y**
5-7y	r= 0.48 p= 0.006	0.74 <0.001	0.71 <0.001	0.48 0.005	0.59 <0.001	0.77 <0.001
8-9y		0.64 <0.001	0.52 0.004	0.11 0.59	0.54 0.004	0.51 0.003
10y			0.75 <0.001	0.48 0.007	0.57 0.001	0.67 <0.001
11y				0.44 0.02	0.82 <0.001	0.57 0.001
12y					0.78 <0.001	0.52 0.002
13y						0.75 <0.001

	CpG +37					
	8-9y	**10y**	**11y**	**12y**	**13y**	**14y**
5-7y	r= 0.48 p= 0.005	0.23 0.19	0.22 0.22	0.16 0.38	0.08 0.68	0.25 0.13
8-9y		0.65 <0.001	0.05 0.81	0.62 <0.001	0.05 0.80	0.42 0.02
10y			0.45 0.009	0.32 0.08	-0.03 0.89	0.02 0.91
11y				0.32 0.09	0.63 <0.001	-0.09 0.63
12y					0.50 0.005	0.29 0.10
13y						0.13 0.49

Chapter 11

References

1. Alberti KGMM, Zimmet P, Shaw J. Metabolic syndrome - a new world-wide definition. A consensus statement from the international diabetes federation. Diabet Med. 2006;23:469–80.

2. Eckel R, Grundy S, Zimmet P. The metabolic syndrome. Lancet. 2005;365:1415–28.

3. Thorleifsson G, Walters GB, Gudbjartsson DF, Steinthorsdottir V, Sulem P, Helgadottir A, et al. Genome-wide association yields new sequence variants at seven loci that associate with measures of obesity. Nat Genet. 2009;41(1):18–24.

4. Hossain P, Kawar B, El Nahas M. Obesity and diabetes in the developing world--a growing challenge. N Engl J Med. 2007;356(3):213–5.

5. Ozanne SE. Epigenetic Signatures of Obesity. N Engl J Med. 2015;372(10):973–4.

6. Godfrey KM, Barker DJP. Fetal programming and adult health. Public Health Nutr. 2001;4:611–24.

7. Ahlqvist E, Ahluwalia TS, Groop L. Genetics of type 2 diabetes. Clin Chem. 2011;57(2):241–54.

8. Medina-Gomez G, Gray S, Vidal-Puig A. Adipogenesis and lipotoxicity: role of peroxisome proliferator-activated receptor gamma (PPARgamma) and PPARgammacoactivator-1 (PGC1). Public Health Nutr. 2007;10(10A):1132–7.

9. Bjørndal B, Burri L, Staalesen V, Skorve J, Berge RK. Different adipose depots: their role in the development of metabolic syndrome and mitochondrial response to hypolipidemic agents. J Obes. 2011;2011:1–15.

10. Thaddeus T. Schug. Sirtuin 1 in lipid metabolism and obesity. Ann Med. 2011;43(3):198–211.

11. Liang P. AdipoQ Is a Novel Adipose-specific Gene Dysregulated in Obesity. J Biol Chem. 1996;271(18):10697–703.

12. Kahn SE, Hull RL, Utzschneider KM. Mechanisms linking obesity to insulin resistance and type 2 diabetes. Nature. 2006;444(14):840–6.

13. Ailhaud G, Massiera F, Weill P, Legrand P, Alessandri J-M, Guesnet P. Temporal changes in dietary fats: role of n-6 polyunsaturated fatty acids in excessive adipose tissue development and relationship to obesity. Prog Lipid Res. 2006;45:203–36.

14. Ailhaud G, Guesnet P, Cunnane SC. An emerging risk factor for obesity: does disequilibrium of polyunsaturated fatty acid metabolism contribute to excessive adipose tissue development? Br J Nutr. 2008;100:461–70.

15. Knittle JL, Timmers K, Ginsberg-Fellner F, Brown RE, Katz DP. The growth of adipose tissue in children and adolescents. Cross-sectional and longitudinal studies of adipose cell number and size. J Clin Invest. 1979;63(February):239–46.

16. Must a, Anderson SE. Body mass index in children and adolescents: considerations for population-based applications. Int J Obes (Lond). 2006;30(4):590–4.

17. Rolland-Cachera MF, Sempé M, Guilloud-Bataille M, Patois E, Péquignot-Guggenbuhl F, Fautrad V. Adiposity indices in children. Am J Clin Nutr. 1982;36(1):178–84.

18. Cole TJ, Bellizzi MC, Flegal KM, Dietz WH. Establishing a standard definition for child overweight and obesity worldwide: international survey. BMJ. 2000;320(7244):1240–3.

19. Onis M De, Onyango AW, Borghi E, Siyam A, Siekmann J. Development of a WHO growth reference for school-aged children and adolescents. Bull World Heal Organsiation. 2007;85(9):660–7.

20. Wells JCK, Fewtrell MS. Measuring body composition. Arch Dis Child. 2006;91(7):612–7.

21. Crozier SR, Inskip HM, Godfrey KM, Cooper C, Harvey NC, Cole A. Weight gain in pregnancy and childhood body composition : findings from the Southampton Women's Survey. Am J Clin Nutr. 2010;91(6):1745–51.

22. Abate N, Garg A, Peshock RM, Stray-Gundersen J, Grundy SM. Relationships of generalized and regional adiposity to insulin sensitivity in men. J Clin Invest. 1995;96:88–98.

23. Janssen I, Katzmarzyk PT, Ross R. Waist circumference and not body mass index explains obesity- related health risk. Am J Clin Nutr. 2004;79:379–84.

24. Anjana M, Sandeep S, Deepa R, Vimaleswaran KS, Farooq S, Mohan V. Visceral and central abdominal fat and anthropometry in relation to diabetes in Asian Indians. Diabetes Care. 2004;27(12):2948–53.

25. Seidell C, Bakker JG. Imaging techniques for measuring adipose-tissue distribution- a comparison between computed tomography and 1.5-T magnetic resonance. Am J Clin Nutr. 1990;51:953–7.

26. Marcus M a, Wang J, Pi-sunyer FX, Thornton JC, Kofopoulou I. Effects of Ethnicity , Gender , Obesity , and Age on Central Fat Distribution : Comparison of Dual X-Ray Absorptiometry Measurements in White , Black , and Puerto Rican Adults. Am J Hum Biol. 1998;10:361–9.

27. Boden G, Shulman GI. Free fatty acids in obesity and type 2 diabetes: defining their role in the development of insulin resistance and beta-cell dysfunction. Eur J Clin Invest. 2002;32 Suppl 3:14–23.

28. Gibson R a, Muhlhausler B, Makrides M. Conversion of linoleic acid and alpha-linolenic acid to long-chain polyunsaturated fatty acids (LCPUFAs), with a focus on pregnancy, lactation and the first 2 years of life. Matern Child Nutr. 2011;7:17–26.

29. Hornstra G. Essential fatty acids in mothers and their neonates. Am J Clin Nutr. 2000;71:1262–9.

30. Massiera F, Saint-Marc P, Seydoux J, Murata T, Kobayashi T, Narumiya S, et al. Arachidonic acid and prostacyclin signaling promote adipose tissue development: a human health concern? J Lipid Res. 2003;44(2):271–9.

31. Flachs P, Horakova O, Brauner P, Rossmeisl M, Pecina P, Franssen-van Hal N, et al. Polyunsaturated fatty acids of marine origin upregulate mitochondrial biogenesis and induce beta-oxidation in white fat. Diabetologia. 2005;48:2365–75.

32. Kabir M, Skurnik G, Naour N, Pechtner V, Meugnier E, Rome S, et al. Treatment for 2 mo with n 3 polyunsaturated fatty acids reduces adiposity and some atherogenic factors but does not improve insulin sensitivity in women with type 2 diabetes: a randomized controlled study. Am J Clin Nutr. 2007;86:1670–9.

33. Gerich JE. Contributions of insulin-resistance and insulin-secretory defects to the pathogenesis of type 2 diabetes mellitus. Mayo Clin Proc. 2003;78(4):447–56.

34. Olefsky JM, Nolan JJ. Insulin resistance and non-insulin-dependent diabetes mellitus: cellular and molecular mechanisms. Am J Clin Nutr. 1995;61:980S – 986S.

35. Pessin JE, Saltiel AR. Signaling pathways in insulin action : molecular targets of insulin resistance. J Clin Endocrinol Metab. 2000;106(2):165–9.

36. Komatsu M, Takei M, Ishii H, Sato Y. Glucose-stimulated insulin secretion: A newer perspective. J Diabetes Investig. 2013;4(6):511–6.

37. Groop LC. Insulin resistance: the fundamental trigger of type 2 diabetes. Diabetes Obes Metab. 1999;1(1):S1–7.

38. Reshef L, Olswang Y, Cassuto H, Blum B, Croniger CM, Kalhan SC, et al. Glyceroneogenesis and the triglyceride/fatty acid cycle. J Biol Chem. 2003;278(33):30413–6.

39. Boden G. Obesity, Insulin Resistance and Free Fatty Acids. Curr Opin Endocrinol diabetes Obes. 2011;18(2):139–43.

40. Kelley DE, Mandarin LJ. Fuel Selection in Human Skeletal Muscle in Insulin Resistance. A Reexamination. Diabetes. 2000;49:677–83.

41. Itani SI, Ruderman NB, Schmieder F, Boden G. Lipid-Induced Insulin Resistance in Human Muscle Is Associated With Changes in Diacylglycerol , Protein Kinase C, and IκB-α. Diabetes. 2002;51:2005–11.

42. Yu C, Chen Y, Cline GW, Zhang D, Zong H, Wang Y, et al. Mechanism by which fatty acids inhibit insulin activation of insulin receptor substrate-1 (IRS-1)-associated phosphatidylinositol 3-kinase activity in muscle. J Biol Chem. 2002;277(52):50230–6.

43. Rakyan VK, Down T a, Balding DJ, Beck S. Epigenome-wide association studies for common human diseases. Nat Rev Genet. Nature Publishing Group; 2011;12(8):529–41.

44. Frayling TM, Timpson NJ, Weedon MN, Freathy RM, Lindgren CM, Perry JRB, et al. A Common Variant in the FTO Gene Is Associated with Body Mass Index and Predisposes to Childhood and Adult Obesity. Science (80-). 2007;316:889–94.

45. Loos RJF, Loos RJF, Lindgren CM, Lindgren CM, Li S, Li S, et al. Common variants near MC4R are associated with fat mass, weight and risk of obesity. Nat Genet. 2008;40(6):768–75.

46. Zillikens MC, Meurs JBJ Van, Rivadeneira F, Amin N, Hofman A, Oostra BA, et al. SIRT1 Genetic Variation Is Related to BMI and Risk of Obesity. Diabetes. 2009;58:2828–34.

47. Peeters A V., Beckers S, Verrijken A, Mertens I, Roevens P, Peeters PJ, et al. Association of SIRT1 gene variation with visceral obesity. Hum Genet. 2008;124(2008):431–6.

48. Crunkhorn S, Dearie F, Mantzoros C, Gami H, da Silva WS, Espinoza D, et al. Peroxisome proliferator activator receptor gamma coactivator-1 expression is reduced in obesity: potential pathogenic role of saturated fatty acids and p38 mitogen-activated protein kinase activation. J Biol Chem. 2007;282(21):15439–50.

49. Ling C, Del Guerra S, Lupi R, Rönn T, Granhall C, Luthman H, et al. Epigenetic regulation of PPARGC1A in human type 2 diabetic islets and effect on insulin secretion. Diabetologia. 2008;51(4):615–22.

50. Ling C, Poulsen P, Carlsson E, Ridderstråle M, Almgren P, Wojtaszewski J, et al. Multiple environmental and genetic factors influence skeletal muscle PGC-1α and PGC-1β gene expression in twins. J Clin Invest. 2004;114(10):1518–26.

51. Barker DJP, Osmond C, Winter PD, Margetts B. Weight in Infancy and Death from Ischaemic Heart Disease. Lancet. 1989;334:577–80.

52. Dabelea D. Birth weight, type 2 diabetes, and insulin resistance in Pima Indian children and young adults. Diabetes Care. 1999;22(6):944–50.

53. Barker DJP. The developmental origins of adult disease. J Am Coll Nutr. 2004;23:588S – 595S.

54. Waterland R a, Michels KB. Epigenetic epidemiology of the developmental origins hypothesis. Annu Rev Nutr. 2007;27:363–88.

55. Schulz LC. The Dutch Hunger Winter and the developmental origins of health and disease. Proc Natl Acad Sci U S A. 2010;107(39):16757–8.

56. Wells JCK. The thrifty phenotype: An adaptation in growth or metabolism? Am J Hum Biol. 2011;23:65–75.

57. Lillycrop K a, Burdge GC. Epigenetic changes in early life and future risk of obesity. Int J Obes (Lond). Nature Publishing Group; 2011;35(1):72–83.

58. Bellinger L, Sculley D V, Langley-Evans SC. Exposure to undernutrition in fetal life determines fat distribution, locomotor activity and food intake in ageing rats. Int J Obes (Lond). 2006;30(5):729–38.

59. Samuelsson AM, Matthews P a., Argenton M, Christie MR, McConnell JM, Jansen EHJM, et al. Diet-induced obesity in female mice leads to offspring hyperphagia, adiposity, hypertension, and insulin resistance: A novel murine model of developmental programming. Hypertension. 2008;51:383–92.

60. Oken E, Gillman MW. Gestational weight gain and child adiposity at age 3 years. Am J Obstet Gynecol. 2007;196(4):322.e1–322.e8.

61. Hillier T a, Pedula KL, Schmidt MM, Mullen J a, Charles M-A, Pettitt DJ. Childhood Obesity and Metabolic Imprinting. Diabetes Care. 2007;30(9):2287–92.

62. Godfrey KM, Lillycrop K a, Burdge GC, Gluckman PD, Hanson M a. Epigenetic mechanisms and the mismatch concept of the developmental origins of health and disease. Pediatr Res. 2007;61(5):5R – 10R.

63. Picard F, Kurtev M, Chung N, Topark-ngarm A, Oliveira RM De, Leid M, et al. Sirt1 promotes fat mobilization in white adipocytes by repressing PPAR-γ. Nature. 2010;429(6993):1–14.

64. Rhee J, Ge H, Yang W, Fan M, Handschin C, Cooper M, et al. Partnership of PGC-1alpha and HNF4alpha in the regulation of lipoprotein metabolism. J Biol Chem. 2006;281(21):14683–90.

65. Lin J and BMS. Transcriptional co-activator PGC-1 a drives the formation of slow-twitch muscle fibres. Nature. 2002;418(15):797–801.

66. Ling C, Del Guerra S, Lupi R, Rönn T, Granhall C, Luthman H, et al. Epigenetic regulation of PPARGC1A in human type 2 diabetic islets and effect on insulin secretion. Diabetologia. 2008;51(4):615–22.

67. Liang F, Kume S, Koya D. SIRT1 and insulin resistance. Nat Rev Endocrinol. 2009;5(7):367–73.

68. Rodgers JT, Lerin C, Haas W, Gygi SP, Spiegelman BM, Puigserver P. Nutrient control of glucose homeostasis through a complex of PGC-1 a and SIRT1. Nature. 2005;434(3):113–8.

69. Cantó C, Auwerx J. PGC-1alpha, SIRT1 and AMPK, an energy sensing network that controls energy expenditure. Curr Opin Lipidol. 2009;20:98–105.

70. Sugden MC, Caton PW, Holness MJ. PPAR control: it's SIRTainly as easy as PGC. J Endocrinol. 2010;204:93–104.

71. Oberkofler H, Linnemayr V, Weitgasser R, Klein K, Xie M, Iglseder B, et al. Complex Haplotypes of the PGC-1a Gene Are Associated With Carbohydrate Metabolism and Type 2 Diabetes. Diabetes. 2004;53:1385–93.

72. Gillum MP, Kotas ME, Erion DM, Kursawe R, Chatterjee P, Nead KT, et al. SirT1 regulates adipose tissue inflammation. Diabetes. 2011;60(12):3235–45.

73. Pedersen SB, Ølholm J, Paulsen SK, Bennetzen MF, Richelsen B. Low Sirt1 expression, which is upregulated by fasting, in human adipose tissue from obese women. Int J Obes (Lond). 2008;32(2008):1250–5.

74. Qiao L, Shao J. SIRT1 regulates adiponectin gene expression through Foxo1-C/enhancer-binding protein alpha transcriptional complex. J Biol Chem. 2006;281(52):39915–24.

75. Puigserver P. Peroxisome Proliferator-Activated Receptor-gamma Coactivator 1alpha (PGC-1alpha): Transcriptional Coactivator and Metabolic Regulator. Endocr Rev. 2003;24(1):78–90.

76. Chalkiadaki A, Guarente L. High-Fat Diet Triggers Inflammation-Induced Cleavage of SIRT1 in Adipose Tissue To Promote Metabolic Dysfunction. Cell Metab. 2012;16(2):180–8.

77. Leone TC, Lehman JJ, Finck BN, Schaeffer PJ, Wende AR, Boudina S, et al. PGC-1alpha deficiency causes multi-system energy metabolic derangements: muscle dysfunction, abnormal weight control and hepatic steatosis. PLoS Biol. 2005;3(4):e101.

78. Braun K, Shanmuganayagam D, Weindruch R, Anderson R. The Role of PGC-1a in Adipocyte Differentiation and Fat Mobilization. Biol Honor Theses Res Pap. 2008;1–7.

79. Chen M, Macpherson A, Owens J, Wittert G, Heilbronn L. Obesity alone or with type 2 diabetes is associated with tissue specific alterations in DNA methylation and gene expression of PPARGC1A and IGF2. J Diabetes Res Clin Metab. 2012;1:1–16.

80. Schenk S, Mccurdy CE, Philp A, Chen MZ, Holliday MJ, Bandyopadhyay GK, et al. Sirt1 enhances skeletal muscle insulin sensitivity in mice during caloric restriction. J Clin Invest. 2011;121(11):4281–8.

81. Ions LJ, Wakeling L a, Bosomworth HJ, Hardyman JE, Escolme SM, Swan DC, et al. Effects of Sirt1 on DNA methylation and expression of genes affected by dietary restriction. Age (Omaha). 2012;

82. Barrès R, Yan J, Egan B, Treebak JT, Rasmussen M, Fritz T, et al. Acute Exercise Remodels Promoter Methylation in Human Skeletal Muscle. Cell Metab. 2012;15:405–11.

83. Ness AR. The Avon Longitudinal Study of Parents and Children (ALSPAC)--a resource for the study of the environmental determinants of childhood obesity. Eur J Endocrinol. 2004;151 Suppl :U141–9.

84. Burke V, Beilin LJ, Simmer K, Oddy WH, Blake K V, Doherty D, et al. Predictors of body mass index and associations with cardiovascular risk factors in Australian children: a prospective cohort study. Int J Obes (Lond). 2005;29(1):15–23.

85. Voss LD, Kirkby J, Metcalf BS, Jeffery AN, O'Riordan C, Murphy MJ, et al. Preventable factors in childhood that lead to insulin resistance, diabetes mellitus and the metabolic syndrome: the EarlyBird diabetes study 1. J Pediatr Endocrinol Metab. 2003;16:1211–24.

86. Wilkin TJ, Voss LD, Metcalf BS, Mallam K, Jeffery a N, Alba S, et al. Metabolic risk in early childhood: the EarlyBird Study. Int J Obes. 2004;28:S64–9.

87. Perez-Pastor EM, Metcalf BS, Hosking J, Jeffery a N, Voss LD, Wilkin TJ. Assortative weight gain in mother-daughter and father-son pairs: an emerging source of childhood obesity. Longitudinal study of trios (EarlyBird 43). Int J Obes. Nature Publishing Group; 2009;33:727–35.

88. Murphy MJ, Metcalf BS, Voss LD, Jeffery AN, Kirkby J, Mallam KM, et al. Girls at five are intrinsically more insulin resistant than boys: The Programming Hypotheses Revisited-The EarlyBird Study (EarlyBird 6). Pediatrics. 2004;113(1):82–6.

89. Inskip HM, Godfrey KM, Robinson SM, Law CM, Barker DJP, Cooper C. Cohort profile: The Southampton Women's Survey. Int J Epidemiol. 2006;35:42–8.

90. Robinson SM, Marriott LD, Crozier SR, Harvey NC, Gale CR, Inskip HM, et al. Variations in infant feeding practice are associated with body composition in childhood: A prospective cohort study. J Clin Endocrinol Metab. 2009;94(8):2799–805.

91. Crozier S, Harvey NC, Inskip HM, Godfrey KM, Cooper C, Robinson S. Maternal vitamin D status in pregnancy is associated with adiposity in the offspring: findings from the Southampton Women's Survey. Am J Clin Nutr. 2012;96(1):57–63.

92. Fuks F. DNA methylation and histone modifications: teaming up to silence genes. Curr Opin Genet Dev. 2005;15(5):490–5.

93. McCarrey JR. The epigenome as a target for heritable environmental disruptions of cellular function. Mol Cell Endocrinol. Elsevier Ireland Ltd; 2012;354:9–15.

94. Jiang Y-H, Bressler J, Beaudet AL. Epigenetics and human disease. Annu Rev Genomics Hum Genet. 2004;5:479–510.

95. Szyf M. The dynamic epigenome and its implications in toxicology. Toxicol Sci. 2007;100(1):7–23.

96. Ling C, Groop L. Epigenetics: a molecular link between environmental factors and type 2 diabetes. Diabetes. 2009;58(12):2718–25.

97. Jenuwein T, Allis CD. Translating the histone code. Science (80-). 2001;293(5532):1074–80.

98. Zhao H, Guan J, Lee H-M, Sui Y, He L, Siu JJ, et al. Up-regulated pancreatic tissue microRNA-375 associates with human type 2 diabetes through beta-cell deficit and islet amyloid deposition. Pancreas. 2010;39(6):843–6.

99. Villeneuve LM, Natarajan R. The role of epigenetics in the pathology of diabetic complications. Am J Physiol Physiol. 2010;299:F14–25.

100. Herder C, Karakas M, Koenig W. Biomarkers for the prediction of type 2 diabetes and cardiovascular disease. Clin Pharmacol Ther. Nature Publishing Group; 2011;90(1):52–66.

101. Richardson B. Impact of aging on DNA methylation. Ageing Res Rev. 2003;2:245–61.

102. Mohn F, Schübeler D. Genetics and epigenetics: stability and plasticity during cellular differentiation. Trends Genet. 2009;25(3):129–36.

103. Singal R, Ginder GD. DNA methylation. Blood. 1999;93:4059–70.

104. Gaston K, Fried M. CpG methylation has differential effects on the binding of YY1 and ETS proteins to the bi-directional promoter of the Surf-1 and Surf-2 genes. Nucleic Acids Res. 1995;23(6):901–9.

105. Jane, D. Gumucio, P. Ney, J. Cunningham, A. Nienhuis S. Methylation-Enhanced Binding of Spl to the Stage Selector Element of the Human y-Globin Gene Promoter May Regulate Developmental Specificity of Expression. Mol Cell Biol. 1993;13(6):3272–81.

106. Hu S, Wan J, Su Y, Song Q, Zeng Y, Nguyen HN, et al. DNA methylation presents distinct binding sites for human transcription factors. Elife. 2013;1–16.

107. Alisch RS, Barwick BG, Chopra P, Myrick LK, Satten G a, Conneely KN, et al. Age-associated DNA methylation in pediatric populations. Genome Res. 2012;22:623–32.

108. Guo H, Zhu P, Yan L, Li R, Hu B, Lian Y, et al. The DNA methylation landscape of human early embryos. Nature. Nature Publishing Group; 2014;511:606–10.

109. Chong S, Whitelaw E. Epigenetic germline inheritance. Curr Opin Genet Dev. 2004;14:692–6.

110. Okano M, Bell DW, Haber D a, Li E. DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. Cell. 1999;99(3):247–57.

111. Burdge GC, Lillycrop K a. Nutrition, epigenetics, and developmental plasticity: implications for understanding human disease. Annu Rev Nutr. 2010;30:315–39.

112. Laubenthal J, Zlobinskaya O, Poterlowicz K, Baumgartner A, Gdula MR, Fthenou E, et al. Cigarette smoke-induced transgenerational alterations in genome stability in cord blood of human F1 offspring. FASEB J. 2012;26:3946–56.

113. Wolff GL, Kodell RL, Moore SR, Cooney C a. Maternal epigenetics and methyl supplements affect agouti gene expression in Avy/a mice. FASEB J. 1998;12(11):949–57.

114. Plagemann A, Harder T, Brunn M, Harder A, Roepke K, Wittrock-Staar M, et al. Hypothalamic proopiomelanocortin promoter methylation becomes altered by early overfeeding: an epigenetic model of obesity and the metabolic syndrome. J Physiol. 2009;587:4963–76.

115. Godfrey KM, Sheppard A, Gluckman PD, Lillycrop K a, Burdge GC, McLean C, et al. Epigenetic gene promoter methylation at birth is associated with child's later adiposity. Diabetes. 2011;60:1528–34.

116. Relton CL, Groom A, St. Pourcain B, Sayers AE, Swan DC, Embleton ND, et al. DNA methylation patterns in cord blood DNA and body size in childhood. PLoS One. 2012;7(3):e31821.

117. Heijmans BT, Tobi EW, Stein AD, Putter H, Blauw GJ, Susser ES, et al. Persistent epigenetic differences associated with prenatal exposure to famine in humans. Proc Natl Acad Sci. 2008;105(44):17046–9.

118. Morales E, Groom A, Lawlor D a, Relton CL. DNA methylation signatures in cord blood associated with maternal gestational weight gain: results from the ALSPAC cohort. BMC Res Notes. 2014;7(278):1–10.

119. Talens RP, Boomsma DI, Tobi EW, Kremer D, Jukema JW, Willemsen G, et al. Variation, patterns, and temporal stability of DNA methylation: considerations for epigenetic epidemiology. FASEB J Fed Am Soc Exp Biol. 2010;24:3135–44.

120. Adalsteinsson BT, Gudnason H, Aspelund T, Harris TB, Launer LJ, Eiriksdottir G, et al. Heterogeneity in white blood cells has potential to confound DNA methylation measurements. PLoS One. 2012;7(10):e46705.

121. Zaldivar F, McMurray RG, Nemet D, Galassetti P, Mills PJ, Cooper DM. Body fat and circulating leukocytes in children. Int J Obes (Lond). 2006;30(6):906–11.

122. Yoo JY, Lee S, Lee H a., Park H, Park YJ, Ha EH, et al. Can Proopiomelanocortin Methylation Be Used as an Early Predictor of Metabolic Syndrome? Diabetes Care. 2014;37:734–9.

123. Nagamura-Inoue T, He H. Umbilical cord-derived mesenchymal stem cells: Their advantages and potential clinical utility. World J Stem Cells. 2014;6(2):195–202.

124. Bieback K, Kern S, Klüter H, Eichler H. Critical parameters for the isolation of mesenchymal stem cells from umbilical cord blood. Stem Cells. 2004;22:625–34.

125. Constantino B, Cogionis B. Nucleated RBCs--Significance in the Peripheral Blood Film. Lab Med. 2000;31(4):223–9.

126. Armstrong D a., Lesseur C, Conradt E, Lester BM, Marsit CJ. Global and gene-specific DNA methylation across multiple tissues in early infancy: Implications for children's health research. FASEB J. 2014;28:2088–97.

127. Ollikainen M, Smith KR, Joo EJ-H, Ng HK, Andronikos R, Novakovic B, et al. DNA methylation analysis of multiple tissues from newborn twins reveals both genetic and intrauterine components to variation in the human neonatal epigenome. Hum Mol Genet. 2010;19(21):4176–88.

128. Ma B, Wilker EH, Willis-Owen S a G, Byun H-M, Wong KCC, Motta V, et al. Predicting DNA methylation level across human tissues. Nucleic Acids Res. 2014;42:3515–28.

129. Christensen BC, Houseman EA, Marsit CJ, Zheng S, Wrensch MR, Wiemels JL, et al. Aging and environmental exposures alter tissue-specific DNA methylation dependent upon CpG island context. PLoS Genet. 2009;5(8):e1000602.

130. Weaver ICG, Cervoni N, Champagne F a, D'Alessio AC, Sharma S, Seckl JR, et al. Epigenetic programming by maternal behavior. Nat Neurosci. 2004;7(8):847–54.

131. Kohli RM, Zhang Y. TET enzymes, TDG and the dynamics of DNA demethylation. Nature. 2013;502:472–9.

132. Wang D, Liu X, Zhou Y, Xie H, Hong X, Tsai H-J, et al. Individual variation and longitudinal pattern of genome-wide DNA methylation from birth to the first two years of life. Epigenetics. 2012;7(6):1–12.

133. Fraga MF, Ballestar E, Paz MF, Ropero S, Setien F, Ballestar ML, et al. Epigenetic differences arise during the lifetime of monozygotic twins. Proc Natl Acad Sci U S A. 2005;102(30):10604–9.

134. Kohli A, Garcia M a, Miller RL, Maher C, Humblet O, Hammond SK, et al. Secondhand smoke in combination with ambient air pollution exposure is associated with increasedx CpG methylation and decreased expression of IFN-γ in T effector cells and Foxp3 in T regulatory cells in children. Clin Epigenetics. 2012;4(1):17.

135. Baccarelli A, Wright RO, Bollati V, Tarantini L, Litonjua A a, Suh HH, et al. Rapid DNA methylation changes after exposure to traffic particles. Am J Respir Crit Care Med. 2009;179:572–8.

136. Wong CCY, Caspi A, Williams B, Craig IW, Houts R, Ambler A, et al. A longitudinal study of epigenetic variation in twins. Epigenetics. 2010;5(6):516–26.

137. Heijmans BT, Kremer D, Tobi EW, Boomsma DI, Slagboom PE. Heritable rather than age-related environmental and stochastic factors dominate variation in DNA methylation of the human IGF2/H19 locus. Hum Mol Genet. 2007;16(5):547–54.

138. Maier S, Olek A. Diabetes : A Candidate Disease for Efficient DNA Methylation Profiling. J Nutr. 2002;132:2440–3.

139. J.Levy, D.Matthews MH. Correct Homeostasis Model Assessment (HOMA) Evaluation Uses the Computer Program. Diabetes Metab. 1998;21(12):2191–2.

140. Lillycrop KA, Phillips ES, Torrens C, Hanson MA, Alan A, Burdge GC. Feeding pregnant rats a protein-restricted diet persistently alters the methylation of specific cytosines in the hepatic PPARα promoter of the offspring. Br J Nutr. 2008;100(2):278–82.

141. Dignamr JD, Lebovitz RM, Roeder RG. Acurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res. 1983;11(5):1475–89.

142. Smith AJP, Humphries SE. Characterization of DNA-binding proteins using multiplexed competitor EMSA. J Mol Biol. Elsevier Ltd; 2009;385:714–7.

143. Shen WF, Rozenfeld S, Lawrence HJ, Largman C. The Abd-B-like Hox homeodomain proteins can be subdivided by the ability to form complexes with Pbx1a on a novel DNA target. J Biol Chem. 1997;272(13):8198–206.

144. Knoepfler PS, Kamps MP. The pentapeptide motif of Hox proteins is required for cooperative DNA binding with Pbx1, physically contacts Pbx1, and enhances DNA binding by Pbx1. Mol Cell Biol. 1995;15(10):5811–9.

145. Sultana H, Neelakanta G, Kantor FS, Malawista SE, Fish D, Montgomery RR, et al. Anaplasma phagocytophilum induces actin phosphorylation to selectively regulate gene transcription in Ixodes scapularis ticks. J Exp Med. 2010;207(8):1727–43.

146. American_Diabetes_Association. Diagnosis and Classification of Diabetes Mellitus. Diabetes Care. 2004;27:S5–10.

147. Welters HJ, Senkel S, Klein-Hitpass L, Erdmann S, Thomas H, Harries LW, et al. Conditional expression of hepatocyte nuclear factor-1beta, the maturity-onset diabetes of the young-5 gene product, influences the viability and functional competence of pancreatic beta-cells. J Endocrinol. 2006;190(1):171–81.

148. Miura A, Yamagata K, Kakei M, Hatakeyama H, Takahashi N, Fukui K, et al. Hepatocyte nuclear factor-4alpha is essential for glucose-stimulated insulin secretion by pancreatic beta-cells. J Biol Chem. 2006;281(8):5246–57.

149. Gupta RK, Gao N, Gorski RK, White P, Hardy OT, Rafiq K, et al. Expansion of adult beta-cell mass in response to increased metabolic demand is dependent on HNF-4alpha. Genes Dev. 2007;21:756–69.

150. Thomas H, Jaschkowitz K, Bulman M, Frayling TM, Mitchell SM, Roosen S, et al. A distant upstream promoter of the HNF-4alpha gene connects the transcription factors involved in maturity-onset diabetes of the young. Hum Mol Genet. 2001;10(19):2089–97.

151. Sandovici I, Smith NH, Nitert MD, Ackers-Johnson M, Uribe-Lewis S, Ito Y, et al. Maternal diet and aging alter the epigenetic control of a promoter-enhancer interaction at the Hnf4a gene in rat pancreatic islets. Proc Natl Acad Sci U S A. 2011;108:5449–54.

152. Hatzis P, Kyrmizi I, Talianidis I. Mitogen-activated protein kinase-mediated disruption of enhancer-promoter communication inhibits hepatocyte nuclear factor 4alpha expression. Mol Cell Biol. 2006;26(19):7017–29.

153. Ribel-Madsen R, Fraga MF, Jacobsen S, Bork-Jensen J, Lara E, Calvanese V, et al. Genome-Wide Analysis of DNA Methylation Differences in Muscle and Fat from Monozygotic Twins Discordant for Type 2 Diabetes. PLoS One. 2012;7(12):e51302.

154. Einstein F, Thompson RF, Bhagat TD, Fazzari MJ, Verma A, Barzilai N, et al. Cytosine methylation dysregulation in neonates following intrauterine growth restriction. PLoS One. 2010;5(1):e8887.

155. Chiu KC, Chuang LM, Yoon C, Saad MF. Hepatic glucokinase promoter polymorphism is associated with hepatic insulin resistance in Asian Indians. BMC Genet. 2000;1(2).

156. Otaegui PJ, Ferre T, Riu E, Bosch F. Prevention of obesity and insulin resistance by glucokinase expression in skeletal muscle of transgenic mice. FASEB J. 2003;17:2097–9.

157. Jiang MH, Fei J, Lan MS, Lu ZP, Liu M, Fan WW, et al. Hypermethylation of hepatic Gck promoter in ageing rats contributes to diabetogenic potential. Diabetologia. 2008;51:1525–33.

158. Wang H, Antinozzi P a, Hagenfeldt K a, Maechler P, Wollheim CB. Molecular targets of a human HNF1 alpha mutation responsible for pancreatic beta-cell dysfunction. EMBO J. 2000;19(16):4257–64.

159. Lovis P, Roggli E, Laybutt DR, Gattesco S, Yang J, Widmann C, et al. Alterations in MicroRNA Expression Contribute to Fatty Acid – Induced Pancreatic beta Cell Dysfunction. Diabetes. 2008;57(October):2728–36.

160. Chen H, Gu X, Su I, Bottino R, Contreras JL, Tarakhovsky A, et al. Polycomb protein Ezh2 regulates pancreatic beta-cell Ink4a/Arf expression and regeneration in diabetes mellitus. Genes Dev. 2009;23(8):975–85.

161. Soll AH, Kahn CR, Neville DM. Insulin Receptor Deficiency in Genetic and Acquired Obesity. J Clin Invest. 1975;56(October):769–80.

162. Carvalho E, Kotani K, Peroni OD, Kahn BB. Adipose-specific overexpression of GLUT4 reverses insulin resistance and diabetes in mice lacking GLUT4 selectively in muscle. Am J Physiol - endocrinology Metab. 2005;289:551–61.

163. Clore JN, Stillman J, Sugerman H. Glucose-6-Phosphatase Flux In Vitro Is Increased in Type 2 Diabetes. Diabetes. 2000;49(June):969–74.

164. Brøns C, Jacobsen S, Nilsson E, Rönn T, Jensen CB, Storgaard H, et al. Deoxyribonucleic acid methylation and gene expression of PPARGC1A in human muscle is influenced by high-fat overfeeding in a birth-weight-dependent manner. J Clin Endocrinol Metab. 2010;95(6):3048–56.

165. Hegele RA, Cao H. Single nucleotide polymorphisms of RXRA encoding retinoid X receptor alpha. J Hum Genet. 2001;46:423–5.

166. Lefebvre B, Benomar Y, Guédin A, Langlois A, Hennuyer N, Dumont J, et al. Proteasomal degradation of retinoid X receptor α reprograms transcriptional activity of PPAR γ in obese mice and humans. J Clin Invest. 2010;120(5):1454–68.

167. Cui Y, Wang H, Chen H, Pang S, Wang L, Liu D, et al. Genetic analysis of the SIRT1 gene promoter in myocardial infarction. Biochem Biophys Res Commun. Elsevier Inc.; 2012;426(2):232–6.

168. Noriega LG, Feige JN, Canto C, Yamamoto H, Yu J, Herman M a, et al. CREB and ChREBP oppositely regulate SIRT1 expression in response to energy availability. EMBO Rep. 2011;12(10):1069–76.

169. Liu T, Liu PY, Marshall GM. The critical role of the class III histone deacetylase SIRT1 in cancer. Cancer Res. 2009;69(5):1702–5.

170. Han L, Zhou R, Niu J, McNutt M a, Wang P, Tong T. SIRT1 is regulated by a PPAR{γ}-SIRT1 negative feedback loop associated with senescence. Nucleic Acids Res. 2010;38(21):7458–71.

171. Gemma C, Sookoian S, Alvariñas J, García SI, Quintana L, Kanevsky D, et al. Maternal pregestational BMI is associated with methylation of the PPARGC1A promoter in newborns. Obesity. 2009;17(5):1032–9.

172. Ait-lounis A, Bonal C, Seguín-este Q, Schmid CD, Bucher P. The Transcription Factor Rfx3 Regulates B-Cell Differentiation, Function, and Glucokinase Expression. Diabetes. 2010;59:1674–85.

173. Dayeh T a, Olsson a H, Volkov P, Almgren P, Rönn T, Ling C. Identification of CpG-SNPs associated with type 2 diabetes and differential DNA methylation in human pancreatic islets. Diabetologia. 2013;56(5):1036–46.

174. Wakeling L a, Ions LJ, Ford D. Could Sirt1-mediated epigenetic effects contribute to the longevity response to dietary restriction and be mimicked by other dietary interventions? Age (Omaha). 2009;31:327–41.

175. Palacios J a, Herranz D, De Bonis ML, Velasco S, Serrano M, Blasco M a. SIRT1 contributes to telomere maintenance and augments global homologous recombination. J Cell Biol. 2010;191(7):1299–313.

176. Ma H, Zhang W, Hu J, Yu Z, Chen Y, Luo Q, et al. Analysis of global DNA methylation levels in human blood using high-performance liquid chromatography/tandem electrospray ionization mass spectrometry. Eur J mass Spectrom. 2009;15:555–61.

177. Muhonen P, Holthofer H. Epigenetic and microRNA-mediated regulation in diabetes. Nephrol Dial Transplant. 2009;24(4):1088–96.

178. Szabova L, Macejova D, Dvorcakova M, Mostbock S, Blazickova S, Zorad S. Expression of nuclear retinoic acid receptor in peripheral blood mononuclear cells (PBMC) of healthy subjects. Life Sci. 2003;72:831–6.

179. Barker DJP. The origins of the developmental origins theory. J Intern Med. 2007;261:412–7.

180. Toperoff G, Aran D, Kark JD, Rosenberg M, Dubnikov T, Nissan B, et al. Genome-wide survey reveals predisposing diabetes type 2-related DNA methylation variations in human peripheral blood. Hum Mol Genet. 2012;21(2):371–83.

181. Mirmira RG. beta -Cell Differentiation Factor Nkx6.1 Contains Distinct DNA Binding Interference and Transcriptional Repression Domains. J Biol Chem. 2000;275(19):14743–51.

182. Comb M, Goodman HM. CpG methylation inhibits proenkephalin gene expression and binding of the transcription factor AP-2. Nucleic Acids Res. 1990;18(13):3975–82.

183. Tfayli H, Arslanian S. The challenge of adolescence: hormonal changes and sensitivity to insulin. Diabetes Voice. 2007;52:28–30.

184. Moran A, Jacobs DR, Steinberger J, Cohen P, Hong CP, Prineas R, et al. Association between the insulin resistance of puberty and the insulin-like growth factor-I/growth hormone axis. J Clin Endocrinol Metab. 2002;87(10):4817–20.

185. Goran M, Gower B. Longitudinal study on pubertal insulin resistance. Diabetes. 2001;50(11):2444–50.

186. MIGUEL C. MONTEIRO. PBX1: A Novel Stage-Specific Regulator of Adipocyte Development. Stem Cells. 2011;29:1837–48.

187. Hawley J a., Lessard SJ. Exercise training-induced improvements in insulin action. Acta Physiol. 2008;192:127–35.

188. Stefan N, Kantartzis K, Machann J, Schick F, Thamer C, Rittig K, et al. Identification and Characterization of Metabolically Benign Obesity in Humans. JAMA Int Med. 2008;168(15):1609–16.

189. Barrès R, Osler ME, Yan J, Rune A, Fritz T, Caidahl K, et al. Non-CpG methylation of the PGC-1alpha promoter through DNMT3B controls mitochondrial density. Cell Metab. 2009;10:189–98.

190. Wolfrum C, Shih DQ, Kuwajima S, Norris AW, Kahn CR, Stoffel M. Role of Foxa-2 in adipocyte metabolism and differentiation. J Clin Invest. 2003;112(3):345–56.

191. Cantile M, Procino A, D'Armiento M, Cindolo L, Cillo C. HOX gene network is involved in the transcriptional regulation of in vivo human adipogenesis. J Cell Physiol. 2002;194:225–36.

192. Stratford EW, Castro R, Daffinrud J, Skårn M, Lauvrak S, Munthe E, et al. Characterization of liposarcoma cell lines for preclinical and biological studies. Sarcoma. 2012;10:1–9.

193. Saleh M, Rambaldi I, Yang XJ, Featherstone MS. Cell signaling switches HOX-PBX complexes from repressors to activators of transcription mediated by histone deacetylases and histone acetyltransferases. Mol Cell Biol. 2000;20(22):8623–33.

194. Abramovich C, Shen WF, Pineault N, Imren S, Montpetit B, Largman C, et al. Functional cloning and characterization of a novel nonhomeodomain protein that inhibits the binding of PBX1-HOX complexes to DNA. J Biol Chem. 2000;275(34):26172–7.

195. Laurent A, Bihan R, Deschamps S, Guerrier D, Dupé V, Omilli F, et al. Identification of a new type of PBX1 partner that contains zinc finger motifs and inhibits the binding of HOXA9-PBX1 to DNA. Mech Dev. 2007;124:364–76.

196. Gesta S, Blüher M, Yamamoto Y, Norris AW, Berndt J, Kralisch S, et al. Evidence for a role of developmental genes in the origin of obesity and body fat distribution. Proc Natl Acad Sci U S A. 2006;103(17):6676–81.

197. Hoile SP, Lillycrop K a., Thomas N a., Hanson M a., Burdge GC. Dietary protein restriction during F0 pregnancy in rats induces transgenerational changes in the hepatic transcriptome in female offspring. PLoS One. 2011;6(7):e21668.

198. Burdge GC, Slater-jefferies J, Torrens C, Phillips ES. Dietary protein restriction of pregnant rats in the F 0 generation induces altered methylation of hepatic gene promoters in the adult male offspring in the F 1 and F 2 generations. Br J Nutr. 2007;97(3):435–9.

199. Painter RC, Osmond C, Gluckman P, Hanson M, Phillips DIW, Roseboom TJ. Transgenerational effects of prenatal exposure to the Dutch famine on neonatal adiposity and health in later life. BJOG An Int J Obstet Gynaecol. 2008;115(10):1243–9.

200. Roseboom TJ, Meulen MH Van Der, Ravelli a. C. Effects of prenatal expossure to the Ductch famine on adult disease in later life: an overview. Mol Cell Endocrinol. 2001;185:93–8.

201. Ravelli a CJ, van der Meulen JHP, Osmond C, Barker DJP, Blecker OP. Obesity at the age of 50y in men and women exposed to famine prenatally. Am J Clin Nutr. 1999;70:811–6.

202. Kaati G, Bygren LO, Edvinsson S. Cardiovascular and diabetes mortality determined by nutrition during parents' and grandparents' slow growth period. Eur J Hum Genet. 2002;10(June):682–8.

203. Pembrey ME, Bygren LO, Kaati G, Edvinsson S, Northstone K, Sjöström M, et al. Sex-specific, male-line transgenerational responses in humans. Eur J Hum Genet. 2006;14:159–66.

204. Northstone K, Golding J, Davey Smith G, Miller LL, Pembrey M. Prepubertal start of father's smoking and increased body fat in his sons: further characterisation of paternal transgenerational responses. Eur J Hum Genet. Nature Publishing Group; 2014;22:1382–6.

205. Soubry A, Schildkraut JM, Murtha A, Wang F, Huang Z, Bernal A, et al. Paternal obesity is associated with IGF2 hypomethylation in newborns: results from a Newborn Epigenetics Study (NEST) cohort. BMC Med. BioMed Central Ltd; 2013;11:11–29.

206. Jablonka E, Raz G. Transgenerational epigenetic inheritance: Prevalence, mechanisms, and implications for the study of heredity and evolution. Q Rev Biol. 2009;84(2):131–76.

207. Skinner MK. Environmental epigenomics and disease susceptibility. EMBO Rep. Nature Publishing Group; 2011;12(7):620–2.

208. Whitaker, J.Wright R. Predicting obesity in young adulthood from childhood and parental obesity. N Engl J Med. 1997;337(13):869–73.

209. Gale CR, Javaid MK, Robinson SM, Law CM, Godfrey KM, Cooper C. Maternal size in pregnancy and body composition in children. J Clin Endocrinol Metab. 2007;92(10):3904–11.

210. Lintsi M, Kaarma H, Kull I. Comparison of hand-to-hand bioimpedance and anthropometry equations versus dual-energy X-ray absorptiometry for the assessment of body fat percentage in 17-18-year-old conscripts. Clin Physiol Funct Imaging. 2004;24:85–90.

211. World Health Organisation 1995. WHO_physical status interpretation of anthropometry.pdf. 1995. p. 1–463.

212. The IDF consensus worldwide definition of the metabolic syndrome Part 1 : Worldwide definition for use in clinical practice.

213. Waist Circumference and Waist-Hip Ratio Report of a WHO Expert Consultation. 2008;

214. Cole Z, Harvey N, Kim M, Ntani G, Robinson S, Inskip H, et al. Increased fat mass is associated with increased bone size but reduced volumetric density in pre pubertal children. Bone. 2012;50(2):562–7.

215. Timpson NJ, Sayers A, Davey-Smith G, Tobias JH. How does body fat influence bone mass in childhood? A Mendelian randomization approach. J Bone Miner Res. 2009;24(9):522–33.

216. Gluckman PD, Hanson M a, Cooper C, Thornburg KL. Effect of in utero and early-life conditions on adult health and disease. N Engl J Med. 2008;359(1):61–73.

217. Reilly JJ, Armstrong J, Dorosty AR, Emmett PM, Ness a, Rogers I, et al. Early life risk factors for obesity in childhood: cohort study. BMJ. 2005;330(7504):1357.

218. Bouchard L, Rabasa-Lhoret R, Faraj M, Lavoie MÈ, Mill J, Pérusse L, et al. Differential epigenomic and transcriptomic responses in subcutaneous adipose tissue between low and high responders to caloric restriction. Am J Clin Nutr. 2010;91:309–20.

219. Finck BN, Kelly DP. PGC-1 coactivators : inducible regulators of energy metabolism in health and disease. J Clin Invest. 2006;116(3):615–22.

220. Faulk C, Dolinoy DC. Timing is everything: The when and how of environmentally induced changes in the epigenome of animals. Epigenetics. 2011;6(7):791–7.

221. Donahue SMA, Rifas-shiman SL, Gold DR, Jouni ZE, Gillman MW, Oken E. Prenatal fatty acid status and child adiposity at age 3 y : results from a US pregnancy cohort. Am J Clin Nutr. 2011;93:780–8.

222. Tobi EW, Lumey LH, Talens RP, Kremer D, Putter H, Stein AD, et al. DNA methylation differences after exposure to prenatal famine are common and timing- and sex-specific. Hum Mol Genet. 2009;18(21):4046–53.

223. Burdge GC, Lillycrop K a. Fatty acids and epigenetics. Curr Opin Clin Nutr Metab Care. 2014;17(2):156–61.

224. Pourmoradian S, Mahdavi R, Mobasseri M, Faramarzi E. Effects of Royal Jelly Supplementation on Body Weight and Dietary Intake in Type 2 Diabetic Females. Heal Promot Perspect. 2012;2(2):231–5.

225. Guo HG, Saiga AS, Sato MS, Miyazawa IM, Shibata MS, Takahata YT, et al. Royal Jelly Supplementation Improves Lipoprotein Metabolism in Humans. J Nutr Sci Vitaminol (Tokyo). 2007;53:345–8.

226. Laker RC, Lillard TS, Okutsu M, Zhang M, Hoehn KL, Connelly JJ, et al. Exercise prevents maternal high-fat diet-induced hypermethylation of the Pgc-1a gene and age-dependent metabolic dysfunction in the offspring. Diabetes. 2014;63:1605–11.

227. Staiger H, Staiger K, Haas C, Weisser M, Machicao F, Häring H-U. Fatty acid-induced differential regulation of the genes encoding peroxisome proliferator-activated receptor-gamma coactivator-1alpha and -1beta in human skeletal muscle cells that have been differentiated in vitro. Diabetologia. 2005;48(10):2115–8.

228. Zhang P, Liu C, Zhang C, Zhang Y, Shen P, Zhang J, et al. Free fatty acids increase PGC-1alpha expression in isolated rat islets. FEBS Lett. 2005;579(6):1446–52.

229. Lim J-H, Gerhart-Hines Z, Dominy JE, Lee Y, Kim S, Tabata M, et al. Oleic acid stimulates complete oxidation of fatty acids through protein kinase A-dependent activation of SIRT1-PGC1α complex. J Biol Chem. 2013;288(10):7117–26.

230. Poulsen LLC, Siersbæk M, Mandrup S. PPARs: Fatty acid sensors controlling metabolism. Semin Cell Dev Biol. Elsevier Ltd; 2012;23:631–9.

231. Varga T, Czimmerer Z, Nagy L. PPARs are a unique set of fatty acid regulated transcription factors controlling both lipid metabolism and inflammation. Biochim Biophys Acta. Elsevier B.V.; 2011;1812:1007–22.

232. Deckelbaum RJ, Worgall TS, Seo T. N− 3 Fatty Acids and Gene Expression. Am J Clin Nutr. 2006;83:1520S – 1525S.

233. Han W, Shi M, Spivack SD. Site-specific methylated reporter constructs for functional analysis of DNA methylation. Epigenetics. 2013;8(11):1176–87.

234. Herskovits Z, Guarente L. SIRT1 in Neurodevelopment and Brain Senescence. Neuron. Elsevier Inc.; 2014;81:471–83.

235. Wrann CD, White JP, Salogiannnis J, Laznik-Bogoslavski D, Wu J, Ma D, et al. Exercise induces hippocampal BDNF through a PGC-1α/FNDC5 pathway. Cell Metab. 2013;18:649–59.

236. Hoile SP, Clarke-Harris R, Huang R-C, Calder PC, Mori T a., Beilin LJ, et al. Supplementation with N-3 Long-Chain Polyunsaturated Fatty Acids or Olive Oil in Men and Women with Renal Disease Induces Differential Changes in the DNA Methylation of FADS2 and ELOVL5 in Peripheral Blood Mononuclear Cells. PLoS One. 2014;9(10):e109896.