Temporal examination of DNA methylation profile reprogramming in the promoter region of PGC-1α during the progression of insulin resistance and type 2 diabetes mellitus in rodent models

Sarah Rebecca Donnelly

Dissertation submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

Doctor of Philosophy
In
Human Nutrition, Foods, and Exercise

Zhiyong Cheng, Committee Co-Chair
Eva M. Schmelz, Committee Co-Chair
Fabio A. Almeida
Matthew W. Hulver
Ryan P. McMillan

July 16th, 2019
Blacksburg, Virginia

Keywords: type 2 diabetes mellitus, epigenetics, mitochondria, methylation

Temporal examination of DNA methylation profile reprogramming in the promoter region of PGC-1α during the progression of insulin resistance and type 2 diabetes mellitus in rodent models

Sarah Rebecca Donnelly

Abstract

Type 2 Diabetes Mellitus (T2DM), a metabolic disorder denoted by elevated blood glucose levels and insufficient insulin action, is growing in prevalence worldwide . Barriers to improving disease outcome resolve primarily around identifying and intervening during the preliminary stages of insulin resistance, a state clinically referred to as pre-diabetes. Emerging evidence suggests that mitochondrial dysfunction may underlie , and potentially precede, progressive insulin resistance, suggesting that biomarkers indicative of mitochondrial dysfunction could predict disease risk and status. In this study, we examined epigenetic modifications, in the form of DNA methylation, in the promoter region of peroxisome proliferator activated receptor gamma coactivator 1 alpha (PGC-1α), a known regulator of mitochondrial biogenesis. Following the initiation of a high fat diet, we observed significant genotypic (DNA methylation) and phenotypic (mitochondrial copy number) alterations in C57/BL6 rodent models. These changes preceded overt disease onset, as classified by clinically utilized indices, which included the homeostatic model assessment for insulin resistance (HOMA-IR), the homeostatic model assessment for β-cell dysfunction (HOMA- β), and the quantitative insulin-sensitivity check index (QUICKI). Our data indicate that methylation analysis may serve as an effective clinical parameter to use in conjunction with physiological criterion for the diagnosis of pre-diabetes and the assessment of T2DM disease risk, and adds to the

growing body of work seeking to elucidate the role of the mitochondria in the manifestations of insulin resistance

Temporal examination of DNA methylation profile reprogramming in the promoter region of PGC-1α during the progression of insulin resistance and type 2 diabetes mellitus in rodent models

Sarah Rebecca Donnelly

General Audience Abstract

High blood glucose, referred to as type 2 diabetes (T2DM), increases the risk for heart and kidney disease, blindness, stroke, and death. Efforts to prevent T2DM have centered primarily around behavioral interventions, which include increased physical activity and decreased caloric intake. Importantly, the interventions are most effective when implemented early on in disease progression. In this study, we sought to examine the effects of a high fat diet on the epigenetic profile of PGC-1α, a gene responsible for maintaining mitochondrial biogenesis. The mitochondria, the powerhouse of the cell, is responsible for maintaining the energy systems in the body. Therefore, we examined how increasing in caloric intake resulted in changes in the epigenetic profile of the PGC-1α promoter, and how these changes impacted mitochondrial number. Further, we sought to examine how hypermethylation of PGC-1α led to changes in gene and protein expression in the mitochondria. Results from our study indicate that DNA methylation changes preceded disease onset, as characterized by the homeostatic model assessment for insulin resistance (HOMA-IR), the homeostatic model assessment for β-cell dysfunction (HOMA- β), and the quantitative insulin-sensitivity check index (QUICKI). Our data indicate that methylation analysis may serve as diagnostic and risk assessment tool for pre-diabetes and T2DM in conjunction with physiological measures.

Acknowledgements

To Dr. Cheng, thank you for giving me the opportunity to pursue my dreams in your lab. You took on an inexperienced and nervous student and bestowed upon me the knowledge and confidence that I could excel as a researcher. Beyond science, you taught me how to engage with colleagues, manage my time effectively, and to take time for myself when it was needed. You always trusted my intuition and pushed me to think beyond the task at hand. I am a better scientist for having had the opportunity to work with you.

To Dr. Schmelz, I couldn't have asked for a better female role model as I was working my way through the weeds of graduate school. Your consistency and honesty gave me a sounding board I could trust, even when that meant that I was in the wrong. I wouldn't be where I am today without your support and belief in me - especially during the times when I was struggling to believe in myself.

To Longhua & Toby, you both were absolutely instrumental in my success as a PhD student. You never failed to answer my questions and guide me in the right direction as a scientist. Every day with you was joyful and meaningful.

To my committee: Dr. Fabio Almeida, Dr. Matthew Hulver, and Dr. Ryan McMillan, thank you for your support and guidance during this process, and for teaching me to think outside the box.

To my teaching mentors: Dr. Donna Westfall-Rudd, Dr. Robert Grange, Dr. Janet Rankin, Dr. Bill Zuti, and Dr. Angela Anderson, I did not expect to fall so deeply in love with teaching when I first began here, but your passion and excitement in the classroom lit a fire in me. Thank you for your mentorship.

To Mom and Dad: thank you for providing me every possible opportunity from the day I was born. You saw in me a curious and hyper little girl, and you constantly gave me new ways to engage that curiosity and work out that energy. The lessens I learned growing up with you two as parents are the only (the *only*) reason I am here today. Thank you for showing me the importance of commitment and ownership of one's actions, and for devoting your life to me.

To Kate and Sean: *"A sibling may be the keeper of one's identity, the only person with the keys to one's unfettered, more fundamental self."* -- Thank you for keeping me company on the lonely days and for your unwavering support in all aspects of my life.

To my family, each and every one of you: I am the luckiest person in the world to be able to call you mine. Thank you for traveling this journey with me.

To Stephen, there are no words to express how grateful I am for you. You came into my life during one of the most challenging periods of my schooling and were steadfast in your support through to the end. Thank you for encouraging me, making me laugh, and keeping me sane.

Finally, to the amazing friends who kept me going: Chris, Josh, Katelyn, Savannah, Mitchell, Kylea, Sadie, Bethany, Justin, Ellen, Emily, Cortney, Mary Elizabeth, Kristen, Kathleen, Douglas and Eleanor. I love you all very much.

Table of Contents

Table of Figures

Table of Tables

Chapter 1: Review of the Literature

1.1 Introduction

Type 2 diabetes mellitus (T2DM) is one of the most pressing health issues in the United States, as nearly 9.4% of the population has been diagnosed with the disease(*1-3*). Further, nearly 33.9% of adults have pre-diabetes (intermediate hyperglycemia), which places them at high risk for developing T2DM and cardiovascular disease (CVD)(*4-6*). Although pharmacological and behavioral changes may allow for the management of the disease after its onset, the lack of a known cure for T2DM underscores the importance of disease prevention through lifestyle interventions. However, due to diagnostic limitations, many of those who are pre-diabetic are not aware that they are affected. It is of critical importance to identify new markers and develop a reliable diagnosis procedure for pre-diabetes to prevent the onset of T2DM and downstream metabolic complications. Dynamic changes in DNA methylation in pre-diabetes and T2DM reveal the potential of DNA methylation as biomarkers for early identification of metabolic abnormalities preceding T2DM(*7, 8*). Therefore, investigation into the potential use of epigenetic analyses in the diagnosis and monitoring of diabetes progression may provide an alternative means for diabetes prevention and amelioration. Future studies will be warranted in order to address the mechanism of epigenetic changes as they relate to nutritional status, age, gender, inflammation, and intervention(*9-11*).

The current clinical diagnosis parameters in pre-diabetes and T2DM lead to discordance, making it challenging to identify pre-diabetic individuals for diabetes prevention(*12, 13*). Thus, it is important to examine the molecular mechanisms underlying the metabolic improvements that

result following lifestyle interventions and to identify possible mechanisms that may contribute to the variable responses to different treatments within populations(*14*).

1.2 Type 2 Diabetes Mellitus

1.2.1 History

The first documentation of physician-recognized diabetes was in 1500 BCE, when it was said that Indian physicians coined the phrase "madhumeha," meaning "honey urine." This would later be replaced by the term "mellitus," meaning "sweet like honey," in reference to the glucose molecules present in the urine. Evidence suggests that diabetes was recognized as a physiological disorder as far back as 250 BCE, when the term "diabainein," or "to pass through" was conceived to describe the excess urination, or polyuria, that occurs in the diabetic state(*15, 16*). In the late-1800's, researchers were able to inadvertently elicit the diabetic phenotype in canines by surgically removing the pancreas in order to study the role of digestive organs during the process of metabolism. Following the identification of the pancreas as one of the primary organs involved in the diabetic phenotype, researchers were able to isolate the beta cells and, using the extracts produced by these cells, ameliorate the phenotype in canines, leading to the discovery of insulin(*15, 17*). Much has been accomplished in the realm of diabetes research since then, including the purification and mass production of insulin used in the treatment of the disease, as well as the universally accepted mechanism whereby environment (diet and exercise) contribute to the treatment of T2DM(*18, 19*).

1.2.2 Pathogenesis

In the healthy state, whereby normal glucose homeostasis is maintained, ingestion of glucose leads to the increase of plasma glucose levels(*20-22*). This increase prompts the pancreatic beta cells to release insulin into the blood stream, leading to concomitant hyper-insulinemia and hyper-glycemia. This physiologic state of increased insulin and glucose in the blood stimulates glucose uptake in tissues and simultaneous suppression of glucose production (gluconeogenesis) in the liver(*22-25*).

The onset of T2DM is typically characterized by two coinciding physiological detriments. These include the impaired ability of insulin to act on the cell (typically skeletal muscle) to localize the GLUT receptors for peripheral glucose uptake and a deficiency in the ability of the beta cells to secrete a sufficient quantity of insulin for efficient glucose uptake into the cell(*22-27*). Initially, the pancreatic beta cells act in a compensatory manner to maintain blood glucose at normal levels, however, this mechanism is soon abolished as beta cell function progressively worsens(*16, 24*). The "incretin defect" refers to the increase in glucagon secretion from the pancreatic alpha cells which leads to a decreased response of the gastrointestinal tract to release incretion hormones in the postprandial period(*20*). In the diabetic state, the liver is insulin resistant and, therefore, unable to sense that the body is in a state of both hyper-insulinemia and glycemia. This results in the continued output of glucose via gluconeogenesis in spite of the inability of the body to properly dispose of the glucose in the peripheral tissues.

Because a staggering majority of T2DM patients are also overweight and exhibit excess adiposity, the role of adipocytes in the pathogenesis of insulin resistance is of great interest(*28*). Lipolysis, the breakdown of triglycerides and subsequent release of free fatty acids (FFA), is activated in the diabetic state due to the suppression of insulin. Chronically elevated FFA levels in the blood, sometimes referred to as "lipotoxicity," have been shown previously to contribute further to the insulin resistance of the skeletal muscle and liver, leading to a cyclical effect of insulin resistance(*28-31*).

1.2.3 Diagnosis

Diagnosis of T2DM in the clinical setting includes different approaches to quantify glucose quantity in the bloodstream and glucose handling by the internal and peripheral tissues. According to the American Diabetes Association (ADA), the World Health Organization (WHO), the European Association for the Study of Diabetes (EASD), and others, glycated hemoglobin (A_{1c}%) ≥ 6.5, fasting glucose ≥ 126 mg/dL, 2-hour glucose ≥ 200 mg/dL and random glucose ≥ 200 mg/dL indicates the presence of T2DM. Pre-diabetes is diagnosed when hemoglobin (A_{1c}%) is between 5.7 and 6.4, fasting glucose is between 100 and 125 mg/dL, and 2-hour glucose is between 140 and 199 mg/dL (**Table 1**)(*32-34*).

	Healthy	Pre-diabetes	T2DM
A1C (%)	> 5.7	5.7-6.4	≥ 6.5
Fasting Plasma Glucose (FPG)	< 100 mg/dL	100-125 mg/dL	≥ 126 mg/dL
Oral Glucose Tolerance Test (OGTT)	> 140 mg/dL	140-199 mg/dL	≥ 200 mg/dL
Random Plasma Glucose Test (RPG)	N/A	N/A	≥ 200 mg/dL

Table 1. Clinical diagnostic parameters for T2DM and pre-diabetes(*33*).

1.2.3.1 Issues with Current Diagnostic Procedures

Importantly, limitations exist regarding the utilization of these tests due to stage-dependent features of each test and the controversy surrounding A_{1c}% as an accurate indicator of insulin resistance(*13, 35-45*). In regard to the progression of decreased insulin sensitivity over time, fasting glucose abruptly increases approximately two to three years prior to the onset of T2DM, while significant changes in glycated hemoglobin (A1c), a hemoglobin molecule with glucose covalently bound, are not observed until one year prior to clinically diagnosed T2D. Though not listed above, HOMA-IR, used to assess beta cell functionality using the following equation: [fasting insulin (microU/L) x fasting glucose (nmol/L)/22.5], shows a significant decrease five years prior to diagnosed disease. Clinical utilization of A1c has been controversial due to limitations in its use as a diagnostic tool. This is primarily due to varying phenotypic hemoglobin traits amongst humans, as well as pregnancy, loss of blood, and hemolytic anemia, and the ability of these conditions to potentially impact interpretations of A1c levels(*38, 46*). Additionally, depending on geographical and socioeconomic characteristics of certain areas of the world, lack of test standardization and resource availability impact the test's universal utilization(*38*).

Of great concern were the results from a previous study in our lab showing that the standards put forth by the ADA for fasting glucose and A1c resulted in significantly different diagnostic outcomes amongst a group of 87 participants

(**Figure 1**). The current clinical diagnosis parameters in pre-diabetes and T2DM lead to discordance depending on physician preference, clinical settings, and variance amongst populations, making it challenging to identify pre-diabetic individuals for diabetes prevention(*12, 13*). Such incongruence may lead to under-diagnosed populations, further increasing the risk of downstream metabolic complications due to the absence of clinical interventions(*13*).

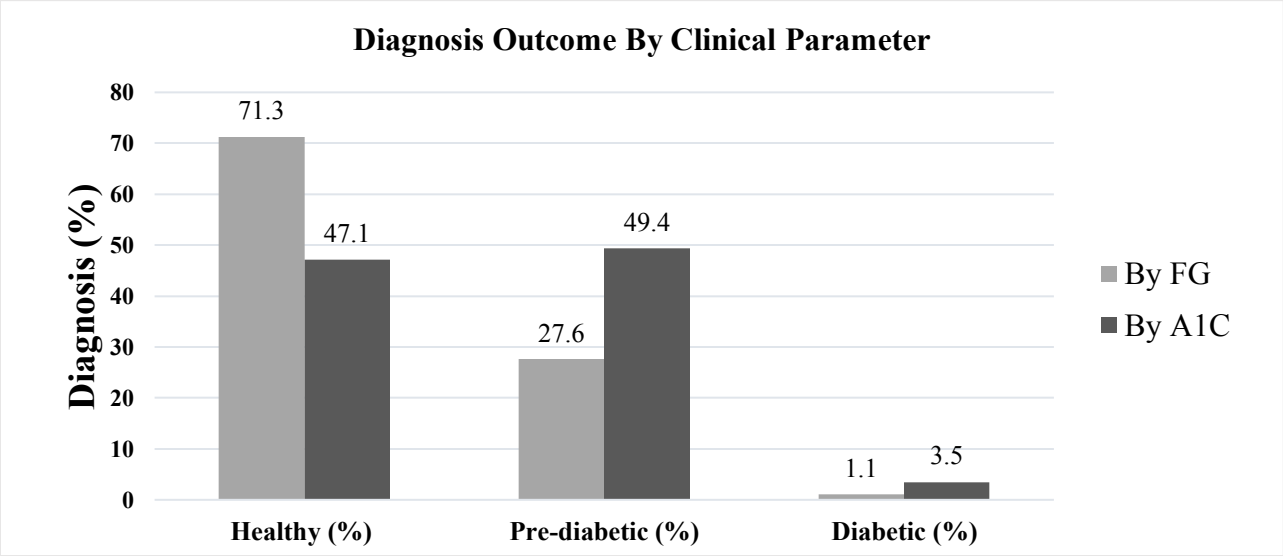


Figure 1. Diagnostic percentage of healthy, pre-diabetes, and T2DM amongst 87 volunteers according to the parameters set forth by the ADA for FG and A1c(*13*).

1.2.4 Treatment

Currently, there are no known cures for T2DM, and effective lifestyle interventions remain the clinical priority in the prevention of progression of pre-diabetes to diabetes (*13, 14, 47, 48*). Increasing evidence demonstrates that the remission of intermediate hyperglycemia to normoglycemia is particularly effective through behavioral

interventions, such as increased physical activity and controlled caloric intake (*14, 48, 49*). In line with modification of lifestyle behaviors associated with positive health outcomes (*50-52*), lifestyle interventions by increasing physical activity can lead to improvements in the clinical characteristics associated with metabolic disorders (*51, 53, 54*). These include body weight, BMI, blood pressure, waist circumference, and waist-to hip ratio. Additionally, lifestyle interventions improve plasma lipid profiles, including triglyceride (TG), low-density lipoprotein (LDL), and high-density lipoprotein (HDL) levels (*51*). Therefore, targeting pre-diabetes as early as possible may be key to preventing T2DM and ameliorating the associated downstream medical complications that accompany the disease. To this end, identification of clinically significant biomarkers coinciding with the presence of early stage pre-diabetes, as well as of progression to the more severe state of T2DM, will be a critical step in combating the disease (*14, 41, 49, 55, 56*).

Environmental factors, such as persistent nutrient surplus and sedentary lifestyle, increase the associated lifetime risk of developing pre-diabetes and T2DM (*14, 48, 49*). Recent epigenetic studies that address the interactions between environmental factors and genetic alterations have identified various molecular mechanisms of lifestyle interventions for T2DM prevention (*12, 14, 57*). Importantly, emerging evidence shows that the changes in the nuclear and mitochondrial DNA methylation profile may reflect early-stage progression of pre-diabetes, as well as the transition from metabolically healthy obesity (MHO) to metabolically unhealthy obesity (MUO) (*13, 14, 58*). Importantly, measuring epigenetic changes may serve to allow for earlier diagnosis of pre-diabetes and timely

assessment of the effectiveness of lifestyle interventions as they relate to diabetes amelioration. Prior scientific reviews have examined the alterations of both histone modifications and miRNAs in the diabetic state, however, comprehensive reviews examining the effects of DNA methylation on early recognition of diabetes, combined with effective intervention strategies as they pertain to the functional response of metabolic functioning to DNA methylation are lacking (*12, 58-67*). In this article, we review the most recent and novel evidence regarding altered DNA methylation in pre-diabetes, T2DM, and examine the changes in the methylome profile following lifestyle intervention.

1.3 Mitochondrial Function in Type 2 Diabetes Mellitus

1.3.1 Mitochondrial Function in Healthy and Diabetic States

Previous studies have shown that mitochondrial function in impaired in insulin resistant states(*68-72*). Mitochondria plays a primary role in energy metabolism and function to maintain energy homeostasis, which requires fusion and fission to take place for adequate ATP generation and thermogenic mechanisms to persist(*14*). Importantly, mitochondria respond to external stimuli (exercise, diet, energy deficit/surplus) via activation of the AMP/ATP-AMPK, NAD+/NADH-SIRT1, and cAMP-PKA/CREB pathways(*14, 73-75*). PGC-1α is considered the master regulator of mitochondrial function due to its primary role as a coactivator responsible for upregulating genes involved in oxidative function. This is accomplished primarily through activation of the NRF1/2-TFAM pathway(*75, 76*). In the diabetic state, mitochondrion experience stress due to the presence of reactive

oxygen species (ROS), as well as circulating free fatty acids and triglycerides, leading to impaired mitochondrial functionality(*14*).

1.3.2 PGC-1α

Mitochondrial biogenesis occurs in response to low ATP levels and involves the increase in both size and number of mitochondria in the cell(*14, 77, 78*). This process is regulated primarily by PGC-1α(*79*). PGC-1α regulates cellular energy metabolism in response to external stimuli, primarily by regulating mitochondrial biogenesis and oxidative metabolism(*80*). Epigenetic regulation of mitochondrial biogenesis and function across tissues in the progression to the diabetic state is of particular interest. Indeed, in metabolically impaired states such as obesity, diabetes, and cardiovascular disease, functional abnormalities in mitochondrial function are common(*77*). Interestingly, increased methylation in the promoter region of PGC-1α has been shown in diabetic islets cells and fat tissue in subjects with T2DM accompanied by subsequent down-regulation of genes involved in mitochondrial biogenesis *(5, 34)*.

A previous study examined if the PGC-1α methylation status in the promoter region could predict obesity and insulin resistance later in life (*81*). It was found that epigenetic marks (specifically, DNA methylation) measured early in life (5-7 years old) remain stable over time and may predict disease risk later in life, concluding that identification of these markers may serve as a tool for early diagnosis and intervention in at-risk cohorts of individuals. An exercise intervention study examining the methylation profile of PGC-1α and subsequent mRNA expression in offspring found that hypermethylation in the

promoter region of the PGC-1α gene in response to maternal high fat feeding was sustained for 12 months and coincided with decreased expression of PGC-1α mRNA (*82*). These effects were ameliorated in offspring from females exposed to exercise during pregnancy, indicating further that lifestyle interventions are effective tools for preventing epigenetic dysregulation and downstream metabolic complications, even when enacted by the mother (intergenerational effect)(*82*).

The identification of epigenetic markers for diagnostic utilization is of great interest, especially in light of the data linking epigenetic reprogramming with the progression of metabolic dysregulation(*83-88*). A study published in 2009 examined the methylation profile of the PGC-1α promoter in skeletal muscle in T2DM subjects in addition to genome-wide promoter analysis of DNA methylation(*89*). They found concurrent hypermethylation of the PGC-1α promoter and reduced expression of the PGC-1α gene. Additionally, they found a reduction in mitochondrial markers, including TFAM, SUO, and CYTc, and subsequent reductions in mitochondrial DNA copy number and mitochondrial size, indicating a potential link between PGC-1α promoter methylation, mitogenesis, and insulin resistance. The hypermethylation of the PGC-1α promoter, therefore, may serve as an early indicator insulin resistance occurring prior to overt symptoms of diabetes progression(*89*).

The modulation of the expression of PGC-1α may be a critical component in the development of T2DM and associated metabolic complications. Although the disease itself is multifaceted and the exact underlying mechanisms are still not entirely

understood, environmental factors such as diet and exercise may contribute to the changes in DNA methylation at the promoter of PGC-1α and may play a critical role in modifying genetic predispositions associated with insulin resistance(*72, 75, 89*). It will be especially important, moving forward, to examine the tissue-specific patterns as compared with those seen in the blood. To this end, it is of interest to examine the temporal changes in DNA in the promoter region of PGC-1α during the progression of insulin resistance and type 2 diabetes mellitus in metabolically active tissues and white blood cells, in order to establish tissue-specific patterns that may lead to the establishment of early diagnostic molecular markers, which would allow for early interventional strategies to be implemented prior to overt disease onset in T2DM.

1.3.2.1 PGC-1α in Adipose Tissue

Adipose tissue serves as the primary energy storage unit in the mammalian body. Adipocytes are responsible for triglyceride synthesis and producing acetyl-CoA, a molecule critical for fatty acid synthesis. These essential metabolic roles deem adipocyte-derive mitochondria extremely critical for physiological processes to persist and for homeostasis to be maintained. Indeed, adipose tissue is critical to the maintenance of glucose homeostasis due to its role in lipid metabolism and turnover of triglycerides, and prior studies have confirmed the relationship between impaired mitochondrial function in adipocytes leading to adverse outcomes associated with T2DM (*90*). Finally, the importance of mitochondrial biogenesis during cellular differentiation in white adipocytes has been confirmed in 3T3-L1 cells. Though PGC-1α exhibits low expression levels in white adipose

tissue, it plays a critical role in this tissue and knockout of PGC-1α results in the onset of insulin resistance in mice (*91*). Choo et al. showed that mitochondrial protein levels, mtDNA, and ability to utilize glucose are severely reduced in db/db mice, suggesting that dysfunction in mitochondrial biogenesis may be a result of insulin resistance in the adipocyte and not due to excess adiposity alone. The effects of reduced mitochondrial biogenesis were alleviated after db/db mice were treated with rosiglitazone and glucose homeostasis was restored. Furthermore, rosiglitazone may induce mitochondrial biogenesis in adipose tissue, leading to increased oxygen consumption and FA oxidation(*92*).

1.3.2.2 PGC-1α in Liver

Proper hepatic function is paramount to the maintenance of macronutrient metabolism (carbohydrate, protein, and fat) and, subsequently, glucose homeostasis. This is due to the responsibility of the liver, in the fed state, to convert glucose to glycogen to be stored in the liver, or lipids to be stored in the adipose tissue. In the fasted state, the liver converts non-carbohydrate sources to glucose (gluconeogenesis), catabolizes glycogen to glucose, and converts fatty acids into ketone bodies (ketogenesis) for energy. Studies have shown that reduced expression of 25 genes encoding genes involved in the oxidative phosphorylation pathway is seen in the obese state (*93-96*). This phenomenon is seen in mice as well, following the initiation of a high-fat diet(*95*). However, PGC-1α has actually been shown to increase in the liver during the diabetic state. Indeed, PGC-1α has been shown to inhibit the hepatic insulin signaling

mechanisms in T2DM and enhance glucose output in the liver, regardless of high blood glucose levels(*12, 97*). The mechanisms of inhibition of insulin action and promotion of glucose output put forth by hepatic PGC-1α led some researchers to begin to refer to PGC-1α in the liver as "pro-diabetic"(*98*) Interestingly, other studies have shown that PGC-1α overexpression coincides with obesity and diabetes, suggesting that PGC-1α promotes gluconeogenesis and alters fatty acid metabolism, leading to sustained impaired glucose homeostasis(*99*).

1.3.2.3 PGC-1α in Skeletal Muscle

Insulin resistance in the skeletal muscle is a principal feature in T2DM, as skeletal muscle plays a key role in insulin-mediated glucose disposal and accounts for almost 80% of glucose uptake (*100, 101*). In the diabetic state, a marked decrease in oxidative slow-twitch fiber types coincides with an increase in glycolytic fast-twitch fiber types. This may be due to the significant reduction in mitochondrial biogenesis and oxidative phosphorylation capacity. As such, it has been of great interest to examine the underlying molecular mechanisms that precede and coincide with worsening insulin sensitivity. Prior studies have shown that PGC-1α is reduced in humans with insulin resistance(*102, 103*). Indeed, a previous study by Mootha et al. examined ~22,000 genes in normal glucose tolerant, impaired glucose tolerant, and T2DM patients using DNA microarrays and found that PGC-1α was significantly decreased in the skeletal muscle of diabetic patients, as compared to their healthy counterparts. Further, these results corresponded with a significant decrease in expression of genes involved in

oxidative phosphorylation, indicating an important downstream effect of decreased PGC-1α expression(*104*). The exact mode whereby PGC-1α expression is regulated is not fully understood, but could be a result of epigenetic control, excess lipid accumulation (characteristic in the obese and diabetic states), or as a result of direct insulin action(*89, 103, 105-107*). It is plausible, too, that these mechanism work in concert resulting in decreased PGC-1α expression in skeletal muscle in the diabetic state.

1.4 Epigenetics

The definition of the term "epigenetics" has evolved over the last 50 years as our understanding of epigenetic mechanisms has improved and the data confirming these pathways have accumulated(*108*). Epigenetic mechanisms, which result in a change in the gene expression without altering the DNA sequence, include histone modification, DNA methylation, and non-coding RNA (ncRNA) (*12, 109, 110*). Importantly, the different epigenetic processes are interrelated and work in coordination to exert molecular effects on genomic transcription and protein translation.

1.4.1 Histone Modification

Activation and silencing of transcription depend on chromatin conformation, which is directly controlled by epigenetic mechanisms at the level of the nucleosome (*111-113*). Nucleosomes consist of DNA wrapped around an octamer of four core histones, which can be modified covalently by phosphorylation, acetylation, ubiquitination, and methylation (*109, 110*). Histone modifications are involved in the regulation of gene

activation and silencing and also play an important role in DNA replication and repair(*113*). Chromatin proteins, too, can be epigenetically regulated to impact normal cell differentiation during the early and late S-phases of cell replication (*114*). As such, histone modification has been identified in various diseases and shown to be an effective prognostic marker (*109*).

1.4.2 Non-coding RNAs

Non-coding RNAs (ncRNAs) are a class of RNA molecules (e.g., long non-coding RNA or lncRNA, and microRNA or miRNA) that regulate gene expression at both the transcriptional and translational levels without coding for a protein themselves (*66, 115-118*). Of the known ncRNAs, miRNA is the most studied and has been shown to mediate both mRNA degradation and mRNA translational repression (*115, 116*).

1.4.3 DNA Methylation

DNA methylation involves the addition of a methyl group to a Cytosine-Guanine (CpG) dinucleotide (**Figure 2**)(*119*). The mechanism of DNA methylation was originally discovered by researchers examining X-chromosome inactivation in rodent models(*120, 121*).

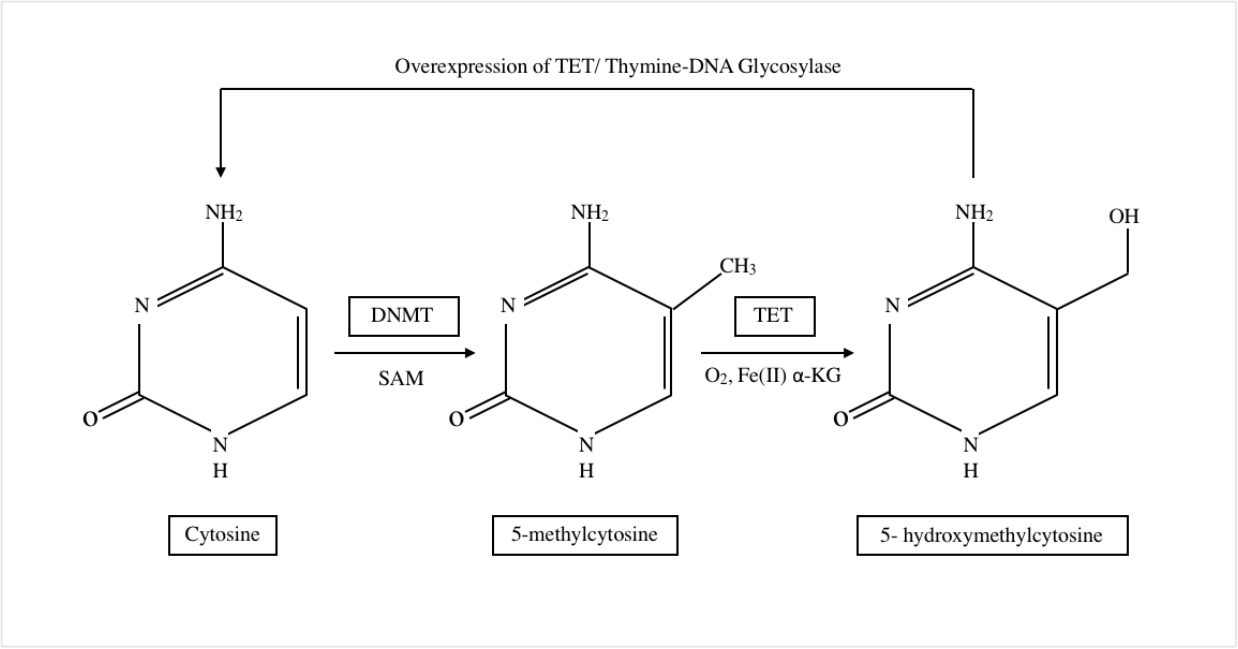


Figure 2. DNA methylation and its role in the regulation of gene expression. DNA methylation at cytosine is controlled by DNA methyltransferase (DNMT) and the methyl donor S-Adenosylmethionine (SAM) to produce 5-methylcytosine. 5-methylcytosine can be oxidized by α-ketoglutarate (α-KG)-dependent TET to produce 5-hydroxymethylsytosine, initiating de-methylation catalyzed by ten-eleven translocation (TET) family enzymes and thymine DNA glycosylase (TDG). Adapted from Zhang et al. (*122*)

DNA methylation is necessary in mammals for survival, as it governs transcriptional regulation and silencing, and is involved in cellular differentiation (*109, 123*). Indeed, DNA methylation leads to the silencing of genes, but may also affect expression of noncoding RNAs, transcriptional elongation, splicing events, and overall genomic stability (*124, 125*). DNA methylation is maintained by DNA methyltransferases, DNMT1, DNMT3A, and/or DNMT3B, which catalyze the transfer of a methyl group

from S-adenosyl methionine (SAM) to DNA (**Figure 3**) (*126*). Interestingly, methylation levels across CpG sites in the genome is bimodal, with CpG islands typically showing hypomethylation and other CpG sites showing hypermethylation. CpG islands are associated with transcription start sites and, because promoter CpG islands must maintain a steady state of hypomethylation, DNA methyltransferases must be actively excluded from these locations (*125-127*). Current data suggests methylation that takes place near the transcription start sites will block initiation of transcriptional elongation (**Figure 3**) (*126*). Gene promoters are a target for DNA methylation and are always unmethylated when a gene is active.

The epigenetic mechanisms mentioned above have been shown be functionally interrelated and to operate in coordination with one another, via a common network, to repress transcription (*128*). For instance, DNMT1, which is responsible for maintaining the methylation of cytosine residues *in vivo*, also exerts effects on histone deacetylase activity via the repression of trithorax-related protein (HRX) (*129*). This is due to the transcription-repressing region of HRX that closely resembles a region in the DNMT1 gene. As mentioned previously, methylation at the level of DNA has also been shown to affect chromatin structure through histone deacetylation, methylation, and local chromatin compaction (*130*). Further, it has been recently revealed that CpG islands that are not methylated may undergo H3K4 and H3K27 tri-methylation via transcriptional modulation, and exclude H3K36 methylation. miRNAs, too, have been shown to exert effects on the DNA methylation patterns in metabolically active tissues, such as the pancreas and heart, as well as CD4+ T cells from peripheral blood mononuclear cells, by

directly targeting DNMT1 (*131-133*), as well as the estrogen receptor-alpha (*118*). Mechanistically, miRNAs may target the 3'UTR of DNMT1 and down-regulate DNMT1 expression, thereby reducing the DNA methylation level and increasing mRNA expression of estrogen receptor α (*118, 134-136*).

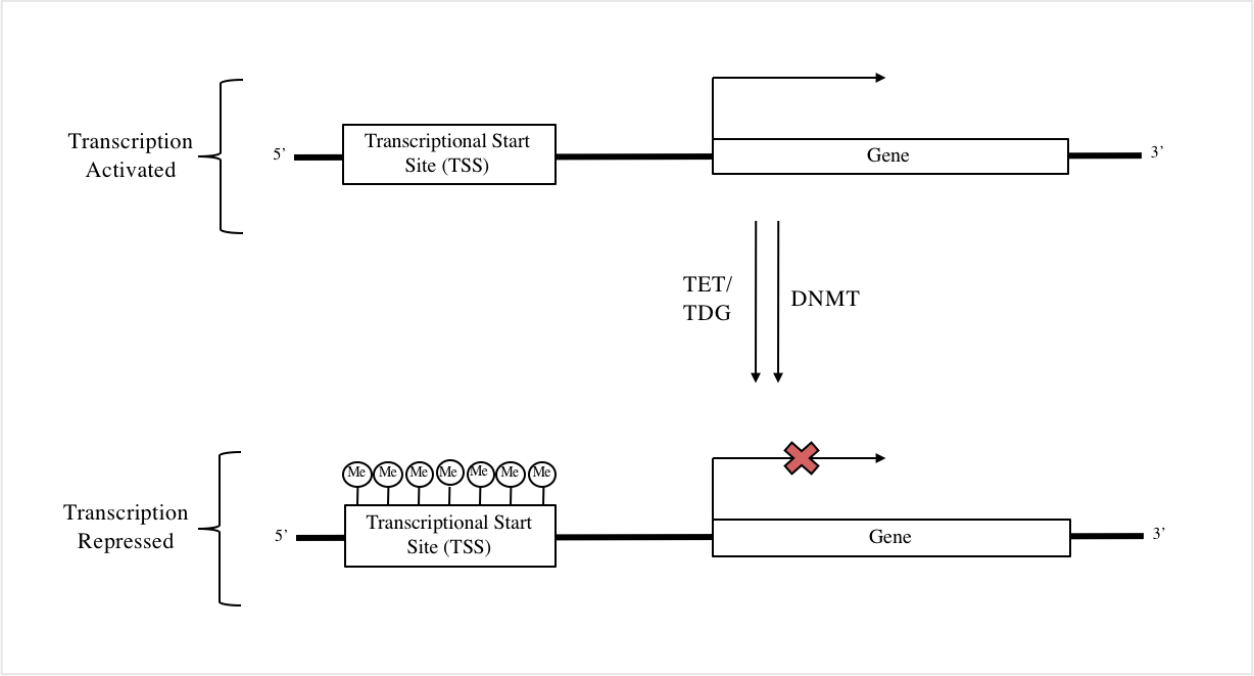


Figure 3. The reversible DNA methylation has been shown to regulate gene expression. Reduced methylation at the transcription start site (TSS) results in transcription initiation and gene expression, while increased methylation leads to gene suppression(*137*).

The dynamic nature of DNA methylation is paramount to the plasticity of the epigenetic mechanism, allowing for cellular adaptations to environmental stimuli, thereby enhancing or silencing gene expression. Specifically, DNA methylation tends to show stability acutely, but is not a permanent molecular phenomenon, which is why organisms can exhibit phenotypic changes in response to environmental alterations(*138*). That is, following treatment protocols and lifestyle intervention, the marker is reversible, adding

to the importance of early diagnosis in metabolic disease states. Methyl-cytosine, upon oxidation by the a-KG-dependent TET family of dioxygenases, yields 5-hydroxymethylcytosine (**Figure 4**). This compound is found in high concentrations in the brain, but relatively small concentrations elsewhere in the body. More importantly, this oxidative step serves as the initiation of active DNA de-methylation (*139*). Over-oxidation of 5-hydroxymethylcytosine leads to the formation of 5-formylcytosine and, eventually, 5-carboxylcytosine. Base-excision repair of 5-carboxylcytosine, mediated by thymine DNA glycosylase, removes the alkyl group and primes the methylation process to begin again (*138, 140*). Not surprisingly, an over- or under- expression of TET enzymes will determine the cellular content of 5-hydroxymethylcytosine, 5-formylcytosine, and 5-carboxylcytosine (*141*). Importantly, because of the CXXC motifs, which are present in the TET family of enzymes, maintain hypomethylation of CpG islands at the transcriptional start sites and Tet1 and 5hmC are present in high quantities at promoter regions of genes(*142*).

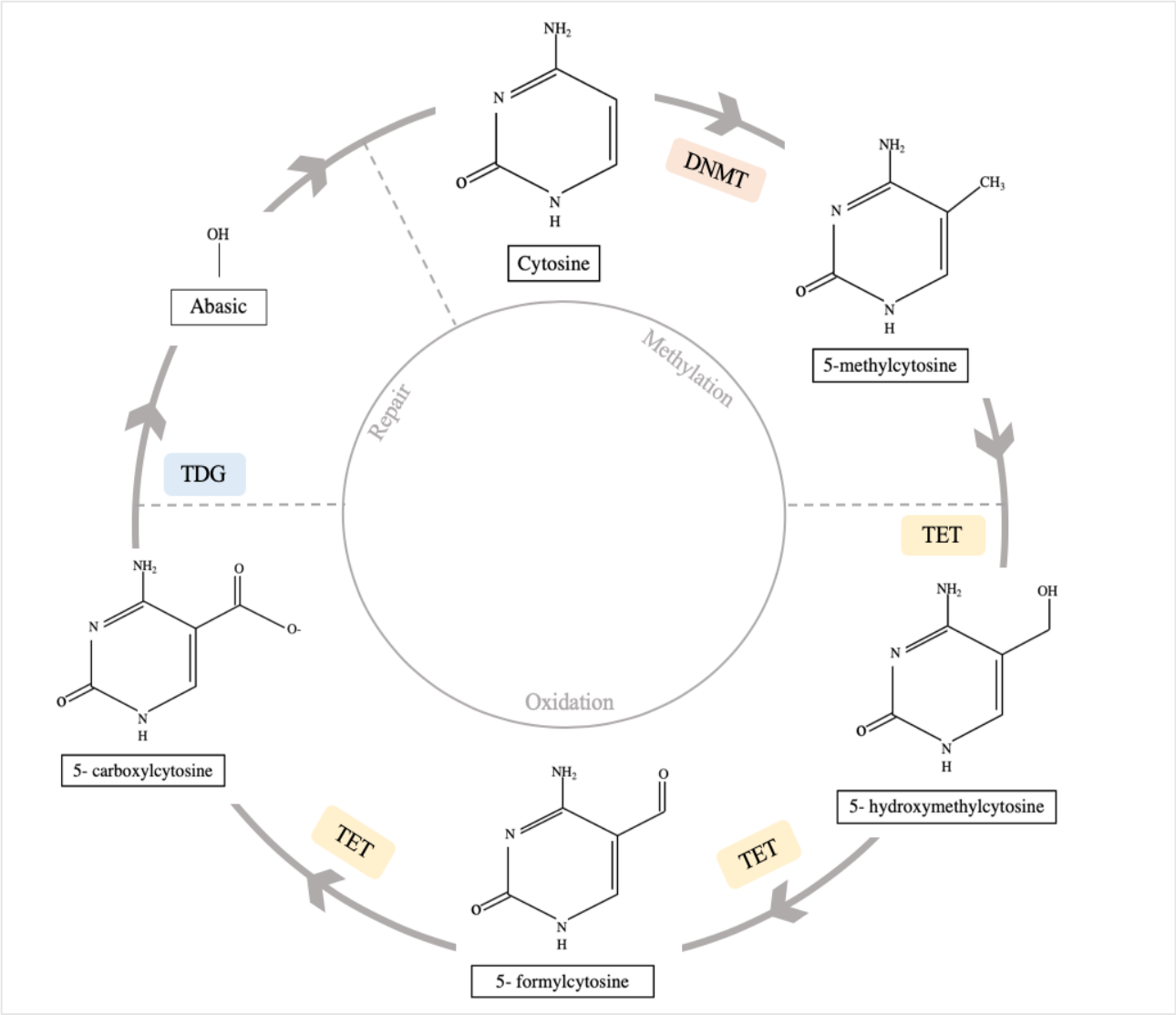


Figure 4. Representation of the process of methylation, oxidation, and repair of the cytosine residue during the process of methylation and subsequent demethylation, resulting in both stability and flexibility at the level of the genome(*140*).

1.4.3.1 Altered DNA Methylation in Type 2 Diabetes Mellitus

Metabolic diseases such as obesity, pre-diabetes, and T2DM have been strongly linked to epigenetic changes (*7, 48, 143, 144*). Altered DNA methylation across tissues has been identified in blood (*81, 145, 146*), pancreas (*147*), liver (*148-150*), adipose tissues (*122, 151, 152*), skeletal muscle (*153*), and diabetic kidney (*154-156*). To define the epigenetic signature of systemic insulin resistance in obese women, Arner et al. conducted genome-wide transcriptome and DNA CpG methylation profiling on subcutaneous (sWAT) and visceral adipose tissue (vWAT) (*157*). This study revealed 336 differentially methylated sites (DMS) that accounted for 223 insulin resistance-associated genes in SAT, and 29 DMS for 18 IR-associated genes (*157*). Using genotypic information as a causal anchor to explore the role of DNA methylation in the etiology of T2DM, Elliott et al. identified DNA methylation in the gene KCNQ1 is likely to be on the causal pathway to disease in later life (*158*). To this end, the key enzyme catalyzing DNA methylation, Dnmt3a, has been shown to prime adipose insulin resistance (*152*).

As one of the effectors of DNA methylation, nutritional status plays a key role in altered DNA methylation. It was shown that high glucose (hyperglycemia) elicits DNA methylation changes that dysregulates gene expression, which persists even after glucose normalization (*159, 160*). The "epigenetic memory" may be passed on to the next generation. Interestingly, fetal exposure to high glucose leads to enlarged subcutaneous adipocytes in adults, which showed lower LEP promoter methylation and higher leptin expression and secretion in adipocytes, thereby increasing risk of developing metabolic disease(*161*). In line with being another effector of DNA methylation, chronic low-grade

inflammation is prevalent in obesity and T2DM (*162-166*). BMI and waist circumference have also been shown to be associated with the global DNA methylation levels in whole blood DNA and CD4+ T-cells (*167, 168*).

Functionally, the liver serves a critical role in maintaining glucose homeostasis and clearing insulin, mechanisms that are impeded in the diabetic state. Because of its functional role in regulating blood glucose levels, tissue-specific DNA methylation changes have been examined in the liver during the diabetic state. Nilsson et al. conducted a genome-wide analysis of DNA methylation in the human liver and, using a linear regression model to identify DNA methylation patterns in diabetic *vs* non-diabetic subjects, found that nearly 94% of CpG sites exhibited reduced DNA methylation in subjects with T2DM *vs* healthy controls (*122*). No significant differences were seen in DNA methylation among non-diabetic patients alone, indicating a potential mechanism whereby DNA methylation in the liver contributes to T2DM risk. These results are similar to those observed by Dayeh et al., who found that 97% of differentially methylated pancreatic CpG sites exhibited reduced DNA methylation in diabetic *vs* normal islet cells (*169*). **Table 2** provides examples of metabolically active tissue-specific changes in DNA methylation, which were associated with dysregulation of gene expression and metabolic homeostasis.

Tissue Type	Medical Conditions Associated	Genes	Alterations in DNA Methylation	Ref
Adipose Tissue	Obesity	HIF3A	DNA methylation in the promoter region of HIF3A was associated positively with BMI in obese (non-diabetic) individuals.	(*8*)
		FTO	Variant-CpG restricted Haplotype-specific methylation within FTO obesity susceptibility locus;	(*170, 171*)
	Type 2 Diabetes	GYS2, ELOVL6, and FADS1	Down-regulated in diabetic state coincided with reduced glucose uptake and impaired lipid handling.	(*122*)
Liver	Non-Alcoholic Fatty Liver Disease	PPARGC1A	Increased DNA methylation in the promoter region of PPARGC1A was correlated with NAFLD progression.	(*172*)
Pancreas	Type 2 Diabetes	PDX-1	Up-regulated DNA methylation in the promoter region of PDX-1 was correlated with decreased mRNA level of PDX-1, insulin expression and glucose-stimulated insulin secretion.	(*173*)
		INS	Increased DNA methylation at the promoter of INS was correlated negatively with INS gene expression in islets.	(*174*)
Skeletal Muscle	Type 2 Diabetes	PPARGC1A	Hypermethylation at the promoter of PPARGC1A was correlated with reduced expression of PPARGC1A and OXPHOS genes and peripheral insulin resistance.	(*79, 89, 175*)
	Aging	COX7A1	Increased DNA methylation at the promoter of COX7A1 was associated with decreased mRNA expression during aging process.	(*176*)
Blood	Obesity	HIF3A	Methylation level at three sites in the first intron of HIF3A was positively associated with BMI.	(*8, 162*)
		SREBF1	Increased methylation at locus cg11024682 demonstrated causal effect on BMI and potentially dyslipidemia and vascular endothelial dysfunction.	(*177*)

Table 2. The changes in DNA methylation associated with pre-diabetes, T2DM and co-morbidities.

HIF3A is a component of the of the heterodimeric transcription factor hypoxia- inducible factor 1 (HIF-1), and increasing evidence has shown that HIF-1 plays a key role in metabolism, energy expenditure, and obesity (*8, 162, 178*). Additionally, HIF3A functions as an accelerator of adipocyte differentiation and plays a role in the cellular response to glucose and insulin. One study showed that BMI was positively correlated with methylation of the promoter region of HIF3A in adipose tissue and in blood (*167*). Interestingly, a recent study by Aslibekyan et al. disputed these conclusions, finding no statistically significant associations between BMI and methylation in HIF3A. However, it is important to note that this could have been due to a difference in cohort and/or tissue type (CD4+ versus whole blood) (*168*). Further investigations are warranted to establish a unifying pathophysiological role of DNA methylation, as indicated by the exception of HIF3A. In obese subjects, rapidly increased adiposity is known to cause local hypoxia in the adipose tissue, which up-regulates HIF3A (*179*). DNA methylation at the promoter of HIF3A in the subcutaneous adipose tissue was positively correlated with BMI of obese individuals, i.e., increased adiposity indicates an elevation of DNA methylation in HIF3A promoter (*8, 162*). However, the increased HIF3A DNA methylation and gene expression in obesity does not support a reciprocal relationship (*8, 50, 162*). In fact, DNA methylation levels in the HIF3A promoter did not show any significant association with the HIF3A gene expression level in adipose tissue (*8*).

The functionality of pancreatic beta cells is paramount for the maintenance of normoglycemia (*180*). Dysfunction of the beta cells leads to insufficient production and secretion of insulin into the blood stream and eventual hyperglycemia, a primary

characteristic of T2DM (*181*). Prior studies have examined the genome-wide DNA methylation patterns in pancreatic islet cells in response to elevated palmitate levels (*in vitro* model representative of physiological presence of circulating FFA, characteristic of the T2DM disease state) (*182*). Global DNA methylation levels were significantly higher in islet cells exposed to the acute elevated palmitate levels. Gene-specific DNA methylation in the islets cells has been examined previously, as well. The insulin gene (INS), for instance, has been shown repeatedly to exhibit hypermethylation of the INS promoter region in T2DM patients compared to healthy controls, and these methylation levels have been shown to be negatively correlated with INS mRNA expression (*174, 183*). Additionally, pancreatic and duodenal homeobox 1 (PDX1), a transcription factor playing a critical role in beta cell maturation, showed significantly increased DNA methylation in islet cells from T2DM patients versus healthy control. Decreased mRNA levels in PDX1 were associated with increased methylation in the enhancer region of the gene (*173*). These results added to additional data showing that PDX1 mRNA expression were correlated with INS mRNA expression, as well as subsequent insulin secretion (**Figure 5**) (*174*).

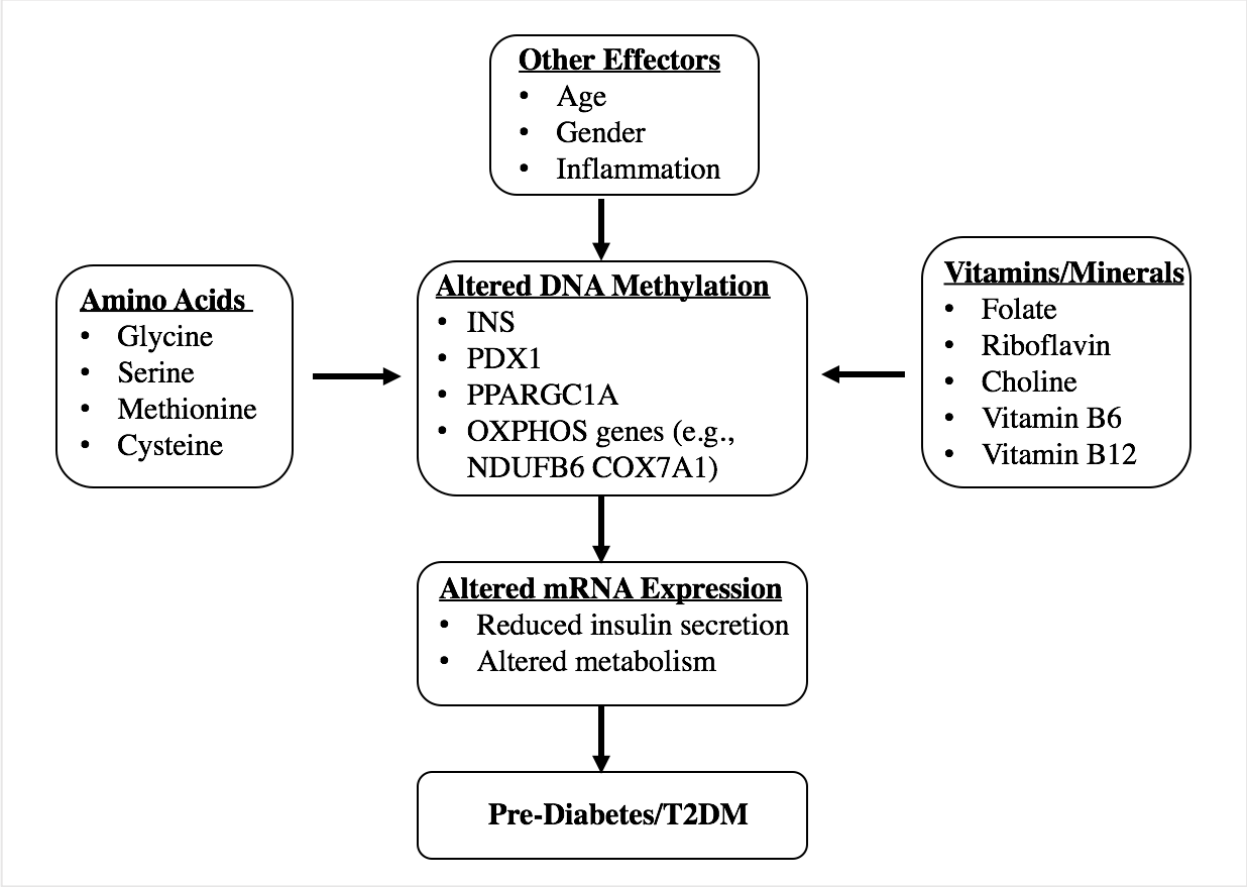


Figure 5. The effectors of DNA methylation and subsequent mRNA expression leading to pre-diabetes and T2DM. Model showing the potential role for DNA methylation in the pathogenesis of T2DM, specifically incorporating known effectors of DNA methylation, including age, gender, inflammation, and nutrient status (amino acids and vitamins/minerals). Impaired insulin secretion and altered metabolism (hallmarks of T2DM) occur as a result of the altered methylation levels at the genes listed, leading to intermediate hyperglycemia and T2DM(*184*).

Mitochondria play the central role in metabolic homeostasis via oxidative phosphorylation (OXPHOS) (*14, 73, 74*). In response to energy demand, the cell activates mitochondrial biogenesis pathways to increase the function and number of mitochondria, where the transcriptional co-activator PGC-1α acts as the master regulator to control genes involved in mitochondrial OXPHOS and metabolic regulation (*77, 78*). In line with the notion that mitochondrial alteration is associated with metabolic derangement (*14, 73, 74, 77*), epigenetic changes have been observed in the gene PPARGC1A that encodes PGC-1α (**Table 3**) (*79, 89, 175*).

Increased methylation in the promoter region of PPARGC1A in diabetic islets cells and fat tissue in subjects with T2DM were accompanied by down-regulation of genes involved in mitochondrial biogenesis (*79, 80*). Examination of the methylation profile of the PPARGC1A gene in skeletal muscle from T2DM subjects revealed hypermethylation of the promoter and reduced expression of the PPARGC1A; consistently, there was downregulation of TFAM, SUO, CYTc, and core 1, and mitochondrial DNA copy number, mitochondrial content and mitochondrial size (*89*). Following high fat feeding, increased methylation was detected in the promoter region of PPARGC1A in skeletal muscle, which was concomitant with down-regulation of PGC-1α and NDUFB6 gene expression, and subsequent insulin resistance (*175*). The reciprocal relationship between DNA methylation and gene expression was also observed for COX7A1, a nuclear encoded OXPHOS gene coding for a subunit of complex IV in the respiratory chain, which shows increased DNA methylation at the promoter and decreased mRNA expression during the progression of aging in human skeletal muscle (*176*).

Hypermethylation of PPARGC1A in the promoter region was also observed in subjects of nonalcoholic fatty liver disease (NAFLD), the medical conditions that are commonly associated with insulin resistance, obesity and T2DM (*172*). The hypermethylation of PPARGC1A and downregulated gene expression (*79, 80, 89*) may account at least in part for altered mitochondrial function in T2DM and pre-diabetes (**Figure 5**).

Though some weight loss therapies have included medication and/or surgery, large-scale studies have shown that behavioral interventions, such as changes in nutritional intake, increasing physical activity, and improving access to educational support, are effective strategies for improving health outcomes (*2, 185*). The interventions targeting lifestyle factors (e.g., caloric restriction and exercise) have shown the potential to prevent the epigenetic dysregulation that accompanies obesity and pre-diabetes (**Table 3**) (*9, 51, 186*). Myocyte-specific enhancer factor 2A (MEF2A), a transcriptional activator involved in the activation of growth factor and stress-induced genes as well as the regulation of exercise-induced GLUT4 expression, shows decreased DNA methylation following an exercise bout (*54*). In addition, the genes encoding thyroid adenomas protein (THADA), mitochondrial ubiquinone oxidoreductase subunit C1 (NDUFC), and mitochondrial NADH:ubiquinone oxidoreductase core subunit 6 (MT-DNA6) undergo DNA hypomethylation, thereby increasing gene expression following exercise (**Table 3**).

Lifestyle Change	Intervention Induced Phenotype	Genes	Alterations in DNA Methylation	Ref
Increased Exercise	Reduction of chronic and persistent inflammation, weight loss, decreased BMI etc.	PPARGC1A	Decreased DNA methylation at the promoter of PPARGC1A was correlated with increased expression of PPARGC1A and OXPHOS genes and improved peripheral insulin sensitivity. Increased mitochondrial biogenesis.	(*51, 79*)
		THADA NDUFC MEF2A	Decreased DNA methylation at the promoter or gene.	(*51*)
		MT-ND6	Decreased DNA methylation at the promoter and improved oxidative capacity.	(*177*)
Calorie Restriction	Improved lipid profile, weight loss, decreased BMI, etc.	PPARGC1A	As BMI decreases, methylation status of probes in intron 1 are decreased in blood and adipose tissue.	(*51*)
		ATP10A	In peripheral blood mononuclear cells (PBMCs), DNA methylation was decreased after hypo-caloric diet at ATP10A	(*145*)
		Leptin	High-fat, hyper-caloric diet altered methylome in leptin promoter.	(*145*)
		TNF-α	Hypo-caloric diet resulted in decreased TNF-α promoter methylation.	(*187*)

Table 3. The improved metabolism and reversed hypermethylation by lifestyle interventions through increased exercise or caloric restriction.

Diet and exercise may also contribute to the changes in DNA methylation in the promoter of PPARGC1A and modify genetic predispositions associated with insulin resistance (*72, 75, 89*). The DNA methylation in PPARGC1A measured early in life (5-7 years old) remains stable over time and may predict disease risk later in life, concluding that identification of these markers may serve as a tool for early diagnosis and intervention in at-risk cohorts of individuals (*81*). Indeed, an exercise intervention study examining the methylation profile of PGC-1α and subsequent mRNA expression in offspring showed that hypermethylation in the promoter region of PPARGC1A in response to maternal high fat feeding was sustained for 12 months and coincided with decreased PPARGC1A gene expression (*82*). These effects were ameliorated in offspring from females exposed to exercise during pregnancy, indicating further that lifestyle interventions are effective tools for preventing epigenetic dysregulation and downstream metabolic complications, even when enacted by the mother (*82*). These findings underscore a mitochondrial link between lifestyle intervention and epigenetic changes, and suggest the key role of mitochondria in metabolic alteration or improvement at the genetic and epigenetic levels.

The current clinical diagnosis parameters in pre-diabetes and T2DM lead to discordance, making it challenging to identify pre-diabetic individuals for diabetes prevention (*13, 42, 44, 45, 47*). Thus, it is important to examine the molecular mechanisms underlying the metabolic improvements following lifestyle interventions and to identify possible mechanisms that may contribute to the variable responses to different treatments within populations.

Given the role for epigenetic changes in T2DM pathogenesis, researcher have started the journey seeking potential use of epigenetic biomarkers to identify individuals of risk of developing T2DM (*7, 48, 144, 188, 189*). By comparing the percentages of dimethylation of H3 histones relative to total H3 histone methylation between diabetic and nondiabetic groups, Michalczyk et al. found that dimethylation of histones H3K27 and H3K4 had the potential as a predictive tool to identify women who have gestational diabetes progressing to T2DM (*189*). In addition, the predictive value of DNA methylation has been demonstrated for PPARGC1A by Clarke-Harris et al., showing that individuals with PPARGC1A hypermethylation at the age of 5-7 years have higher risk of developing obesity and metabolic syndrome at the age of 9-14 years (*81*). Of interest, examination of platelet mitochondrial DNA methylation indicated the potential of mitochondrial epigenetic markers for cardiovascular disease (CVD) risk(*190*). Hypermethylation was observed in CVD patients compared to healthy controls, including the genes encoding mitochondrial cytochrome c oxidase (MT-CO1, MT-CO2, and MT-CO3) and tRNA leucine 1 (MT-TL1), underlying mitochondrial DNA methylation potentially as non-invasive and easy-to-obtain markers for CVD, known to be diabetic complications (*190*).

To define the diagnostic value of mitochondrial epigenetic markers, our lab compared fasting glucose (FG) and hemoglobin A1c with mitochondrial DNA methylation for the outcomes of T2DM and pre-diabetes diagnosis (*13*). In contrast to FG and A1c that produce discordance to reflect T2DM and pre-diabetes progression, mitochondrial DNA methylation levels (MT-ND6, and MT-DLOOP) work in concert with parameters of impaired insulin sensitivity (HOMA-IR) to indicate early stage of pre-diabetes. In

addition, by stratifying health status of obese participants per metabolic syndrome scores, we observed a consistent increase in DNA methylation in PPARGC1A and the mitochondrial genes MT-ND6 as metabolically healthy obesity (MHO) progresses to metabolically unhealthy obesity (MUO) (*13*). Specifically, DNA methylation was upregulated 2.22 times ($p<0.01$) in MT-ND6 and 2.18 times ($p<0.01$) in PPARGC1A when the metabolic syndrome score increased from 0 to 4. Further larger-scale studies to examine the potential of mitochondrial epigenetic marker in pre-diabetes diagnosis will be of critical importance to develop consistent markers for T2D prevention.

Mitochondrial homeostasis is critical for metabolic health. In line with pre-diabetes and T2DM associated with mitochondrial dysfunction, altered DNA methylation profiles are increasingly observed in genes that regulate mitochondrial biogenesis (e.g., PPARGC1A) and mitochondrial respiration chain function (e.g., MT-ND6, MT-COX1). These changes may arise from environmental or lifestyle factors (e.g., nutritional status, physical activities, aging, gender, and inflammation). As such, lifestyle intervention targeting physical activity have shown promise to prevent T2DM or slow down disease progression, concomitant with DNA methylation reprograming. Importantly, emerging evidence suggests that mitochondrial DNA methylation level may reflect early-stage progression of pre-diabetes, underscoring its potential as a useful biomarker for pre-diabetes diagnosis and T2DM prevention. However, outstanding questions remain as to the molecular mechanism underlying the altered DNA methylation in pre-diabetes and T2DM, and the pathways by which the effectors (e.g., nutritional status and physical activity) induce the reversal of epigenetic changes and disease remission (*48*). Future

studies in these aspects, particularly in larger scale and including monozygotic twins, are of critical importance. Mechanistic studies that advance our understanding of epigenetic reprograming may lay the foundation for the development of epigenetic approaches in diagnosis of pre-diabetes and T2DM, and for sustainable and personalized treatment or intervention.

1.4.3.2 Effectors of DNA Methylation

Nutritional status: Nutritional status has been shown to effectively mediate the methylation status of genes, including those involved in metabolism (*186, 191-202*). Further, global markers of DNA methylation change in response to dietary factors, and energy intake is positively correlated with DNA methylation (*203*). Therefore, important variables to consider when utilizing DNA methylome data as an indicator for disease include fed/fasted status as well as nutrient intake. As mentioned above, SAM is responsible for the donation of methyl groups to the cytosine residues during the process of DNA methylation. Dietary amino acids (glycine, serine, methionine, cysteine) and vitamins/minerals (folate, riboflavin, choline, and Vitamin B6 and B12) are known methyl donors required for the generation of SAM. Not surprisingly, DNA methylation patterns change in response to the intake status of these various methyl donors via dietary and supplementary means (*204*). A previous study which sought to examine the epigenetic response to meal composition found that dynamic alterations in DNA methylation and gene expression were seen 160 minutes after a standardized meal (*191*). However, this effect was attenuated when accounted for changes in leukocyte fractions between the fasted and fed states. Further, plasma folate status has been shown to be

positively correlated with methylation in the PCA1 in males and females, positively correlated with methylation of SFRP1 in males only, and negatively correlated with methylation of PF only in males, shedding light on the complexity of methylation in different genders at different nutritional states (*205*). Circulating homocysteine concentrations, too, have been shown to associate with DNA methylation, however there are discrepancies in the data amongst groups showing both elevated and reduced global DNA methylation levels. Nevertheless, DNA methylation and gene expression show plasticity and variability as nutrition status changes in both males and females, and additional studies are needed to examine the tissue-specific and nutrient-specific effects of nutritional status on DNA methylation (**Figure 6**).

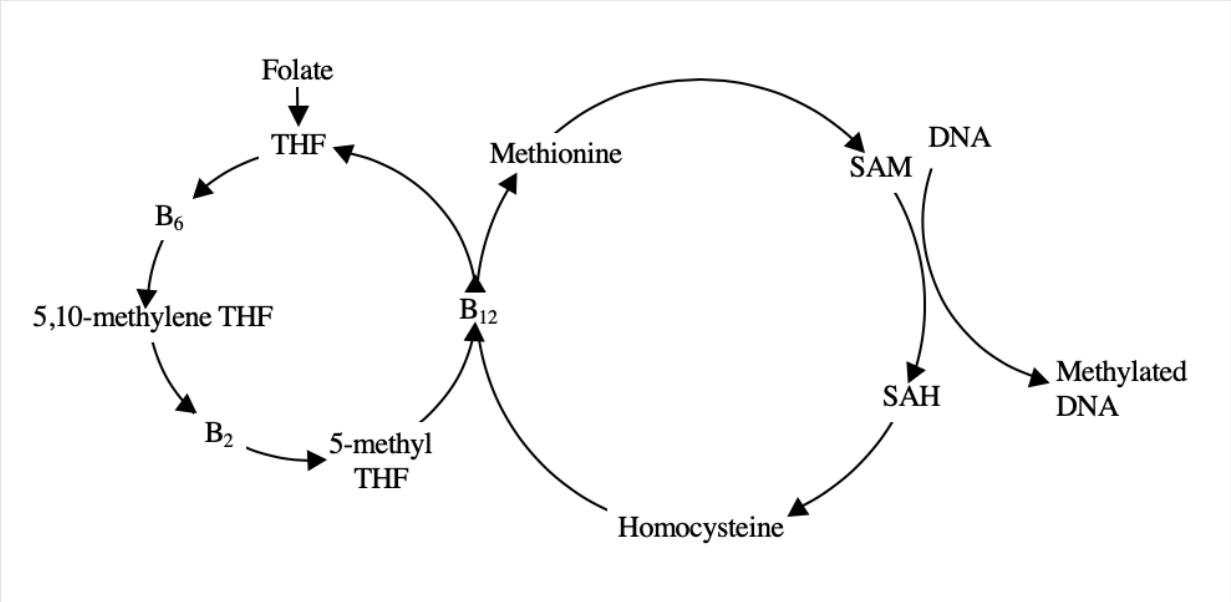


Figure 6. Schematic representation of how nutritional factors contribute to the SAM cycle. SAM is responsible for providing the methyl groups during the process of DNA methylation, which is catalyzed by DNMT(*184*).

Age: DNA methylation has been shown to be age-dependent (*192, 206*). Indeed, the term "epigenetic drift" refers to the substantial and significant global epigenetic variations that take place as animals age, as shown in both aging and identical twin human and animal studies (*207, 208*). An additional phenomenon, referred to as the "epigenetic clock" refers to site-specific age-related changes at the level of the gene, which may also be tissue specific(*119, 209*). The mechanisms underlying the epigenetic shifts seen in twin studies are not completely understood, adding to the difficulty interpreting whether alterations in gene expression are adaptive and can be reversed, or are solely due to inherent biological aging mechanisms (*210*). Longitudinal studies have also confirmed increases in inter-individual and within-pair methylation discordance with age (*211, 212*). Hypomethylation in LINE-1, a repetitive element used to measure global DNA methylation, is seen in both aging and various types of cancer (*213-216*). As cancer is considered a "disease of aging," it is not surprising that trends of global hypomethylation and gene-specific promoter hypermethylation patterns are similar in both cancer and aging (*217*). Age-dependent methylome alterations could have powerful implications in disease development.

Sex: Physiological levels of DNA methylation are not uniform amongst men in women. Many studies have revealed that global DNA methylation is higher in men than women, regardless of disease status (*133, 149, 170, 192, 218, 219*). For instance, the mean difference in DNA methylation level at the autosomal loci was significantly different between males and females(*212*). The potential mechanism for this discordance is likely due to the X-inactivation dosage compensation mechanism in females, an innate

mechanism used across species to equalize the number of expressed genes in females as compared to males (*220*). Interestingly, a sex-specific methylation profile was reported for human liver in both the X-chromosome and autosomes, which associates with altered hepatic gene expression and HDL-cholesterol(*149*). In human islets, however, sex differences were identified in chromosome-wide and gene-specific DNA methylation, and these differences were correlated with changes in β-cell function (i.e., insulin expression and secretion)(*133*). DNA methylation may play a role in the differentiation or maintenance of sexual dimorphisms. Our methylome mapping of the effects of sex may be useful to understanding the molecular mechanism involved in both normal development and diseases(*219*). This mechanism should be examined further in future studies to elucidate the discrepancy in DNA methylation patterns between male and female humans.

Inflammation: Subclinical presence of inflammation, as well as the pattern of inflammatory cytokines, is important in the progression of pre-diabetes to T2DM (*221*). DNA methylation has been shown to be an important cellular mechanism modulating gene expression associated with inflammation (*222*). Indeed, in both chronic gastritis and gastric cancer, chronic inflammation has been associated with increased DNA methylation (*137, 223*). Studies investigating the effect of inflammation on global DNA methylation have shown that in peripheral blood leukocytes, chronic inflammation is associated with increased DNA methylation (*222, 224*). An additional study confirmed these results, and found that in additional to inflammation, global DNA hypermethylation was associated with chronic kidney disease and atherosclerosis (*203*). Further,

inflammatory cytokine IL-6 may directly impact the epigenetic changes by regulating the DNMTase family, while simultaneously maintaining promoter methylation (*224*).

1.5 Dissertation Proposal

Type 2 diabetes mellitus (T2DM) is a growing epidemic and affects nearly 10% of the population in the United States. Effective prevention of T2DM through lifestyle intervention requires early identification of individuals with prediabetes (a state of high risk to develop T2DM), underscoring the significance and urgency to discover diagnostic markers. Previous studies from our lab and others have identified epigenetic changes that regulate mitochondrial biogenesis and function. Although mitochondrion-related epigenetic changes and metabolic derangements are observed in obesity and T2DM, the kinetics of these epigenetic and metabolic changes have not been studied, and the temporal correlation during disease progression remains poorly defined. Therefore, investigation into the potential use of epigenetic analyses in the diagnosis and monitoring of diabetes progression may provide an alternative means for diabetes prevention and amelioration.

We hypothesize that epigenetic changes precede intermediate hyperglycemia, and will test this hypothesis in mice (C57BL/6J) fed a high fat diet (HFD). As the key regulator of mitochondria, PGC-1α will be the focus of the study, and we hypothesize that the response to a dietary intervention (implementation of a high fat diet) will result in hypermethylation of the promoter region of PGC-1α, leading to downregulation of the PGC-1α gene transcription and subsequent detriments in mitochondrial copy number.

Results from this project will establish the temporal correlation of epigenetic changes with metabolic syndrome, and depict the potential of mitochondrion-related epigenetic markers in the assessment of disease progression and intervention. We will test this hypothesis through the pursuit of the following specific aims:

1.5.1 Specific Aim 1: Determine the inflection point of metabolic changes during high fat diet (HFD) challenge.

Hypothesis: Changes of body composition, glucose tolerance (GTT), and insulin sensitivity (ITT) over the duration of dietary challenges will correlate to the progression from a healthy state to a diabetic state.

These measurements will determine the inflection point(s) of metabolic changes.

1.5.2 Specific Aim 2: Determine the kinetics of DNA methylation (DNAme) in the promoter region of PGC-1α gene and mitochondrial changes during dietary challenges.

Hypothesis: Hypermethylation of the promoter region of PGC-1α will coincide with decreased mitochondrial DNA copy number and decreased protein expression involved in mitochondrial biogenesis and oxidative phosphorylation. Correlation analysis between leukocytes and metabolically active tissue will show significant temporal correlations of epigenetic changes in PGC-1α with insulin resistance, which will additionally reflect changes in anthropometric and metabolic measures.

The proposed research will shed light on how epigenetic reprogramming, specifically DNA methylation in genes associated with mitochondrial function, correlates with insulin resistance and prediabetes progression and remission in response to dietary INT. It may potentially lead to the design and implementation of novel diagnostic approach to identify early-stage pre-diabetes for effective intervention.

1.6 Summary

Early intervention is critical to prevent T2DM onset and associated complications. It is, therefore, essential to identify markers of early-stage dysregulation in glucose handling. Due to the genetic, epigenetic, and phenotypical alterations that occur in the mitochondria during the obese and insulin resistant states, it is of particular importance to examine the mechanisms responsible for these changes(*73, 74, 225, 226*). Since PGC-1α has a critical regulatory capacity in controlling mitochondrial biogenesis, the identification of epigenetic methylation in the promoter region of PGC-1α, which may underpin the mitochondrial dysfunction that coincides with disease onset, are crucial for the establishment of early prognostic markers. In order to elucidate the molecular alterations occurring in the pre-diabetic state, prior to overt disease onset, I will examine the temporal methylation reprogramming at the promoter region of PGC-1α, in addition to subsequent gene expression alterations. I will further investigate how these changes relate to changes in mitochondrial content and mitochondrial protein content. In conducting these experiments, I hope to identify a potential mechanism whereby methylation profiles of PGC-1α may improve diagnostic timing in order to ensure an "as-early-as possible" intervention time and prevent the onset of T2DM in patients currently in a pre-diabetic state.

1.7 References

1. N. C. D. R. F. Collaboration, Worldwide trends in diabetes since 1980: a pooled analysis of 751 population-based studies with 4.4 million participants. *Lancet* **387**, 1513-1530 (2016).
2. S. L. Norris *et al.*, Long-term non-pharmacological weight loss interventions for adults with type 2 diabetes mellitus. *Sao Paulo Med J* **134**, 184 (2016).
3. J. X. Rong *et al.*, Rosiglitazone Induces Mitochondrial Biogenesis in Differentiated Murine 3T3-L1 and C3H/10T1/2 Adipocytes. *PPAR Res* **2011**, 179454 (2011).
4. S. E. Kahn, M. E. Cooper, S. Del Prato, Pathophysiology and treatment of type 2 diabetes: perspectives on the past, present, and future. *Lancet* **383**, 1068-1083 (2014).
5. C. L. Ogden, M. D. Carroll, K. M. Flegal, Prevalence of obesity in the United States. *JAMA* **312**, 189-190 (2014).
6. D. W. Haslam, W. P. James, Obesity. *Lancet* **366**, 1197-1209 (2005).
7. C. Davegardh, S. Garcia-Calzon, K. Bacos, C. Ling, DNA methylation in the pathogenesis of type 2 diabetes in humans. *Mol Metab*, (2018).
8. A. M. Main *et al.*, DNA methylation and gene expression of HIF3A: cross-tissue validation and associations with BMI and insulin resistance. *Clin Epigenetics* **8**, 89 (2016).
9. T. Ronn *et al.*, Impact of age, BMI and HbA1c levels on the genome-wide DNA methylation and mRNA expression patterns in human adipose tissue and identification of epigenetic biomarkers in blood. *Hum Mol Genet* **24**, 3792-3813 (2015).
10. C. Ling *et al.*, Multiple environmental and genetic factors influence skeletal muscle PGC-1alpha and PGC-1beta gene expression in twins. *J Clin Invest* **114**, 1518-1526 (2004).

11. T. Dayeh, C. Ling, Does epigenetic dysregulation of pancreatic islets contribute to impaired insulin secretion and type 2 diabetes? *Biochem Cell Biol* **93**, 511-521 (2015).

12. C. Ling, L. Groop, Epigenetics: a molecular link between environmental factors and type 2 diabetes. *Diabetes* **58**, 2718-2725 (2009).

13. L. D. Zheng *et al.*, Mitochondrial Epigenetic Changes Link to Increased Diabetes Risk and Early-Stage Prediabetes Indicator. *Oxid Med Cell Longev* **2016**, 5290638 (2016).

14. Z. Cheng, F. A. Almeida, Mitochondrial alteration in type 2 diabetes and obesity: an epigenetic link. *Cell Cycle* **13**, 890-897 (2014).

15. S. R. Leeder, The history of insulin: the mystery of diabetes. *Med J Aust* **199**, 227 (2013).

16. A. M. Ahmed, History of diabetes mellitus. *Saudi Med J* **23**, 373-378 (2002).

17. R. Lakhtakia, The history of diabetes mellitus. *Sultan Qaboos Univ Med J* **13**, 368-370 (2013).

18. K. F. Eriksson, F. Lindgarde, Prevention of type 2 (non-insulin-dependent) diabetes mellitus by diet and physical exercise. The 6-year Malmo feasibility study. *Diabetologia* **34**, 891-898 (1991).

19. J. Tuomilehto *et al.*, Prevention of type 2 diabetes mellitus by changes in lifestyle among subjects with impaired glucose tolerance. *N Engl J Med* **344**, 1343-1350 (2001).

20. E. Cersosimo, C. Triplitt, C. Solis-Herrera, L. J. Mandarino, R. A. DeFronzo, in *Endotext,* K. R. Feingold *et al.*, Eds. (South Dartmouth (MA), 2000).

21. E. Cersosimo, P. Garlick, J. Ferretti, Insulin regulation of renal glucose metabolism in humans. *Am J Physiol* **276**, E78-84 (1999).

22. L. D. Katz, M. G. Glickman, S. Rapoport, E. Ferrannini, R. A. DeFronzo, Splanchnic and peripheral disposal of oral glucose in man. *Diabetes* **32**, 675-679 (1983).

23. R. A. DeFronzo, R. Gunnarsson, O. Bjorkman, M. Olsson, J. Wahren, Effects of insulin on peripheral and splanchnic glucose metabolism in noninsulin-dependent (type II) diabetes mellitus. *J Clin Invest* **76**, 149-155 (1985).

24. M. Nauck, F. Stockmann, R. Ebert, W. Creutzfeldt, Reduced incretin effect in type 2 (non-insulin-dependent) diabetes. *Diabetologia* **29**, 46-52 (1986).

25. A. Mari, J. Wahren, R. A. DeFronzo, E. Ferrannini, Glucose absorption and production following oral glucose: comparison of compartmental and arteriovenous-difference methods. *Metabolism* **43**, 1419-1425 (1994).

26. E. Ferrannini *et al.*, The disposal of an oral glucose load in healthy subjects. A quantitative study. *Diabetes* **34**, 580-588 (1985).

27. K. Ekberg *et al.*, Contributions by kidney and liver to glucose production in the postabsorptive state and after 60 h of fasting. *Diabetes* **48**, 292-298 (1999).

28. G. M. Reaven, Role of insulin resistance in human disease (syndrome X): an expanded definition. *Annu Rev Med* **44**, 121-131 (1993).

29. D. Thiebaud *et al.*, Effect of long chain triglyceride infusion on glucose metabolism in man. *Metabolism* **31**, 1128-1136 (1982).

30. A. Golay, J. P. Felber, E. Jequier, R. A. DeFronzo, E. Ferrannini, Metabolic basis of obesity and noninsulin-dependent diabetes mellitus. *Diabetes Metab Rev* **4**, 727-747 (1988).

31. D. E. Kelley, L. J. Mandarino, Fuel selection in human skeletal muscle in insulin resistance: a reexamination. *Diabetes* **49**, 677-683 (2000).

32. C. S. Fox *et al.*, Update on Prevention of Cardiovascular Disease in Adults With Type 2 Diabetes Mellitus in Light of Recent Evidence: A Scientific Statement From the

American Heart Association and the American Diabetes Association. *Diabetes Care* **38**, 1777-1803 (2015).

33. A. American Diabetes, 2. Classification and Diagnosis of Diabetes. *Diabetes Care* **39 Suppl 1**, S13-22 (2016).

34. A. American Diabetes, Standards of medical care in diabetes-2015 abridged for primary care providers. *Clin Diabetes* **33**, 97-111 (2015).

35. J. B. Meigs, D. M. Nathan, L. A. Cupples, P. W. Wilson, D. E. Singer, Tracking of glycated hemoglobin in the original cohort of the Framingham Heart Study. *J Clin Epidemiol* **49**, 411-417 (1996).

36. E. Selvin, C. M. Crainiceanu, F. L. Brancati, J. Coresh, Short-term variability in measures of glycemia and implications for the classification of diabetes. *Arch Intern Med* **167**, 1545-1551 (2007).

37. E. Selvin *et al.*, Glycated hemoglobin and the risk of kidney disease and retinopathy in adults with and without diabetes. *Diabetes* **60**, 298-305 (2011).

38. E. Selvin, M. W. Steffes, E. Gregg, F. L. Brancati, J. Coresh, Performance of A1C for the classification and prediction of diabetes. *Diabetes Care* **34**, 84-89 (2011).

39. E. Selvin *et al.*, Glycated hemoglobin, diabetes, and cardiovascular risk in nondiabetic adults. *N Engl J Med* **362**, 800-811 (2010).

40. D. B. Sacks, A1C versus glucose testing: a comparison. *Diabetes Care* **34**, 518-523 (2011).

41. A. G. Tabak, C. Herder, W. Rathmann, E. J. Brunner, M. Kivimaki, Prediabetes: a high-risk state for diabetes development. *Lancet* **379**, 2279-2290 (2012).

42. J. Ghouse *et al.*, Visit-to-Visit Variability of Hemoglobin A1c in People Without Diabetes and Risk of Major Adverse Cardiovascular Events and All-Cause Mortality. *Diabetes Care*, (2018).

43. I. B. Hirsch, M. Brownlee, Beyond hemoglobin A1c--need for additional markers of risk for diabetic microvascular complications. *JAMA* **303**, 2291-2292 (2010).

44. R. M. Cohen, S. Haggerty, W. H. Herman, HbA1c for the diagnosis of diabetes and prediabetes: is it time for a mid-course correction? *J Clin Endocrinol Metab* **95**, 5203-5206 (2010).

45. R. M. Cohen, E. P. Smith, Frequency of HbA1c discordance in estimating blood glucose control. *Curr Opin Clin Nutr Metab Care* **11**, 512-517 (2008).

46. R. R. Little, W. L. Roberts, A review of variant hemoglobins interfering with hemoglobin A1c measurement. *J Diabetes Sci Technol* **3**, 446-451 (2009).

47. P. Tuso, Prediabetes and lifestyle modification: time to prevent a preventable disease. *Perm J* **18**, 88-93 (2014).

48. Z. Cheng, L. Zheng, F. A. Almeida, Epigenetic reprogramming in metabolic disorders: nutritional factors and beyond. *J Nutr Biochem* **54**, 1-10 (2018).

49. P. Schwandt, On the occasion of the world diabetes day: diabetes mellitus - a globally increasing health problem. *Int J Prev Med* **3**, 747-748 (2012).

50. R. S. Bhopal *et al.*, Effect of a lifestyle intervention on weight change in south Asian individuals in the UK at high risk of type 2 diabetes: a family-cluster randomised controlled trial. *Lancet Diabetes Endocrinol* **2**, 218-227 (2014).

51. M. D. Nitert *et al.*, Impact of an exercise intervention on DNA methylation in skeletal muscle from first-degree relatives of patients with type 2 diabetes. *Diabetes* **61**, 3322-3332 (2012).

52. R. Nesto, R. Fain, Y. Li, W. Shanahan, Evaluation of lorcaserin on progression of prediabetes to type 2 diabetes and reversion to euglycemia. *Postgrad Med* **128**, 364-370 (2016).

53. E. S. Schellenberg, D. M. Dryden, B. Vandermeer, C. Ha, C. Korownyk, Lifestyle interventions for patients with and at risk for type 2 diabetes: a systematic review and meta-analysis. *Ann Intern Med* **159**, 543-551 (2013).

54. K. M. Narayan, J. P. Boyle, T. J. Thompson, E. W. Gregg, D. F. Williamson, Effect of BMI on lifetime risk for diabetes in the U.S. *Diabetes Care* **30**, 1562-1566 (2007).

55. J. E. Shaw, R. A. Sicree, P. Z. Zimmet, Global estimates of the prevalence of diabetes for 2010 and 2030. *Diabetes Res Clin Pract* **87**, 4-14 (2010).

56. D. L. Blackwell, J. W. Lucas, T. C. Clarke, Summary health statistics for U.S. adults: national health interview survey, 2012. *Vital Health Stat 10*, 1-161 (2014).

57. M. Pigeyre, F. T. Yazdi, Y. Kaur, D. Meyre, Recent progress in genetics, epigenetics and metagenomics unveils the pathophysiology of human obesity. *Clin Sci (Lond)* **130**, 943-986 (2016).

58. J. Sun *et al.*, Role of Epigenetic Histone Modifications in Diabetic Kidney Disease Involving Renal Fibrosis. *J Diabetes Res* **2017**, 7242384 (2017).

59. J. Feng, W. Xing, L. Xie, Regulatory Roles of MicroRNAs in Diabetes. *Int J Mol Sci* **17**, (2016).

60. D. Sekar, B. Venugopal, P. Sekar, K. Ramalingam, Role of microRNA 21 in diabetes and associated/related diseases. *Gene* **582**, 14-18 (2016).

61. B. F. Belgardt *et al.*, The microRNA-200 family regulates pancreatic beta cell survival in type 2 diabetes. *Nat Med* **21**, 619-627 (2015).

62. M. K. Skinner, Environmental epigenomics and disease susceptibility. *EMBO Rep* **12**, 620-622 (2011).

63. S. E. Pinney, R. A. Simmons, Epigenetic mechanisms in the development of type 2 diabetes. *Trends Endocrinol Metab* **21**, 223-229 (2010).

64. M. Wegner, D. Neddermann, M. Piorunska-Stolzmann, P. P. Jagodzinski, Role of epigenetic mechanisms in the development of chronic complications of diabetes. *Diabetes Res Clin Pract* **105**, 164-175 (2014).

65. J. A. Hanover, M. W. Krause, D. C. Love, Bittersweet memories: linking metabolism to epigenetics through O-GlcNAcylation. *Nat Rev Mol Cell Biol* **13**, 312-321 (2012).

66. N. Hashimoto, T. Tanaka, Role of miRNAs in the pathogenesis and susceptibility of diabetes mellitus. *J Hum Genet* **62**, 141-150 (2017).

67. X. Li, C. Li, G. Sun, Histone Acetylation and Its Modifiers in the Pathogenesis of Diabetic Nephropathy. *J Diabetes Res* **2016**, 4065382 (2016).

68. P. Maechler, C. B. Wollheim, Mitochondrial function in normal and diabetic beta-cells. *Nature* **414**, 807-812 (2001).

69. P. Maechler, Mitochondrial function and insulin secretion. *Mol Cell Endocrinol* **379**, 12-18 (2013).

70. C. Bonnard *et al.*, Mitochondrial dysfunction results from oxidative stress in the skeletal muscle of diet-induced insulin-resistant mice. *J Clin Invest* **118**, 789-800 (2008).

71. M. Sadagurski *et al.*, IRS2 increases mitochondrial dysfunction and oxidative stress in a mouse model of Huntington disease. *J Clin Invest* **121**, 4070-4081 (2011).

72. A. Martin-Montalvo, R. de Cabo, Mitochondrial metabolic reprogramming induced by calorie restriction. *Antioxid Redox Signal* **19**, 310-320 (2013).

73. Z. Cheng, Y. Tseng, M. F. White, Insulin signaling meets mitochondria in metabolism. *Trends Endocrinol Metab* **21**, 589-598 (2010).

74. Z. Cheng, E. M. Schmelz, D. Liu, M. W. Hulver, Targeting mitochondrial alterations to prevent type 2 diabetes--evidence from studies of dietary redox-active compounds. *Mol Nutr Food Res* **58**, 1739-1749 (2014).

75. C. Handschin, B. M. Spiegelman, The role of exercise and PGC1alpha in inflammation and chronic disease. *Nature* **454**, 463-469 (2008).

76. P. S. Eisele, R. Furrer, M. Beer, C. Handschin, The PGC-1 coactivators promote an anti-inflammatory environment in skeletal muscle in vivo. *Biochem Biophys Res Commun* **464**, 692-697 (2015).

77. J. A. Villena, New insights into PGC-1 coactivators: redefining their role in the regulation of mitochondrial function and beyond. *FEBS J* **282**, 647-672 (2015).

78. N. A. Stephens *et al.*, A transcriptional signature of "exercise resistance" in skeletal muscle of individuals with type 2 diabetes mellitus. *Metabolism* **64**, 999-1004 (2015).

79. C. Ling *et al.*, Epigenetic regulation of PPARGC1A in human type 2 diabetic islets and effect on insulin secretion. *Diabetologia* **51**, 615-622 (2008).

80. T. L. Lochmann, R. R. Thomas, J. P. Bennett, Jr., S. M. Taylor, Epigenetic Modifications of the PGC-1alpha Promoter during Exercise Induced Expression in Mice. *PLoS One* **10**, e0129647 (2015).

81. R. Clarke-Harris *et al.*, PGC1alpha promoter methylation in blood at 5-7 years predicts adiposity from 9 to 14 years (EarlyBird 50). *Diabetes* **63**, 2528-2537 (2014).

82. R. C. Laker *et al.*, Exercise prevents maternal high-fat diet-induced hypermethylation of the Pgc-1alpha gene and age-dependent metabolic dysfunction in the offspring. *Diabetes* **63**, 1605-1611 (2014).

83. J. C. Schell, J. Rutter, Mitochondria link metabolism and epigenetics in haematopoiesis. *Nat Cell Biol* **19**, 589-591 (2017).

84. K. S. Gill, Strategies for nutritional improvement. *Indian J Matern Child Health* **2**, 70-71 (1991).

85. S. T. Keating, J. A. van Diepen, N. P. Riksen, A. El-Osta, Epigenetics in diabetic nephropathy, immunity and metabolism. *Diabetologia* **61**, 6-20 (2018).

86. D. M. Silberman, Metabolism, neurodegeneration and epigenetics: Emerging role of Sirtuins. *Neural Regen Res* **13**, 417-418 (2018).

87. L. J. Hawkins, R. Al-Attar, K. B. Storey, Transcriptional regulation of metabolism in disease: From transcription factors to epigenetics. *PeerJ* **6**, e5062 (2018).

88. E. Tzika, T. Dreker, A. Imhof, Epigenetics and Metabolism in Health and Disease. *Front Genet* **9**, 361 (2018).

89. R. Barres *et al.*, Non-CpG methylation of the PGC-1alpha promoter through DNMT3B controls mitochondrial density. *Cell Metab* **10**, 189-198 (2009).

90. H. J. Choo *et al.*, Mitochondria are impaired in the adipocytes of type 2 diabetic mice. *Diabetologia* **49**, 784-791 (2006).

91. S. Kleiner *et al.*, Development of insulin resistance in mice lacking PGC-1alpha in adipose tissues. *Proc Natl Acad Sci U S A* **109**, 9635-9640 (2012).

92. L. Wilson-Fritch *et al.*, Mitochondrial biogenesis and remodeling during adipogenesis and in response to the insulin sensitizer rosiglitazone. *Mol Cell Biol* **23**, 1085-1094 (2003).

93. H. Misu *et al.*, Genes involved in oxidative phosphorylation are coordinately upregulated with fasting hyperglycaemia in livers of patients with type 2 diabetes. *Diabetologia* **50**, 268-277 (2007).

94. T. Takamura *et al.*, Obesity upregulates genes involved in oxidative phosphorylation in livers of diabetic patients. *Obesity (Silver Spring)* **16**, 2601-2609 (2008).

95. J. Pihlajamaki *et al.*, Thyroid hormone-related regulation of gene expression in human fatty liver. *J Clin Endocrinol Metab* **94**, 3521-3529 (2009).

96. M. Perez-Carreras *et al.*, Defective hepatic mitochondrial respiratory chain in patients with nonalcoholic steatohepatitis. *Hepatology* **38**, 999-1007 (2003).

97. S. H. Koo *et al.*, PGC-1 promotes insulin resistance in liver through PPAR-alpha-dependent induction of TRB-3. *Nat Med* **10**, 530-534 (2004).

98. K. Sharabi, C. D. Tavares, A. K. Rines, P. Puigserver, Molecular pathophysiology of hepatic glucose production. *Mol Aspects Med* **46**, 21-33 (2015).

99. E. M. Morris *et al.*, PGC-1alpha overexpression results in increased hepatic fatty acid oxidation with reduced triacylglycerol accumulation and secretion. *Am J Physiol Gastrointest Liver Physiol* **303**, G979-992 (2012).

100. A. D. Attie, C. M. Kendziorski, PGC-1alpha at the crossroads of type 2 diabetes. *Nat Genet* **34**, 244-245 (2003).

101. M. E. Patti, S. Corvera, The role of mitochondria in the pathogenesis of type 2 diabetes. *Endocr Rev* **31**, 364-395 (2010).

102. M. E. Patti *et al.*, Coordinated reduction of genes of oxidative metabolism in humans with insulin resistance and diabetes: Potential role of PGC1 and NRF1. *Proc Natl Acad Sci U S A* **100**, 8466-8471 (2003).

103. R. J. Southgate *et al.*, PGC-1alpha gene expression is down-regulated by Akt- mediated phosphorylation and nuclear exclusion of FoxO1 in insulin-stimulated skeletal muscle. *FASEB J* **19**, 2072-2074 (2005).

104. V. K. Mootha *et al.*, PGC-1alpha-responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes. *Nat Genet* **34**, 267-273 (2003).

105. M. Lagouge *et al.*, Resveratrol improves mitochondrial function and protects against metabolic disease by activating SIRT1 and PGC-1alpha. *Cell* **127**, 1109-1122 (2006).

106. C. Lerin *et al.*, GCN5 acetyltransferase complex controls glucose metabolism through transcriptional repression of PGC-1alpha. *Cell Metab* **3**, 429-438 (2006).

107. C. M. Frederiksen *et al.*, Transcriptional profiling of myotubes from patients with type 2 diabetes: no evidence for a primary defect in oxidative phosphorylation genes. *Diabetologia* **51**, 2068-2077 (2008).

108. A. D. Riggs, Z. Xiong, Methylation and epigenetic fidelity. *Proc Natl Acad Sci U S A* **101**, 4-5 (2004).

109. S. J. van Dijk *et al.*, Epigenetics and human obesity. *Int J Obes (Lond)* **39**, 85-97 (2015).

110. A. Lennartsson, K. Ekwall, Histone modification patterns and epigenetic codes. *Biochim Biophys Acta* **1790**, 863-868 (2009).

111. R. Jaenisch, A. Bird, Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. *Nat Genet* **33 Suppl**, 245-254 (2003).

112. R. Karlic, H. R. Chung, J. Lasserre, K. Vlahovicek, M. Vingron, Histone modification levels are predictive for gene expression. *Proc Natl Acad Sci U S A* **107**, 2926-2931 (2010).

113. S. I. Grewal, D. Moazed, Heterochromatin and epigenetic control of gene expression. *Science* **301**, 798-802 (2003).

114. A. J. McNairn, D. M. Gilbert, Epigenomic replication: linking epigenetics to DNA replication. *Bioessays* **25**, 647-656 (2003).

115. H. Zhu, S. W. Leung, Identification of microRNA biomarkers in type 2 diabetes: a meta-analysis of controlled profiling studies. *Diabetologia* **58**, 900-911 (2015).

116. X. Luo, B. Yang, S. Nattel, MicroRNAs and atrial fibrillation: mechanisms and translational potential. *Nat Rev Cardiol* **12**, 80-90 (2015).

117. C. Wahlestedt, Targeting long non-coding RNA to therapeutically upregulate gene expression. *Nat Rev Drug Discov* **12**, 433-446 (2013).

118. Y. S. Wang *et al.*, MicroRNA-152 mediates DNMT1-regulated DNA methylation in the estrogen receptor alpha gene. *PLoS One* **7**, e30635 (2012).

119. M. J. Jones, S. J. Goodman, M. S. Kobor, DNA methylation and healthy human aging. *Aging Cell* **14**, 924-932 (2015).

120. S. Ohno, W. D. Kaplan, R. Kinosita, Conjugation of the heteropycnotic X and Y chromosomes of the rat spermatocyte. *Exp Cell Res* **12**, 395-397 (1957).

121. M. F. Lyon, Gene action in the X-chromosome of the mouse (Mus musculus L.). *Nature* **190**, 372-373 (1961).

122. E. Nilsson *et al.*, Altered DNA methylation and differential expression of genes influencing metabolism and inflammation in adipose tissue from subjects with type 2 diabetes. *Diabetes* **63**, 2962-2976 (2014).

123. Z. D. Smith, A. Meissner, DNA methylation: roles in mammalian development. *Nat Rev Genet* **14**, 204-220 (2013).

124. A. How Kit, H. M. Nielsen, J. Tost, DNA methylation based biomarkers: practical considerations and applications. *Biochimie* **94**, 2314-2337 (2012).

125. M. Volkmar *et al.*, DNA methylation profiling identifies epigenetic dysregulation in pancreatic islets from type 2 diabetic patients. *EMBO J* **31**, 1405-1426 (2012).

126. P. A. Jones, Functions of DNA methylation: islands, start sites, gene bodies and beyond. *Nat Rev Genet* **13**, 484-492 (2012).

127. S. Sharma, D. D. De Carvalho, S. Jeong, P. A. Jones, G. Liang, Nucleosomes containing methylated DNA stabilize DNA methyltransferases 3A/3B and ensure faithful epigenetic inheritance. *PLoS Genet* **7**, e1001286 (2011).

128. X. Nan *et al.*, Transcriptional repression by the methyl-CpG-binding protein MeCP2 involves a histone deacetylase complex. *Nature* **393**, 386-389 (1998).

129. F. Fuks, W. A. Burgers, A. Brehm, L. Hughes-Davies, T. Kouzarides, DNA methyltransferase Dnmt1 associates with histone deacetylase activity. *Nat Genet* **24**, 88-91 (2000).

130. N. R. Rose, R. J. Klose, Understanding the relationship between DNA methylation and histone lysine methylation. *Biochim Biophys Acta* **1839**, 1362-1372 (2014).

131. V. Chavali, S. C. Tyagi, P. K. Mishra, MicroRNA-133a regulates DNA methylation in diabetic cardiomyocytes. *Biochem Biophys Res Commun* **425**, 668-672 (2012).

132. S. Zhao *et al.*, MicroRNA-126 regulates DNA methylation in CD4+ T cells and contributes to systemic lupus erythematosus by targeting DNA methyltransferase 1. *Arthritis Rheum* **63**, 1376-1386 (2011).

133. E. Hall *et al.*, Sex differences in the genome-wide DNA methylation pattern and impact on gene expression, microRNA levels and insulin secretion in human pancreatic islets. *Genome Biol* **15**, 522 (2014).

134. M. Fabbri *et al.*, MicroRNA-29 family reverts aberrant methylation in lung cancer by targeting DNA methyltransferases 3A and 3B. *Proc Natl Acad Sci U S A* **104**, 15805-15810 (2007).

135. R. Garzon *et al.*, MicroRNA-29b induces global DNA hypomethylation and tumor suppressor gene reexpression in acute myeloid leukemia by targeting directly DNMT3A and 3B and indirectly DNMT1. *Blood* **113**, 6411-6418 (2009).

136. L. Sinkkonen *et al.*, MicroRNAs control de novo DNA methylation through regulation of transcriptional repressors in mouse embryonic stem cells. *Nat Struct Mol Biol* **15**, 259-267 (2008).

137. M. Teitell, B. Richardson, DNA methylation in the immune system. *Clin Immunol* **109**, 2-5 (2003).

138. N. Zhang, Epigenetic modulation of DNA methylation by nutrition and its mechanisms in animals. *Anim Nutr* **1**, 144-151 (2015).

139. C. X. Song *et al.*, Genome-wide profiling of 5-formylcytosine reveals its roles in epigenetic priming. *Cell* **153**, 678-691 (2013).

140. R. M. Kohli, Y. Zhang, TET enzymes, TDG and the dynamics of DNA demethylation. *Nature* **502**, 472-479 (2013).

141. S. Ito *et al.*, Tet proteins can convert 5-methylcytosine to 5-formylcytosine and 5-carboxylcytosine. *Science* **333**, 1300-1303 (2011).

142. A. J. Wood, R. J. Oakey, Genomic imprinting in mammals: emerging themes and established theories. *PLoS Genet* **2**, e147 (2006).

143. E. Nilsson, C. Ling, DNA methylation links genetics, fetal environment, and an unhealthy lifestyle to the development of type 2 diabetes. *Clin Epigenetics* **9**, 105 (2017).

144. Z. Zhou, B. Sun, X. Li, C. Zhu, DNA methylation landscapes in the pathogenesis of type 2 diabetes mellitus. *Nutr Metab (Lond)* **15**, 47 (2018).

145. M. M. Mendelson *et al.*, Association of Body Mass Index with DNA Methylation and Gene Expression in Blood Cells and Relations to Cardiometabolic Disease: A Mendelian Randomization Approach. *PLoS Med* **14**, e1002215 (2017).

146. G. Toperoff *et al.*, Genome-wide survey reveals predisposing diabetes type 2-related DNA methylation variations in human peripheral blood. *Hum Mol Genet* **21**, 371-383 (2012).

147. P. Volkov *et al.*, Whole-Genome Bisulfite Sequencing of Human Pancreatic Islets Reveals Novel Differentially Methylated Regions in Type 2 Diabetes Pathogenesis. *Diabetes* **66**, 1074-1085 (2017).

148. C. Baumeier *et al.*, Hepatic DPP4 DNA Methylation Associates With Fatty Liver. *Diabetes* **66**, 25-35 (2017).

149. S. Garcia-Calzon, A. Perfilyev, V. D. de Mello, J. Pihlajamaki, C. Ling, Sex Differences in the Methylome and Transcriptome of the Human Liver and Circulating HDL-Cholesterol Levels. *J Clin Endocrinol Metab* **103**, 4395-4408 (2018).

150. V. D. de Mello *et al.*, Human liver epigenetic alterations in non-alcoholic steatohepatitis are related to insulin action. *Epigenetics* **12**, 287-295 (2017).

151. M. Keller *et al.*, Genome-wide DNA promoter methylation and transcriptome analysis in human adipose tissue unravels novel candidate genes for obesity. *Mol Metab* **6**, 86-100 (2017).

152. D. You *et al.*, Dnmt3a is an epigenetic mediator of adipose insulin resistance. *Elife* **6**, (2017).

153. C. Davegardh *et al.*, Abnormal epigenetic changes during differentiation of human skeletal muscle stem cells from obese subjects. *BMC Med* **15**, 39 (2017).

154. B. P. Larkin, S. J. Glastras, H. Chen, C. A. Pollock, S. Saad, DNA methylation and the potential role of demethylating agents in prevention of progressive chronic kidney disease. *FASEB J* **32**, 5215-5226 (2018).

155. N. R. van Zuydam *et al.*, A Genome-Wide Association Study of Diabetic Kidney Disease in Subjects With Type 2 Diabetes. *Diabetes* **67**, 1414-1427 (2018).

156. S. Oba *et al.*, Aberrant DNA methylation of Tgfb1 in diabetic kidney mesangial cells. *Sci Rep* **8**, 16338 (2018).

157. P. Arner *et al.*, The epigenetic signature of systemic insulin resistance in obese women. *Diabetologia* **59**, 2393-2405 (2016).

158. H. R. Elliott *et al.*, Role of DNA Methylation in Type 2 Diabetes Etiology: Using Genotype as a Causal Anchor. *Diabetes* **66**, 1713-1722 (2017).

159. E. Hall *et al.*, The effects of high glucose exposure on global gene expression and DNA methylation in human pancreatic islets. *Mol Cell Endocrinol* **472**, 57-67 (2018).

160. J. Zheng, J. Cheng, Q. Zhang, X. Xiao, Novel insights into DNA methylation and its critical implications in diabetic vascular complications. *Biosci Rep* **37**, (2017).

161. N. S. Hansen *et al.*, Fetal Hyperglycemia Changes Human Preadipocyte Function in Adult Life. *J Clin Endocrinol Metab* **102**, 1141-1150 (2017).

162. K. J. Dick *et al.*, DNA methylation and body-mass index: a genome-wide analysis. *Lancet* **383**, 1990-1998 (2014).

163. F. B. Hu, J. B. Meigs, T. Y. Li, N. Rifai, J. E. Manson, Inflammatory markers and risk of developing type 2 diabetes in women. *Diabetes* **53**, 693-700 (2004).

164. J. C. Pickup, M. A. Crook, Is type II diabetes mellitus a disease of the innate immune system? *Diabetologia* **41**, 1241-1248 (1998).

165. M. I. Schmidt *et al.*, Markers of inflammation and prediction of diabetes mellitus in adults (Atherosclerosis Risk in Communities study): a cohort study. *Lancet* **353**, 1649-1652 (1999).

166. A. E. Harvey, L. M. Lashinger, S. D. Hursting, The growing challenge of obesity and cancer: an inflammatory issue. *Ann N Y Acad Sci* **1229**, 45-52 (2011).

167. M. K. Shin *et al.*, Metabolic consequences of high-fat diet are attenuated by suppression of HIF-1alpha. *PLoS One* **7**, e46562 (2012).

168. S. Aslibekyan *et al.*, Epigenome-wide study identifies novel methylation loci associated with body mass index and waist circumference. *Obesity (Silver Spring)* **23**, 1493-1501 (2015).

169. T. Dayeh *et al.*, Genome-wide DNA methylation analysis of human pancreatic islets from type 2 diabetic and non-diabetic donors identifies candidate genes that influence insulin secretion. *PLoS Genet* **10**, e1004160 (2014).

170. C. G. Bell *et al.*, Integrated genetic and epigenetic analysis identifies haplotype-specific methylation in the FTO type 2 diabetes and obesity susceptibility locus. *PLoS One* **5**, e14040 (2010).

171. T. M. Frayling *et al.*, A common variant in the FTO gene is associated with body mass index and predisposes to childhood and adult obesity. *Science* **316**, 889-894 (2007).

172. T. Hardy *et al.*, Plasma DNA methylation: a potential biomarker for stratification of liver fibrosis in non-alcoholic fatty liver disease. *Gut* **66**, 1321-1328 (2017).

173. B. T. Yang *et al.*, Increased DNA methylation and decreased expression of PDX-1 in pancreatic islets from patients with type 2 diabetes. *Mol Endocrinol* **26**, 1203-1212 (2012).

174. B. T. Yang *et al.*, Insulin promoter DNA methylation correlates negatively with insulin gene expression and positively with HbA(1c) levels in human pancreatic islets. *Diabetologia* **54**, 360-367 (2011).

175. C. Brons *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* **95**, 3048-3056 (2010).

176. T. Ronn *et al.*, Age influences DNA methylation and gene expression of COX7A1 in human skeletal muscle. *Diabetologia* **51**, 1159-1168 (2008).

177. C. J. Pirola *et al.*, Epigenetic modification of liver mitochondrial DNA is associated with histological severity of nonalcoholic fatty liver disease. *Gut* **62**, 1356-1363 (2013).

178. A. Serman, M. Vlahovic, L. Serman, F. Bulic-Jakus, DNA methylation as a regulatory mechanism for gene expression in mammals. *Coll Antropol* **30**, 665-671 (2006).

179. K. Sun, N. Halberg, M. Khan, U. J. Magalang, P. E. Scherer, Selective inhibition of hypoxia-inducible factor 1alpha ameliorates adipose tissue dysfunction. *Mol Cell Biol* **33**, 904-917 (2013).

180. S. Z. Hasnain *et al.*, Glycemic control in diabetes is restored by therapeutic manipulation of cytokines that regulate beta cell stress. *Nat Med* **20**, 1417-1426 (2014).

181. C. A. Aspinwall, J. R. Lakey, R. T. Kennedy, Insulin-stimulated insulin secretion in single pancreatic beta cells. *J Biol Chem* **274**, 6360-6365 (1999).

182. E. Hall *et al.*, Effects of palmitate on genome-wide mRNA expression and DNA methylation patterns in human pancreatic islets. *BMC Med* **12**, 103 (2014).

183. A. Kuroda *et al.*, Insulin gene expression is regulated by DNA methylation. *PLoS One* **4**, e6953 (2009).

184. J. A. Law, S. E. Jacobsen, Establishing, maintaining and modifying DNA methylation patterns in plants and animals. *Nat Rev Genet* **11**, 204-220 (2010).

185. S. L. Norris *et al.*, Long-term non-pharmacological weight loss interventions for adults with prediabetes. *Cochrane Database Syst Rev*, CD005270 (2005).

186. R. Feil, M. F. Fraga, Epigenetics and the environment: emerging patterns and implications. *Nat Rev Genet* **13**, 97-109 (2012).

187. J. Campion, F. I. Milagro, E. Goyenechea, J. A. Martinez, TNF-alpha promoter methylation as a predictive biomarker for weight-loss response. *Obesity (Silver Spring)* **17**, 1293-1297 (2009).

188. L. Gillberg, C. Ling, The potential use of DNA methylation biomarkers to identify risk and progression of type 2 diabetes. *Front Endocrinol (Lausanne)* **6**, 43 (2015).

189. A. A. Michalczyk *et al.*, Epigenetic Markers to Predict Conversion From Gestational Diabetes to Type 2 Diabetes. *J Clin Endocrinol Metab* **101**, 2396-2404 (2016).

190. A. A. Baccarelli, H. M. Byun, Platelet mitochondrial DNA methylation: a potential new marker of cardiovascular disease. *Clin Epigenetics* **7**, 44 (2015).

191. M. Rask-Andersen *et al.*, Postprandial alterations in whole-blood DNA methylation are mediated by changes in white blood cell composition. *Am J Clin Nutr* **104**, 518-525 (2016).

192. B. C. Christensen *et al.*, Aging and environmental exposures alter tissue-specific DNA methylation dependent upon CpG island context. *PLoS Genet* **5**, e1000602 (2009).

193. W. A. Loenen, S-adenosylmethionine: jack of all trades and master of everything? *Biochem Soc Trans* **34**, 330-333 (2006).

194. J. A. McKay, J. C. Mathers, Diet induced epigenetic changes and their implications for health. *Acta Physiol (Oxf)* **202**, 103-118 (2011).

195. A. J. McKnight, G. J. McKay, A. P. Maxwell, Genetic and epigenetic risk factors for diabetic kidney disease. *Adv Chronic Kidney Dis* **21**, 287-296 (2014).

196. B. M. Murdoch, G. K. Murdoch, S. Greenwood, S. McKay, Nutritional Influence on Epigenetic Marks and Effect on Livestock Production. *Front Genet* **7**, 182 (2016).

197. I. P. Pogribny *et al.*, Irreversible global DNA hypomethylation as a key step in hepatocarcinogenesis induced by dietary methyl deficiency. *Mutat Res* **593**, 80-87 (2006).

198. I. P. Pogribny, V. P. Tryndyak, L. Muskhelishvili, I. Rusyn, S. A. Ross, Methyl deficiency, alterations in global histone modifications, and carcinogenesis. *J Nutr* **137**, 216S-222S (2007).

199. I. P. Pogribny *et al.*, Role of DNA damage and alterations in cytosine DNA methylation in rat liver carcinogenesis induced by a methyl-deficient diet. *Mutat Res* **669**, 56-62 (2009).

200. A. Starlard-Davenport *et al.*, Dietary methyl deficiency, microRNA expression and susceptibility to liver carcinogenesis. *World Rev Nutr Diet* **101**, 123-130 (2010).

201. A. Starlard-Davenport *et al.*, Dietary methyl deficiency, microRNA expression and susceptibility to liver carcinogenesis. *J Nutrigenet Nutrigenomics* **3**, 259-266 (2010).

202. I. P. Pogribny, S. J. James, F. A. Beland, Molecular alterations in hepatocarcinogenesis induced by dietary methyl deficiency. *Mol Nutr Food Res* **56**, 116-125 (2012).

203. P. Stenvinkel *et al.*, Impact of inflammation on epigenetic DNA methylation - a novel risk factor for cardiovascular disease? *J Intern Med* **261**, 488-499 (2007).

204. M. B. Glier, T. J. Green, A. M. Devlin, Methyl nutrients, DNA methylation, and cardiovascular disease. *Mol Nutr Food Res* **58**, 172-182 (2014).

205. H. S. Tapp *et al.*, Nutritional factors and gender influence age-related DNA methylation in the human rectal mucosa. *Aging Cell* **12**, 148-155 (2013).

206. H. T. Bjornsson *et al.*, Intra-individual change over time in DNA methylation with familial clustering. *JAMA* **299**, 2877-2883 (2008).

207. Y. L. Ottaviano *et al.*, Methylation of the estrogen receptor gene CpG island marks loss of estrogen receptor expression in human breast cancer cells. *Cancer Res* **54**, 2552-2555 (1994).

208. G. M. Martin, Epigenetic drift in aging identical twins. *Proc Natl Acad Sci U S A* **102**, 10413-10414 (2005).

209. S. Bocklandt *et al.*, Epigenetic predictor of age. *PLoS One* **6**, e14821 (2011).

210. M. F. Fraga *et al.*, Epigenetic differences arise during the lifetime of monozygotic twins. *Proc Natl Acad Sci U S A* **102**, 10604-10609 (2005).

211. R. P. Talens *et al.*, Epigenetic variation during the adult lifespan: cross-sectional and longitudinal data on monozygotic twin pairs. *Aging Cell* **11**, 694-703 (2012).

212. M. P. Boks *et al.*, The relationship of DNA methylation with age, gender and genotype in twins and healthy controls. *PLoS One* **4**, e6767 (2009).

213. D. T. Hsiung *et al.*, Global DNA methylation level in whole blood as a biomarker in head and neck squamous cell carcinoma. *Cancer Epidemiol Biomarkers Prev* **16**, 108-114 (2007).

214. L. E. Moore *et al.*, Genomic DNA hypomethylation as a biomarker for bladder cancer susceptibility in the Spanish Bladder Cancer Study: a case-control study. *Lancet Oncol* **9**, 359-366 (2008).

215. J. Y. Choi *et al.*, Association between global DNA hypomethylation in leukocytes and risk of breast cancer. *Carcinogenesis* **30**, 1889-1897 (2009).

216. L. Hou *et al.*, Blood leukocyte DNA hypomethylation and gastric cancer risk in a high-risk Polish population. *Int J Cancer* **127**, 1866-1874 (2010).

217. T. Yuan *et al.*, An integrative multi-scale analysis of the dynamic DNA methylation landscape in aging. *PLoS Genet* **11**, e1004996 (2015).

218. A. E. Handel, G. C. Ebers, S. V. Ramagopalan, Epigenetics: molecular mechanisms and implications for disease. *Trends Mol Med* **16**, 7-16 (2010).

219. M. Inoshita *et al.*, Sex differences of leukocytes DNA methylation adjusted for estimated cellular proportions. *Biol Sex Differ* **6**, 11 (2015).

220. N. Brockdorff, B. M. Turner, Dosage compensation in mammals. *Cold Spring Harb Perspect Biol* **7**, a019406 (2015).

221. D. R. Hodge *et al.*, Interleukin-6 regulation of the human DNA methyltransferase (HDNMT) gene in human erythroleukemia cells. *J Biol Chem* **276**, 39508-39511 (2001).

222. V. K. Rakyan *et al.*, Human aging-associated DNA hypermethylation occurs preferentially at bivalent chromatin domains. *Genome Res* **20**, 434-439 (2010).

223. G. H. Kang *et al.*, Aberrant CpG island hypermethylation of chronic gastritis, in relation to aging, gender, intestinal metaplasia, and chronic inflammation. *Am J Pathol* **163**, 1551-1556 (2003).

224. J. Zhao, J. Goldberg, J. D. Bremner, V. Vaccarino, Global DNA methylation is associated with insulin resistance: a monozygotic twin study. *Diabetes* **61**, 542-546 (2012).

225. L. D. Zheng *et al.*, Insulin resistance is associated with epigenetic and genetic regulation of mitochondrial DNA in obese humans. *Clin Epigenetics* **7**, 60 (2015).

226. A. Sleigh *et al.*, Mitochondrial dysfunction in patients with primary congenital insulin resistance. *J Clin Invest* **121**, 2457-2461 (2011).

Chapter 2

Identification of temporal metabolic and anthropometric alterations following a high-fat diet treatment in C57BL/6 male mice

Sarah Donnelly[1], Longhua Liu[2], Louise Zhang[1], Zhipeng Tao[1], Fabio Almeida[3], and Zhiyong Cheng[4]

[1]Department of Human Nutrition, Foods, and Exercise, Virginia Tech, Blacksburg, VA;

[2]Department of Pathology and Cell Biology, Columbia University, New York, NY;

[3]Department of Health Promotion, University of Nebraska Medical Center, Omaha NE

[4]Department of Human Nutrition, University of Florida, Gainesville, FL

Corresponding Author
Zhiyong Cheng, Ph.D.
572 Newell Drive
Gainesville, Florida 32611-1907
540-235-4889
z.cheng@ufl.edu

2.1 Abstract

In order to comparatively assess the junctures in which phenotypical (anthropometrical and metabolic) changes parallel with significant epigenetic changes (DNA methylation), determination of the temporal inflection points of significant metabolic and physiological alterations must betide. In this study, measurements of body composition, glucose fasting glucose, tolerance (GTT), fasting insulin, and insulin sensitivity (ITT) were analyzed over the duration of dietary challenges, demonstrating the progression from a healthy state to a prediabetic state, and finally to diabetic state. The goal of the current study was to clearly define how anthropometric data, glucose tolerance, and insulin sensitivity change with continuing consumption of a HFD and, primarily, how these changes associate with the changing methylation profile of PGC-1α. As early as day 3, significant differences were seen between chow and HFD groups in body weight (19.28 ± 0.25 gm vs. 21.24 ± 0.20 gm, $p<0.05$), fat mass (6.63 ± 0.27 % vs. 8.22 ± 0.21%, $p<0.001$), and fasting glucose (90.25 ± 7.80 mg/dL vs. 104.625 ± 5.34 mg/dL, $p<0.001$), indicating a particularly early inflection point of metabolic irregularities that may follow more acute upstream molecular changes. In Chapter 3, we will use these inflection points comparatively as we examine significant alterations in methylation levels at the promoter region of PGC-1α and subsequent mitochondrial protein alterations.

2.2 Introduction

Murine models for Type 2 Diabetes Mellitus (T2DM) have proven indispensable in the study of the underlying molecular mechanisms in insulin resistant states, due primarily to the similarities that exist between the genome of the mouse versus the human(*1*). Prior studies examining the kinetics of HFD-induced diabetic traits have shown that molecular changes are seen as early as 3 days following the initiation of a HFD(*2, 3*), indicating the potential for diagnostic mechanisms to be developed and utilized prior to overt disease onset(*4-10*). A previous study by Park et al. sought to examine the temporal alterations in insulin signaling that occurred during high fat feeding and saw that glucose metabolism was altered as early as 1.5 weeks following diet initiation(*6*). Interestingly, they also found that insulin resistance developed simultaneously in the liver, skeletal muscle, and adipose tissue following 3 weeks of a HFD. Obesity, which is closely linked to T2DM, significantly increased in their study following 1.5 weeks of HFD as well, further shedding light on the importance of early identification of high-risk individuals.

The field of epigenetic blood based biomarkers is still in its early stages, however, research in this area is gaining traction(*11*). Biomarkers in blood are typically viewed as the gold standard, as compared to those obtained from tissue samples, due to the non-invasive nature of such samples. Importantly, previous studies have shown that insulin resistance in metabolically active tissues, such as the liver, skeletal muscle, and adipose tissue, are highly predictive of T2DM risk. Because analysis of these tissues is expensive and invasive, the ideal mechanism would be to identify biomarkers that exhibit dynamic parallels between the blood and metabolically active tissues.

The potential of blood-based DNA methylation biomarkers for early diagnosis of T2DM has been examined previously(*12-15*). This includes global DNA methylation (*16-28*)and genome-wide (*18, 20*)methylation analyses; however, gene-specific DNA methylation analysis in a time-specific manner has been lacking. Prior studies have examined the relationship between both DNA methylation of PGC-1α as well as PGC-1α gene polymorphisms with T2DM risk; however, these studies have not examined the temporal changes or whether the changes are mirrored in the tissue versus the blood(*11, 29-34*).

Preclinical and clinical data in human diabetic patients indicates that there are changes in mitochondrial copy number and function at different stages of the disease and at progressive severities of insulin resistance, however, the mechanisms for these alterations are not clearly understood. PGC-1α is a potentially interesting candidate to examine during disease progression, as upregulation of PGC-1α has been linked to improved insulin signaling and mitochondrial homeostasis in both skeletal muscle and adipose tissue, the two primary tissues responsible for glucose uptake. Therefore, we will analyze the temporal methylation changes that occur in the promoter region of PGC-1α over the disease time-course and quantify the subsequent changes in mitochondrial proteins and mitochondrial copy number. Importantly, in order to utilize these methylation data accurately and appropriately, we must first identify the stages of insulin resistance and anthropometric changes for downstream comparisons to methylation profiles. Therefore, in the following study, we will examine the time-course of insulin resistance onset and progressive severity in order to properly quantify whether or how the DNA methylation profile at the promoter region of PGC-1α mirrors these metabolic and anthropometric changes.

2.3 Methods

2.3.1 Mice

All procedures were conducted in accordance of the Virginia Polytechnic Institute and State University Animal Care and Use Committee (IACUC). C57BL/6J male mice (4 weeks) were purchased from Jackson Laboratory (Bar Harbor, Maine) and were housed in plastic cages on a 12 hours light/dark cycle (22–25°C) and *ad libitum* access to water and food. Animals were fasted for 12-16 hours overnight prior to sacrifice and subsequent tissue harvest and were sacrificed according to approved guidelines put forth by IACUC.

2.3.2 Diet

Male C57BL/6 mice (n=96) were randomly assigned either to a normal chow diet (10% kcal from fat) or a high fat diet (HFD) (60% kcal from fat, 20% kcal from carbohydrates, and 20% kcal from protein)(D12492, Research Diets Inc., New Brunswick, NJ).

2.3.3 Body Weight & Fat Mass Quantification

Both groups of mice (chow and HFD) were weighed and body mass quantified at day 0, 3, 6, 14, 21, 28, 42, 56, 70, and 84. Body weight and fat mass were measured prior to blood/tissue collection using a basic laboratory scale and the Bruker Minispec LF90 NMR Analyzer ((Bruker Optics, Inc). Cages were changed every 2-3 days and harvested tissue was snap frozen in liquid nitrogen for downstream molecular analyses at day 6, 14, 28, 56, and 84. Experimental procedures were approved by the Virginia Tech Institutional Animal Care and Use Committee (IACUC).

2.3.4 Blood Glucose & Insulin Monitoring

Fasting blood glucose was assessed following a 12 hour overnight fast. TA glucometer (Kroger, Cincinnati, OH) was used to measure tail vein blood glucose levels. Glucose tolerance (GTT) was measured using the following method: mice were fasted for 12 hours followed by an intraperitoneal injection of a single bolus of glucose (2 g/kg BW). Blood glucose levels were measured at point 0 as well as 15, 30, 60, and 120 minutes following injection. Insulin tolerance (ITT) was measured using the following method: mice were fasted for 4 hours followed by an intraperitoneal injection of insulin (0.75 units/kg BW). Blood insulin levels were measured at point 0 as well as 15, 30, and 60 minutes following injection. Blood insulin levels were confirmed using an ultrasensitive 96 mouse insulin ELISA kit (Mercodia, Inc., Uppsala, Sweden). Following completion of the assigned dietary intervention, mice were fasted overnight and euthanized. Metabolically active tissues (list) were collected, snap-frozen in liquid nitrogen, and stored at -80°C for downstream analyses.

2.3.5 Measures of Insulin Resistance

The homeostatic model assessment for insulin resistance (HOMA-IR) and β-cell dysfunction (HOMA- β) are often used in the clinic to assess varying degrees of insulin resistant and β-cell dysfunction using the equations $[G_0 \times I_0/22.5]$ and $[20 \times I_0/(G_0 - 3.5)]$, respectively. The quantitative insulin-sensitivity check index (QUICKI) is used as a surrogate index to assess insulin sensitivity and incorporates fasting glucose and insulin values in its equation $1/[\log(I_0) + \log(G_0)]$, where I_0 and G_0 represent fasting insulin and glucose, respectively. All above clinical indices were used to characterize the severity of

disease state throughout the time course of the study in order to gain clinical insight into disease progression.

2.3.6 Statistical Analysis

Data are presented as mean ± SE. Differences were examined by a two-tailed t-test (for 2-group comparison) or one-way ANOVA with the least significant difference post hoc test to detect statistical differences (for more than 2-group comparison). A value of $p < 0.05$ was considered statistically significant.

2.4 Results

2.4.1 Body Weight and Fat Mass

At baseline, the average body weight was similar between chow and HFD groups (18.22 ± 0.23 gm vs. 18.14 ± 0.21 gm, p=0.85) (**Figure 1a**). Significant differences in body weight change were seen as early as day 3 between the chow and HFD groups (19.28 ± 0.25 gm vs. 21.24 ± 0.20 gm, $p<0.05$), and these significant differences in body weight were maintained for the remainder of the feeding study. At the final time point, day 84, differences between groups remained highly significant (28.21 ± 0.46 gm vs. 40.46 ± 1.30 gm, $p<0.001$). Fat mass % was similar between chow and HFD groups at baseline (2.84 ± 0.17% vs. 2.40 ± 0.16%, p=0.16) (**Figure 1b**). On day 3, significant differences were seen between the chow and HFD groups (6.63 ± 0.27 % vs. 8.22 ± 0.21%, $p<0.001$), and these differences persisted through the final time point (day 84) (6.63 ± 1.65% vs. 22.14 ± 2.53%, $p<0.001$).

2.4.2 Fasting Glucose & Glucose Tolerance Test

Fasting glucose measurements were conducted at day 0, 3, 6, 14, 21, 28, 42, 56, 70, and 84. At baseline, fasting glucose levels were not significantly different between chow and HFD groups (64.65 ± 19.1 mg/dL vs. 71.13 ± 11.1mg/dL, p=0.17) (Figure 2). Significant differences were seen on day 3 and day 6 (90.25 ± 7.80 mg/dL vs. 104.625 ± 5.34 mg/dL, $p<0.001$; and 95.25 ± 3.28 mg/dL vs 104.57 ± 9.34 mg/dL, $p<0.05$, respectfully), and persisted through the entirety of the study to day 84 (100.75 ± 6.31 mg/dL vs. 137.25 ± 15.75 mg/dL, $p<0.001$). Significant differences in glucose tolerance tests (GTT) were seen as early as day 14 and day 28 ($p<0.01$)(**Figure 3a and 3b**).

2.4.3 Fasting Insulin & Insulin Tolerance Test

Fasting insulin levels were significantly different between groups at baseline (6.47 ± 2.32 mU/L vs. 12.45 ± 3.15 mU/L, $p<0.001$) but normalized on day 3 (8.17 ± 0.70 mU/L vs. 8.21 ± 2.72 mU/L, p=0.97)(Figure 4a). The HFD treated group had significantly higher fasting insulin at day 28 than the chow diet group (16.01 ± 4.61 mU/L vs. 26.07 ± 8.26 mU/L, $p<0.001$). This trend was maintained through day 84 (9.82 ± 4.87 mU/L vs. 30.33 ± 23.10 mU/L, $p<0.05$). Significant differences in insulin tolerance tests (ITT) were seen as early as day 14 ($p<0.01$)(**Figure 4b**).

2.4.4 Measures of Insulin Sensitivity

HOMA-IR between chow and HFD groups were significantly different between groups at baseline (1.40 ± 0.46 vs. 3.09 ± 0.93, $p<0.001$) but normalized on day 3 (1.81 ± 0.20 mU/L vs. 2.11 ± 0.68, p=0.25) (Figure 5a). From day 28, the groups differed significantly in terms

of HOMA-IR values (3.84 ± 1.00 vs. 7.02 ± 2.21, $p<0.001$) through day 84 (2.41 ± 1.21 vs. 10.67 ± 8.44, $p<0.05$). HOMA-β between chow and HFD groups were not significantly different between groups at baseline (105.06 ± 68.89 vs. 133.94 ± 54.90, $p<0.37$) and only showed significant differences between groups at day 14 and day 21 (173.48 ± 96.35 vs. 62.75 ± 42.61, $p<0.01$ and 88.45 ± 27.13 vs. 49.51 ± 31.75, $p<0.01$, respectively) (Figure 5b). QUICKI between chow and HFD groups were significantly different between groups at baseline (0.73 ± 0.30 vs. 0.47 ± 0.05, $p<0.05$) but normalized on day 3 (0.56 ± 0.03 vs. 0.57 ± 0.09, $p=0.92$). At day 28, the groups differed significantly in terms of QUICKI values (0.43 ± 0.04 vs. 0.35 ± 0.03, $p<0.001$), and this was maintained through day 84(0.56 ± 0.25 vs. 0.37 ± 0.10, $p<0.01$) (**Figure 5c**).

2.5 Discussion

In this study, we identified a distinct inflection point of body weight, fat mass (%), and fasting blood glucose concentration. Our first study examined the physiological changes in BMI and glucose metabolism over a longer timeline. However, upon observing the significant physiological changes occurring at day 14, we chose to incorporate an additional cohort of mice in order to analyze the early stages of body compositional changes and glucose dysregulation. Important to note, C57BL/6 mice are useful for the examination of diet-induced obesity (DIO), as they show marked changes in body weight and fat mass %, as well as intermediate measures of insulin resistance. However, these murine models typically do not develop basal hyperglycemia sufficient for diabetes diagnosis, therefore they are ideal models for pre-diabetic phenotypes, which was important in our study as we sought to examine molecular changes

occurring during the progression from the healthy state to the more severe state of impaired glucose handling, with an intentional focus on changes occurring prior to overt disease onset.

As early as day 3, we observed significant increases in body weight, fat mass (%), and fasting blood glucose levels in the HFD group, indicating that metabolic dysregulation is occurring very early in the disease progression process. We did not see this phenomenon reflected in the fasting insulin patterns or insulin scoring indices until day 28, further emphasizing the need for mechanisms whereby "as-early-as-possible" diagnosis or risk assessment in a clinical setting.

In order to characterize the stage of insulin resistance in our mouse model, we utilized surrogate markers for insulin resistance, which included HOMA-IR, HOMA-β, and QUICKI, each of which incorporates fasting insulin and fasting glucose levels in their respective equations. The HOMA indices are considered simple and accurate surrogate indices, as they only require a fasting glucose and insulin measurement, whereas indices that require oral glucose tolerance tests are more time-consuming, but may provide more accurate indications of disease state(*35, 36*). In a previous study, QUICKI, which also incorporates baseline glucose and insulin measurements into its equation, was significantly better at predicting the glucose clamp index of insulin sensitivity that the more simple minimal model index of insulin sensitivity. Unlike measurements of body composition and blood glucose, these indices were insufficient at early-identification of metabolic irregularities. Previous studies have highlighted some of the diagnostic shortcomings of using insulin levels to predict insulin resistance(*37-39*). Most agree that due to the fact that fasting insulin values tend to more closely associate with hepatic insulin resistance and steady state secretion of insulin, the latter of which tends to reflect later stages of

beta cell dysfunction and does not reflect earlier stages of insulin secretion(*37, 38*). Finally, populations at high risk for developing diabetes show a marked failure to increase and maintain insulin secretion to levels appropriate to maintain normoglycemia, indicating that cross-sectional insulin data may not be optimal for defining the disease state.

Previous studies have examined various prediction models for accurately and appropriately diagnosing diabetes and risk for diabetes in both animal and human cohorts(*40-44*). However, prediction models that identify disease predictors or elevated risk levels have shown to have limited ability to accurately predict risk(*40*). The primary clinical concern should, ideally, be to identify the individuals at highest risk for the disease, while simultaneously excluding low-risk populations and eliminating the burden of treatment for those who are not likely to be diagnosed with diabetes in the long term.

Elevated body weight levels over time is associated independently with diabetes risk(*41, 42*). This is likely due to the understood phenomenon that elevated body weight and obesity coincides with increases in lipolysis, leading to increased fatty acid production and dysfunctional adipokine secretion, leading to increases in visceral fat, increases in hepatic glucose output, decreased insulin-mediated glucose disposal, and decreased insulin secretion. Additionally, elevated body weight and fat mass (%) are both associated with impaired glucose metabolism and elevated fasting blood glucose levels(*45*).

Limitations to the work in this study include lack of HbA1c information, an additional commonly used surrogate marker for the diabetic phenotype. Additionally, acute physiological

measurements in the time range of hours to days may add more details to the immerging picture of metabolic irregularities prior to disease onset. There are many mouse models of type 2 diabetes, and this study utilized C57BL/6 male mice. An important point to note, C57BL/6 mice exhibit moderate degrees of obesity and insulin resistance, compared to genetically modified counterparts (ex. B6.V-Lep^{ob}/J and BKS.Cg-Dock7^{m} +/+ Lepr^{db}/J). We chose to utilize the C57BL/6 murine model due to its representative characteristics to the pre-diabetic state, and due to the overarching goal of the study, which was to elucidate epigenetic molecular mechanisms (DNA methylation) prior to overt disease onset.

In conclusion, we were able to identify a distinct inflection point of body weight, fat mass, and fasting blood glucose levels, which were not reflected in commonly used surrogate indices for diagnosis of insulin resistance. Future studies will examine these changes in concert with cellular and genetic alterations.

2.6 Figures

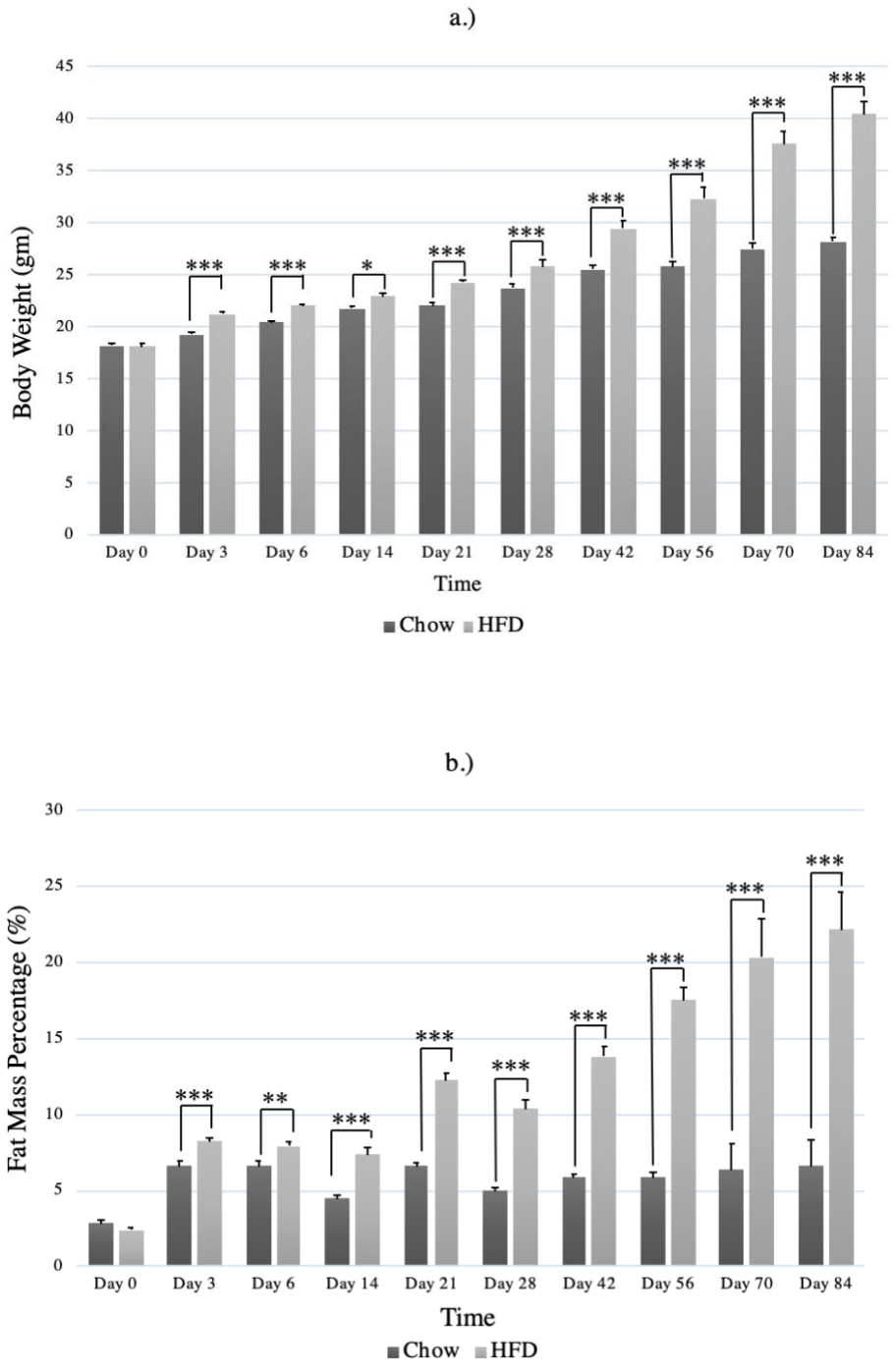


Figure 1. The changes in body weight (a) and fat mass (b) in C57BL/6J mice fed on control (chow) and high fat diet (HFD). The mice were 4-5 week old at week 0 (n=4-12).

* $p<0.05$; ** $p<0.01$; ***$p<0.001$.

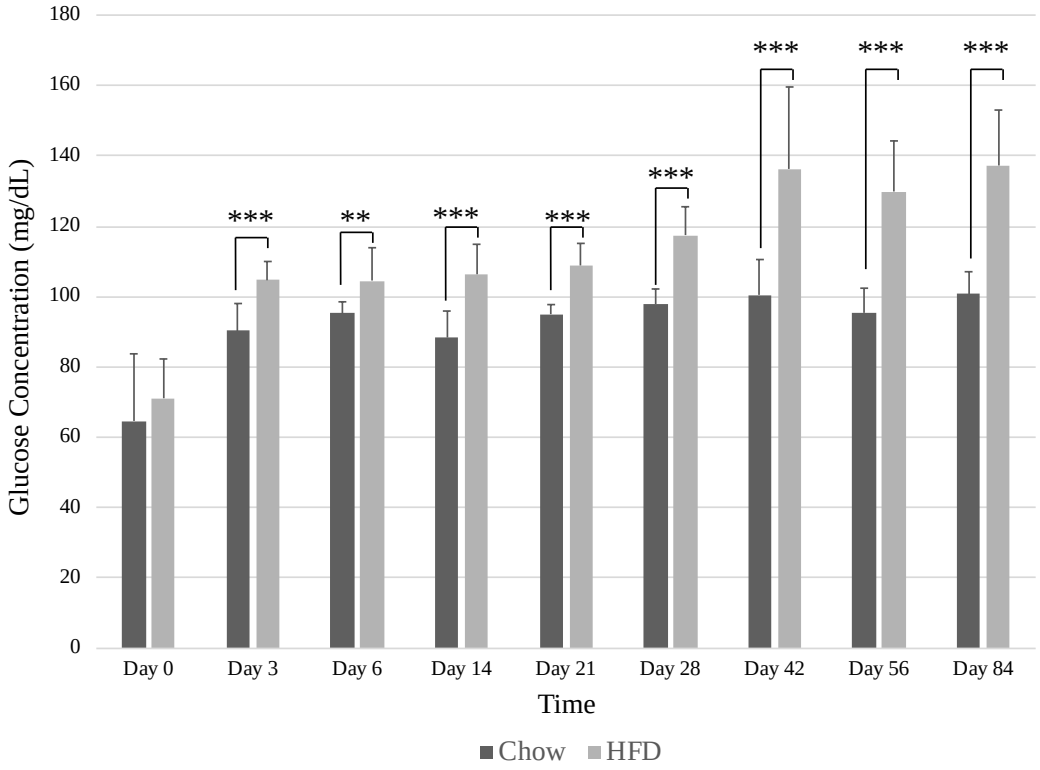
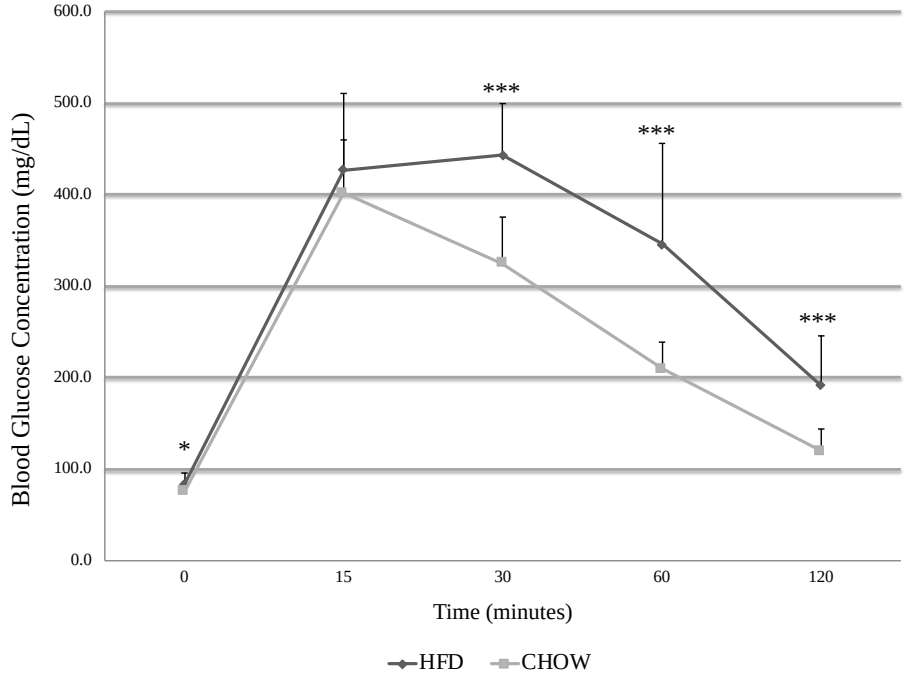


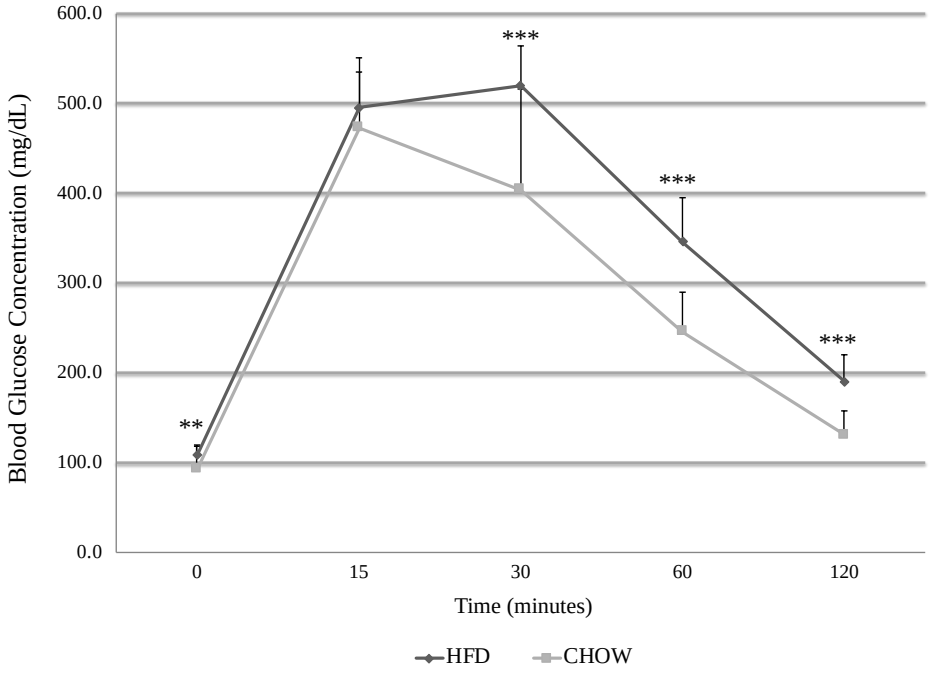
Figure 2. The changes in fasting blood glucose levels in C57BL/6J mice fed on control (chow) and high fat diet (HFD). The mice were 4-5 week old at day 0 (n=4-12).

** $p<0.01$; ***$p<0.001$.

a.)


600.0
500.0
400.0
300.0
200.0
100.0
0.0
Blood Glucose Concentration (mg/dL)
*
***
***
***
0
15
30
60
120
Time (minutes)
HFD
CHOW

b.)


600.0
500.0
400.0
300.0
200.0
100.0
0.0
Blood Glucose Concentration (mg/dL)
**
***
***
***
0
15
30
60
120
Time (minutes)
HFD
CHOW

c.)

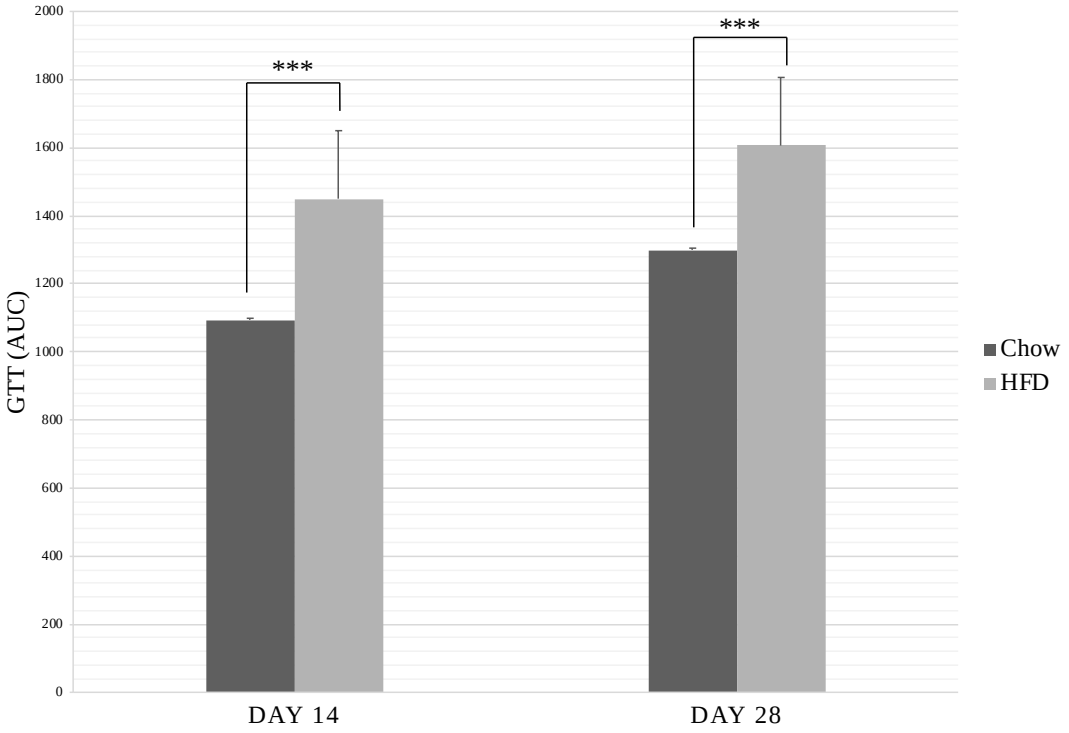


Figure 3 (a-c) . The changes in glucose tolerance in C57BL/6J mice fed on control (chow) and high fat diet (HFD) on day 14 (a) and day 28 (b). Figure 3c shows the area under the curve (AUC) for the respective GTT.

***p<0.001.

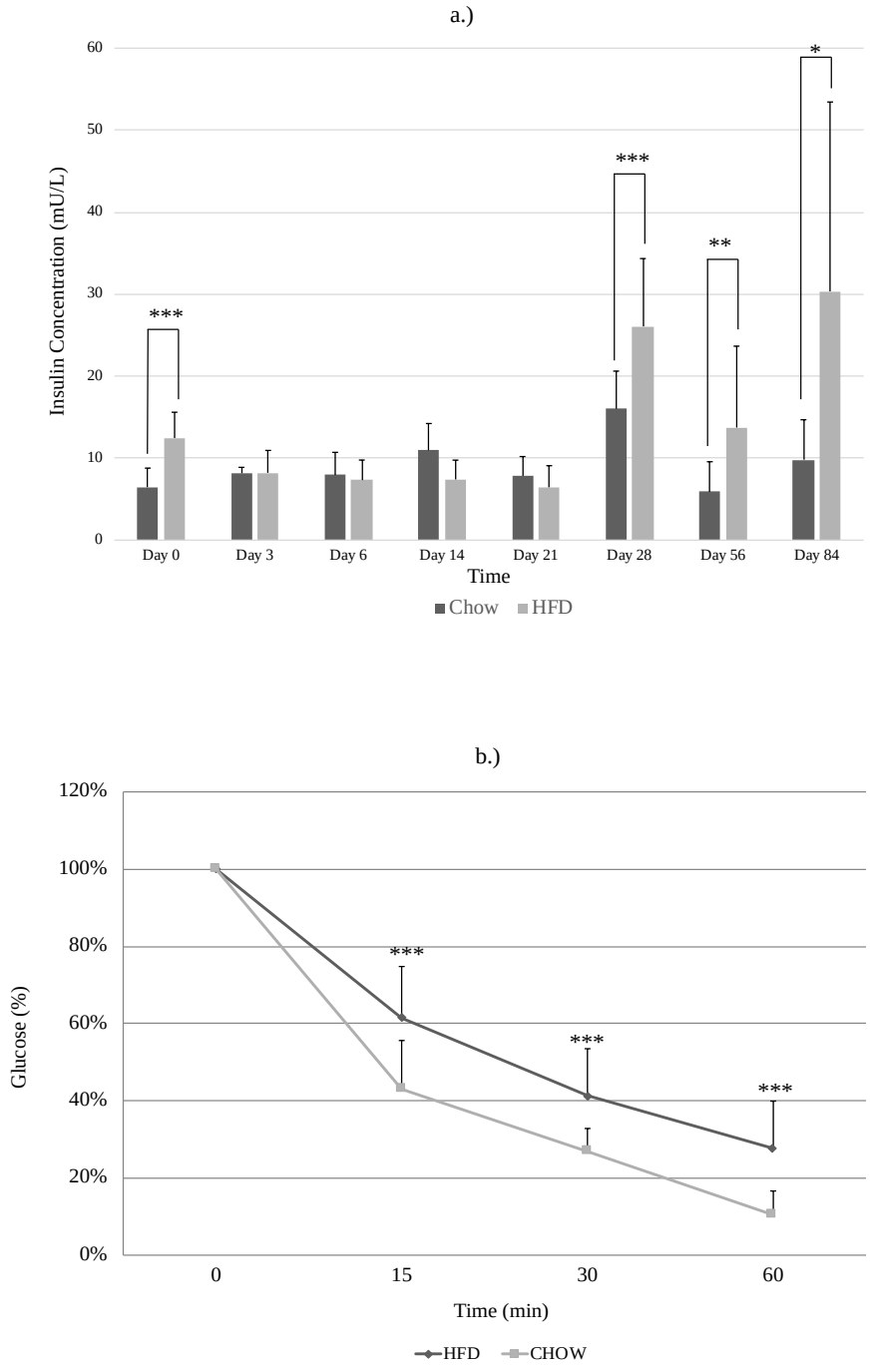


Figure 4 (a,b). The changes in fasting insulin (a) throughout the study time course and insulin tolerance test (ITT) values (b) at day 14 in C57BL/6J mice fed on control (chow) and high fat diet (HFD).

* $p<0.05$; ** $p<0.01$; ***$p<0.001$.

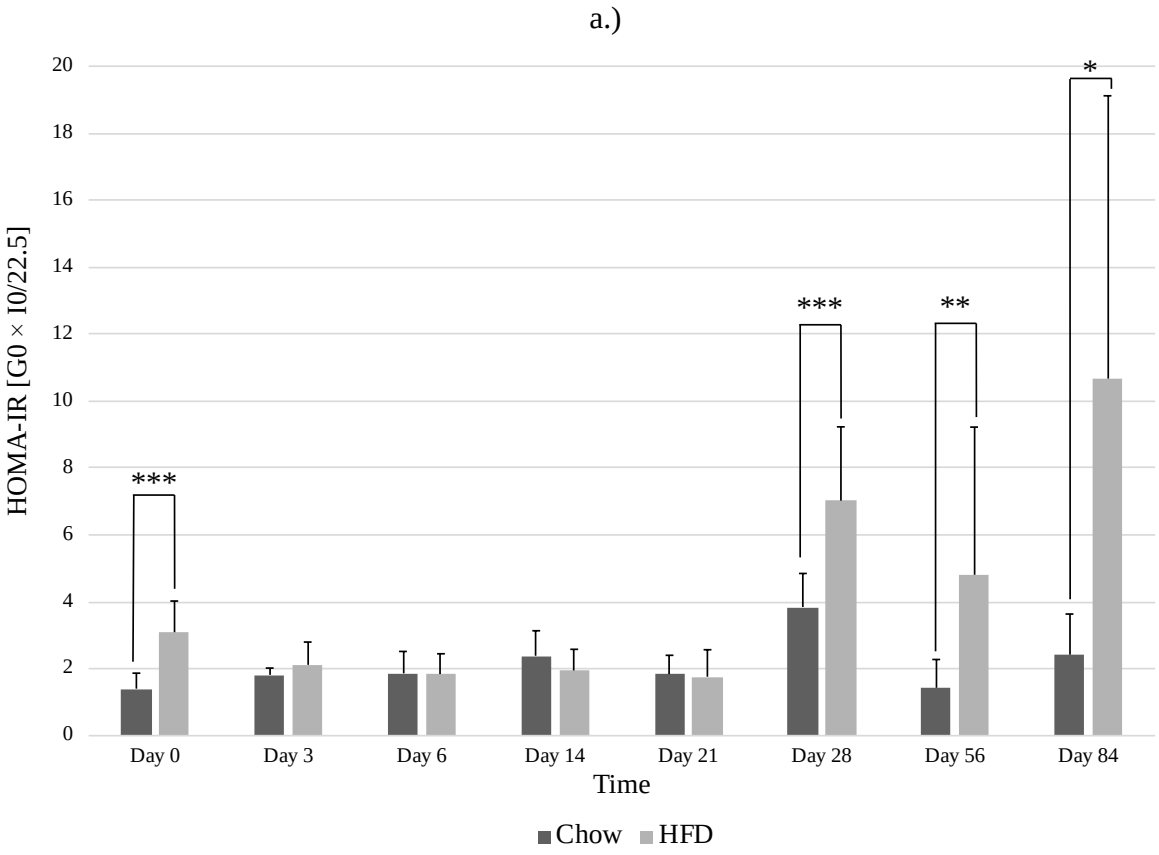


Figure 5a . The variability in HOMA-IR values shown across the time course of the study in C57BL/6J mice fed on control (chow) and high fat diet (HFD). The mice were 4-5 week old at day 0 (n=4-12).

* $p<0.05$; ** $p<0.01$; ***$p<0.001$.

b.)

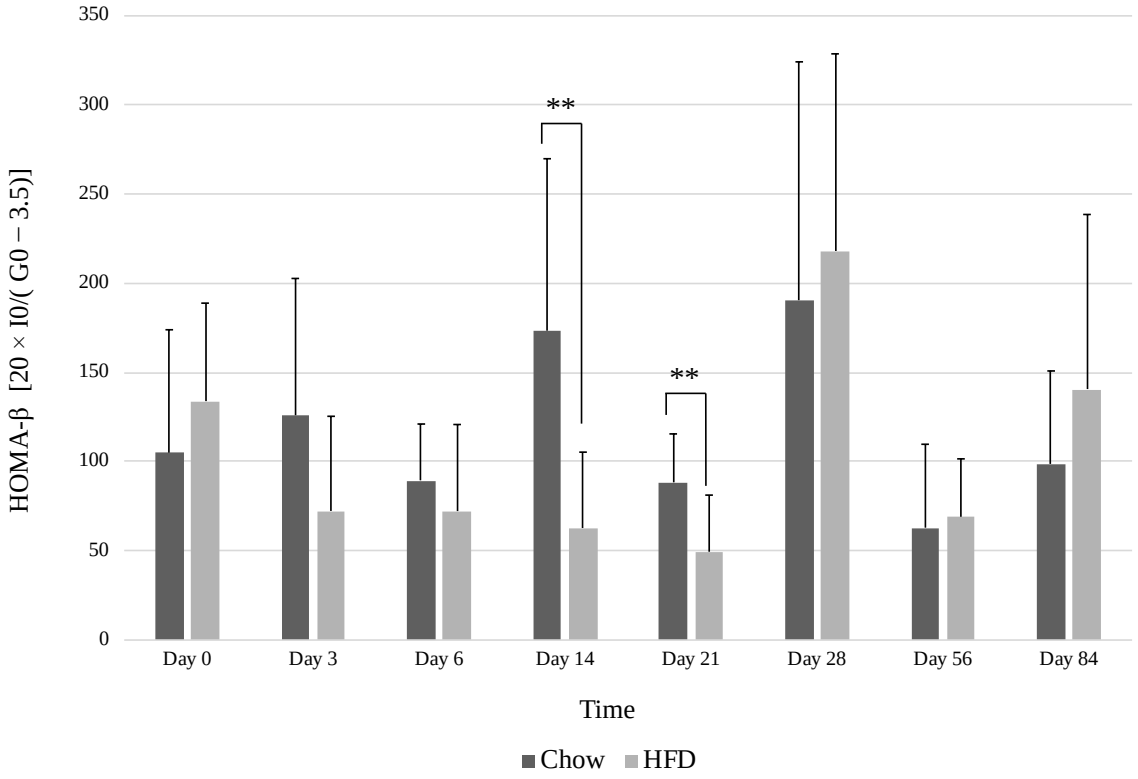


Figure 5b . The variability in HOMA-β values shown across the time course of the study in C57BL/6J mice fed on control (chow) and high fat diet (HFD). The mice were 4-5 week old at day 0 (n=4-12).

* p<0.05; ** p<0.01; ***p<0.001.

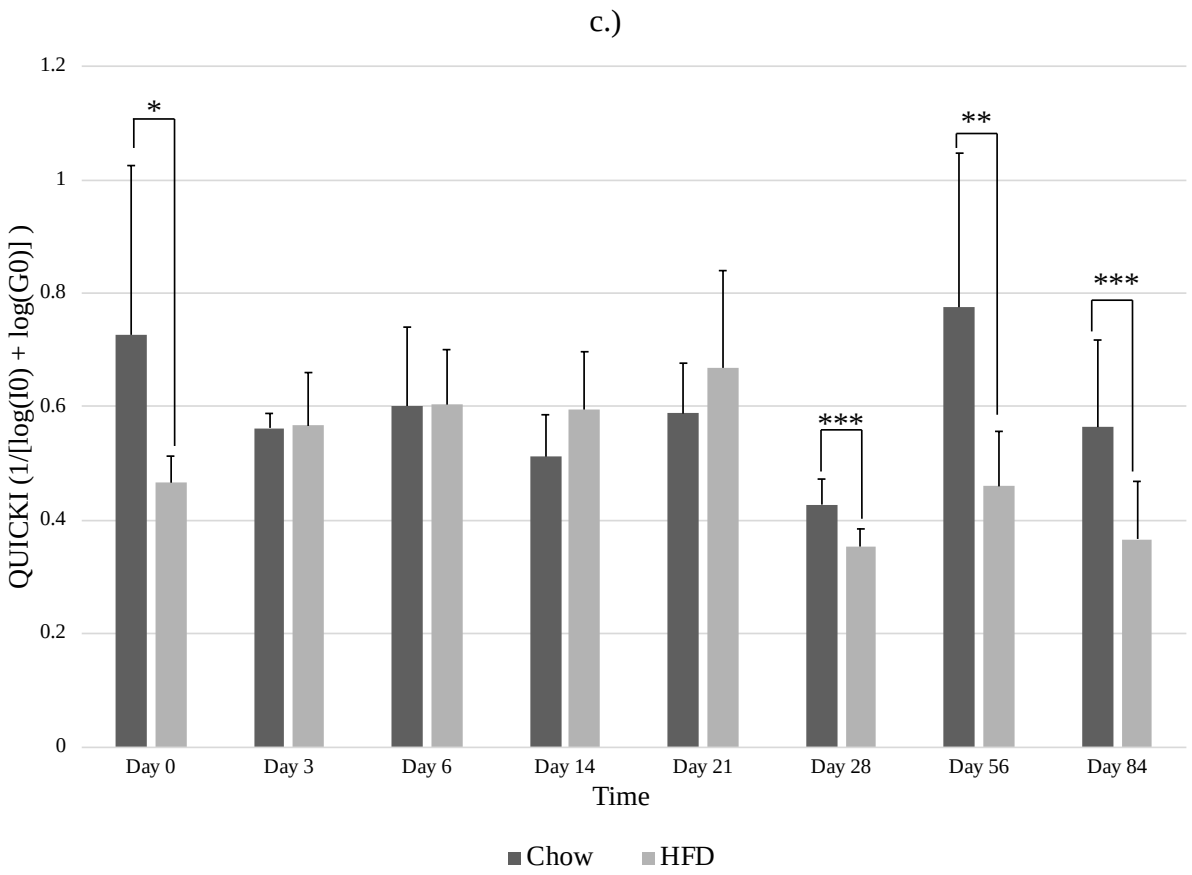


Figure 5c. The variability in QUICKI values shown across the time course of the study in C57BL/6J mice fed on control (chow) and high fat diet (HFD). The mice were 4-5 week old at day 0 (n=4-12).

* $p<0.05$; ** $p<0.01$; ***$p<0.001$.

2.7 References

1. A. Heydemann, An Overview of Murine High Fat Diet as a Model for Type 2 Diabetes Mellitus. *J Diabetes Res* **2016**, 2902351 (2016).
2. R. E. Mosser *et al.*, High-fat diet-induced beta-cell proliferation occurs prior to insulin resistance in C57Bl/6J male mice. *Am J Physiol Endocrinol Metab* **308**, E573-582 (2015).
3. N. Turner *et al.*, Distinct patterns of tissue-specific lipid accumulation during the induction of insulin resistance in mice by high-fat feeding. *Diabetologia* **56**, 1638-1648 (2013).
4. B. Ahren, G. Pacini, Insufficient islet compensation to insulin resistance vs. reduced glucose effectiveness in glucose-intolerant mice. *Am J Physiol Endocrinol Metab* **283**, E738-744 (2002).
5. O. P. McGuinness, J. E. Ayala, M. R. Laughlin, D. H. Wasserman, NIH experiment in centralized mouse phenotyping: the Vanderbilt experience and recommendations for evaluating glucose homeostasis in the mouse. *Am J Physiol Endocrinol Metab* **297**, E849-855 (2009).
6. S. Y. Park *et al.*, Unraveling the temporal pattern of diet-induced insulin resistance in individual organs and cardiac dysfunction in C57BL/6 mice. *Diabetes* **54**, 3530-3540 (2005).
7. U. S. Pettersson, T. B. Walden, P. O. Carlsson, L. Jansson, M. Phillipson, Female mice are protected against high-fat diet induced metabolic syndrome and increase the regulatory T cell population in adipose tissue. *PLoS One* **7**, e46057 (2012).
8. A. A. Toye *et al.*, A genetic and physiological study of impaired glucose homeostasis control in C57BL/6J mice. *Diabetologia* **48**, 675-686 (2005).

9. R. S. Surwit, C. M. Kuhn, C. Cochrane, J. A. McCubbin, M. N. Feinglos, Diet-induced type II diabetes in C57BL/6J mice. *Diabetes* **37**, 1163-1167 (1988).

10. R. S. Surwit, M. F. Seldin, C. M. Kuhn, C. Cochrane, M. N. Feinglos, Control of expression of insulin resistance and hyperglycemia by different genetic factors in diabetic C57BL/6J mice. *Diabetes* **40**, 82-87 (1991).

11. L. Gillberg, C. Ling, The potential use of DNA methylation biomarkers to identify risk and progression of type 2 diabetes. *Front Endocrinol (Lausanne)* **6**, 43 (2015).

12. T. Willmer, R. Johnson, J. Louw, C. Pheiffer, Blood-Based DNA Methylation Biomarkers for Type 2 Diabetes: Potential for Clinical Applications. *Front Endocrinol (Lausanne)* **9**, 744 (2018).

13. H. Heyn, M. Esteller, DNA methylation profiling in the clinic: applications and challenges. *Nat Rev Genet* **13**, 679-692 (2012).

14. T. Geach, Epigenetics: Blood-based markers for T2DM. *Nat Rev Endocrinol* **12**, 311 (2016).

15. N. S. Shenker *et al.*, Epigenome-wide association study in the European Prospective Investigation into Cancer and Nutrition (EPIC-Turin) identifies novel genetic loci associated with smoking. *Hum Mol Genet* **22**, 843-851 (2013).

16. R. Luttmer *et al.*, Metabolic syndrome components are associated with DNA hypomethylation. *Obes Res Clin Pract* **7**, e106-e115 (2013).

17. J. A. Pinzon-Cortes *et al.*, Effect of diabetes status and hyperglycemia on global DNA methylation and hydroxymethylation. *Endocr Connect* **6**, 708-725 (2017).

18. T. E. Matsha *et al.*, Genome-Wide DNA Methylation in Mixed Ancestry Individuals with Diabetes and Prediabetes from South Africa. *Int J Endocrinol* **2016**, 3172093 (2016).

19. T. E. Matsha, C. Pheiffer, T. Mutize, R. T. Erasmus, A. P. Kengne, Glucose Tolerance, MTHFR C677T and NOS3 G894T Polymorphisms, and Global DNA Methylation in Mixed Ancestry African Individuals. *J Diabetes Res* **2016**, 8738072 (2016).

20. C. Pheiffer, R. T. Erasmus, A. P. Kengne, T. E. Matsha, Differential DNA methylation of microRNAs within promoters, intergenic and intragenic regions of type 2 diabetic, pre-diabetic and non-diabetic individuals. *Clin Biochem* **49**, 433-438 (2016).

21. D. Simar *et al.*, DNA methylation is altered in B and NK lymphocytes in obese and type 2 diabetic human. *Metabolism* **63**, 1188-1197 (2014).

22. F. F. Zhang *et al.*, Significant differences in global genomic DNA methylation by gender and race/ethnicity in peripheral blood. *Epigenetics* **6**, 623-629 (2011).

23. N. Zhang, Epigenetic modulation of DNA methylation by nutrition and its mechanisms in animals. *Anim Nutr* **1**, 144-151 (2015).

24. G. M. Martin-Nunez *et al.*, Type 2 diabetes mellitus in relation to global LINE-1 DNA methylation in peripheral blood: a cohort study. *Epigenetics* **9**, 1322-1328 (2014).

25. M. S. Pearce *et al.*, Global LINE-1 DNA methylation is associated with blood glycaemic and lipid profiles. *Int J Epidemiol* **41**, 210-217 (2012).

26. Y. Wu, W. Cui, D. Zhang, W. Wu, Z. Yang, The shortening of leukocyte telomere length relates to DNA hypermethylation of LINE-1 in type 2 diabetes mellitus. *Oncotarget* **8**, 73964-73973 (2017).

27. J. Zhao, J. Goldberg, J. D. Bremner, V. Vaccarino, Global DNA methylation is associated with insulin resistance: a monozygotic twin study. *Diabetes* **61**, 542-546 (2012).

28. J. Thongsroy, M. Patchsung, A. Mutirangura, The association between Alu hypomethylation and severity of type 2 diabetes mellitus. *Clin Epigenetics* **9**, 93 (2017).

29. C. Ling *et al.*, Epigenetic regulation of PPARGC1A in human type 2 diabetic islets and effect on insulin secretion. *Diabetologia* **51**, 615-622 (2008).

30. C. Ling *et al.*, Multiple environmental and genetic factors influence skeletal muscle PGC-1alpha and PGC-1beta gene expression in twins. *J Clin Invest* **114**, 1518-1526 (2004).

31. R. Ribel-Madsen *et al.*, Genome-wide analysis of DNA methylation differences in muscle and fat from monozygotic twins discordant for type 2 diabetes. *PLoS One* **7**, e51302 (2012).

32. R. Barres *et al.*, Non-CpG methylation of the PGC-1alpha promoter through DNMT3B controls mitochondrial density. *Cell Metab* **10**, 189-198 (2009).

33. C. Brons *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* **95**, 3048-3056 (2010).

34. L. Gillberg, S. C. Jacobsen, T. Ronn, C. Brons, A. Vaag, PPARGC1A DNA methylation in subcutaneous adipose tissue in low birth weight subjects--impact of 5 days of high-fat overfeeding. *Metabolism* **63**, 263-271 (2014).

35. R. A. DeFronzo, Pathogenesis of type 2 diabetes mellitus. *Med Clin North Am* **88**, 787-835, ix (2004).

36. K. F. Eriksson, F. Lindgarde, Prevention of type 2 (non-insulin-dependent) diabetes mellitus by diet and physical exercise. The 6-year Malmo feasibility study. *Diabetologia* **34**, 891-898 (1991).

37. T. M. Wallace, J. C. Levy, D. R. Matthews, Use and abuse of HOMA modeling. *Diabetes Care* **27**, 1487-1495 (2004).

38. A. Mari, B. Ahren, G. Pacini, Assessment of insulin secretion in relation to insulin resistance. *Curr Opin Clin Nutr Metab Care* **8**, 529-533 (2005).

39. A. Mari, J. Wahren, R. A. DeFronzo, E. Ferrannini, Glucose absorption and production following oral glucose: comparison of compartmental and arteriovenous-difference methods. *Metabolism* **43**, 1419-1425 (1994).

40. A. Abbasi *et al.*, Prediction models for risk of developing type 2 diabetes: systematic literature search and independent external validation study. *BMJ* **345**, e5900 (2012).

41. D. Vistisen *et al.*, Patterns of obesity development before the diagnosis of type 2 diabetes: the Whitehall II cohort study. *PLoS Med* **11**, e1001602 (2014).

42. A. G. Tabak, C. Herder, W. Rathmann, E. J. Brunner, M. Kivimaki, Prediabetes: a high-risk state for diabetes development. *Lancet* **379**, 2279-2290 (2012).

43. N. Abate, M. Chandalia, Risk of Obesity-Related Cardiometabolic Complications in Special Populations: A Crisis in Asians. *Gastroenterology* **152**, 1647-1655 (2017).

44. C. Power, C. Thomas, Changes in BMI, duration of overweight and obesity, and glucose metabolism: 45 years of follow-up of a birth cohort. *Diabetes Care* **34**, 1986-1991 (2011).

45. S. G. Wannamethee, A. G. Shaper, Weight change and duration of overweight and obesity in the incidence of type 2 diabetes. *Diabetes Care* **22**, 1266-1272 (1999).

Chapter 3

Determination of DNA methylation kinetics in the promoter region of PGC-1α and subsequent gene expression alterations during progressing insulin resistance.

Sarah Donnelly[1], Longhua Liu[2], Louise Zhang[1], Zhipeng Tao[1], Fabio Almeida[3], and Zhiyong Cheng[4]

[1]Department of Human Nutrition, Foods, and Exercise, Virginia Tech, Blacksburg, VA;

[2]Department of Pathology and Cell Biology, Columbia University, New York, NY;

[3]Department of Health Promotion, University of Nebraska Medical Center, Omaha NE

[4]Department of Human Nutrition, University of Florida, Gainesville, FL

Corresponding Author
Zhiyong Cheng, Ph.D.
572 Newell Drive
Gainesville, Florida 32611-1907
540-235-4889
z.cheng@ufl.edu

3.1 Abstract

Metabolic disorders, such as obesity and T2DM, tend to coincide with aberrant mitochondrial functioning(*1-6*). Though it is known that T2DM is a result of impaired glucose and insulin signaling, the molecular mechanisms that underlie these phenomena are not fully understood(*1*). PGC-1α is a master regulator of mitochondria, as it serves as a transcriptional coactivator for many of the genes involved in mitochondrial biogenesis, therefore, it is of interest to further exam PGC-1α and the epigenetic controls that may result in decreased transcription of the gene(*7-11*).

In order to identify parallels whereby anthropometric and metabolic changes coincide with changes in the DNA methylome of PGC-1α and changes in mitochondrial content, we will collect and analyze metabolically active tissue and leukocytes and utilize correlation analysis to determine the relationship between changes seen at the level of the tissue with those seen in the blood. Results from blood samples, the most commonly used surrogate tissue, will be contrasted with that from other tissues including adipose tissues, liver, and skeletal muscle. Temporal correlation of epigenetic changes in PGC-1α with metabolic and anthropometric alterations, in addition to measures of insulin resistance, will be examined to determine the temporal inflection point whereby epigenetic marker begin to reflect metabolic changes.

3.2 Introduction

Mitochondrial biogenesis is the process whereby mitochondria grow and divide, allowing for adequate ATP synthesis to meet the energy demands of the cell(*12-16*). Environmental stressors, such as caloric restriction and exercise, prompt mitochondrial biogenesis by activating upstream genes such as PGC-1α and mitochondrial transcription factor A (TFAM). Indeed, transcriptional regulatory mechanisms control the biogenesis of mitochondria and, therefore, may contribute to diseased states and metabolic dysfunction. TFAM, a gene encoded by the nuclear genome, prompts the replication of mitochondrial DNA and activates transcription of mitochondrial encoded genes(*13*). Included in the TFAM promoter region is a binding site for nuclear respiratory factors 1 and 2 (NRF1 and NRF2), thereby connecting nuclear and mitochondrial activation, as the role of TFAM is primarily inside the mitochondrial matrix.

At the center of the transcriptional regulatory network of mitochondrial biogenesis is PGC-1α. PGC-1α is a transcriptional coactivator, meaning it serves to enhance transcriptional activity via protein-protein interaction with transcriptional activators (*13*). In other words, transcription of certain genes cannot take place in the absence of PGC-1α, if PGC-1α is required as a coactivator. PGC-1α responds to external stimuli and exerts subsequent effects on various aspects of energy metabolism. Prior studies have linked upregulation of PGC-1α to improved insulin signaling and mitochondrial homeostasis in both skeletal muscle and adipose tissue, the two primary tissues responsible for glucose uptake(*17*). Not surprisingly, deletion of PGC-1α in C2C12 myotubes results in an increase in fast-twitch glycolytic fibers and a subsequent reduction in slow-twitch oxidative fiber, a phenotypic phenomenon associated with skeletal muscle insulin resistance and impaired glucose disposal(*18-24*).

Insulin resistance is characterized by dysfunctional glucose uptake, insulin signaling, and glycogenolysis, adding credence to the long accepted theory that impaired mitochondrial activity may underlie insulin resistance(*17*). Indeed, prior studies have shown that increases in fatty acyl CoA and diacylglycerol, subsequent of impaired fatty acid oxidation in the mitochondria, disrupts insulin signaling. Elevated intracellular fatty acid metabolites in the cell result in the activation of protein kinase C, thereby activating a serine kinase cascade that subsequently results in the inhibition of phosphatidyl inositol 3-kinase, leading to the suppression of insulin mediated glucose uptake in the cell(*17*).

Additionally, glucose sensing in the pancreatic beta cells is impaired in the diabetic state, likely due in part to detriments in mitochondrial oxidative metabolism(*17, 25, 26*). In a healthy state, the presence of glucose leads to glucose oxidation and increases in production of ATP from the electron transport chain (ETC), leading to an increased ratio of ATP/ADP, closure of the ATP/ADP regulated potassium channel, membrane depolarization, and finally, calcium-stimulated insulin secretion. Dysfunctional mitochondrial biogenesis, characteristic of the insulin resistant state, does not allow for this mechanism to materialize appropriately, leading to impairments in secretion of insulin by the beta cells(*25, 26*).

Given the critical role of mitochondria in regulating glucose sensing and insulin responsiveness, and the role of PGC-1α in maintaining properly functioning mitochondria, it is of particular interest to examine the temporal alterations in DNA methylation occurring in the promoter region of PGC-1α, as these may dictate downstream phenotypic characteristics associated with diabetes. Further,

it will be of interest to elucidate the response of PGC-1α downregulation in the pathogenesis of the disease, and examine whether it's downregulation is a primary or secondary event to overt disease onset.

3.3 Methods

3.3.1 Mice

C57BL/6J male mice (n=48) were purchased from Jackson Laboratory (Bar Harbor, Maine) and were housed in plastic cages on a 12 hours light/dark cycle (22–25°C) and *ad libitum* access to water and food. At the onset of the study, the mice were placed on either a normal chow diet (10% kcal from fat) or a high fat diet (HFD) (60% kcal from fat, 20% kcal from carbohydrates, and 20% kcal from protein)(D12492, Research Diets Inc., New Brunswick, NJ). Animals were fasted for 12-16 hours overnight prior to sacrifice and subsequent tissue harvest and were sacrificed according to approved guidelines put forth by IACUC. At the completion of the feeding study, mice were euthanized by placing the animals in a clean cage and introducing 100% carbon dioxide at a fill rate of approximately 10-30% of the chamber volume per minute. Cervical dislocation was performed to ensure that the animal was deceased. All procedures were conducted in accordance of the Virginia Polytechnic Institute and State University Animal Care and Use Committee (IACUC).

3.3.2 Tissue Collection

Following euthanasia, multiple metabolically active tissues (liver, skeletal muscle, visceral adipose tissue (eWAT), and subcutaneous adipose tissue (sWAT,)) were isolated and snap-frozen in liquid nitrogen. Those samples not used immediately were stored at -80 °C.

3.3.3 DNA Extraction & mtDNA Quantification

DNA was extracted from tissue and blood using the QIAamp® DNA Mini and Blood Mini kit (QIAGEN, Hilden, Germany). Briefly, lysate buffers allow for binding of the DNA to the membrane of the spin column, the membrane is then cleaned through a series of washing steps, and nucleic acids are eluted in the final step. Quantity and quality of DNA was measured using the Syngergy H4 Hybrid Multi-Mode Microplate Reader (BioTek Instruments, Winooski, VT, USA). Relative amounts of nuclear DNA and mitochondrial DNA (mtDNA) were measured by qRT-PCR as shown previously(*27-29*), using GAPDH as a housekeeping gene (specific to nuclear DNA) and Cytochrome B (a component of respiratory chain complex III) as a mitochondrial-specific gene. The primer set information is listed in **Table 1**. The following conditions were used for amplification: 1*(95°C/10min), 40*(95°C/0:15sec, 58°C/0:45sec), 1*(95°C/0:15sec, 60°C/1:00, 95°C/0:15sec). The ratio of mtDNA:nDNA reflects the tissue concentration of mitochondria per cell. Primer information is listed in **Table 1**.

	Target	Sequence (5' to 3')
mtDNAn Analysis	mGAPDH-f	ACAGTCCATGCCATCACTGCC
	mGAPDH-r	GCCTGCTTCACCACCTTCTTG
	mGAPDH-f	AGTTCAACGGCACAGTCAAG
	mGAPDH-r	GTGGTGAAGACGCCAGTAGA
	mCytb-f	GCTTTCCACTTCATCTTACCATTTA
	mCyt-r	TGTTGGGTTGTTTGATCCTG

Table 1. Primer information for mitochondrial DNA quantification

3.3.4 Bisulphite Conversion & Methylation Analysis

Bisulphite conversion of DNA was performed using the EpiTect Bisulfite Kit (QIAGEN), whereby methylated cytosines were conserved and unmethylated cytosines were converted to uracils. DNA quality/quantity was examined using the Synergy H4 Hybrid Multi-Mode Microplate Reader (BioTek Instruments, Winooski, VT, USA) and stored at −20 °C. qPCR was performed to examine methylation profile at mPGC-1α and mTFAM as described previously(*30*).The following conditions were used for amplification: 1*(95°C/15min), 40*(94°C/0:15sec, 57°C/0:45sec), 1*(95°C/0:15sec, 60°C/1:00, 95°C/0:15sec). Primer information is located in Table 2.

	Target	Sequence (5' to 3')
Methylation Analysis	mPGC-1α-Mf1	ATTGAGTTTTAGTGAATTAAAACGT
	mPGC-1α-Mr	AAAATATAAAAAAAATACCTCCGAT
	mPGC-1α-Uf	ATTGAGTTTTAGTGAATTAAAATGT
	mPGC-1α-Ur	AAAATATAAAAAAAATACCTCCAAT
	mPGC-1α-Mf	TTTTACGTTTATATTTGGTTGAGATC
	mPGC-1α-Mr	AACAAAAACTTACACCAACTACGTT
	mPGC-1α-Uf	TATGTTTATATTTGGTTGAGATTGG
	mPGC-1α-Ur	AACAAAAACTTACACCAACTACATT

Table 2. Primer information for methylation analysis.

3.3.5 RNA Extraction & cDNA Synthesis

RNA was extracted from tissue samples using the RNeasy Miki Kit (QIAGEN) according to the manufacturer's instructions. Upon extraction, RNA samples were used to synthesize cDNA by reverse transcription polymerase chain reaction (rtPCR). The iScriptTM cDNA Synthesis kit (BioRad) was utilized for the synthesis of cDNA. The following conditions

were used for amplification of qPCR of synthesized cDNA: 1*(95°C/5min), 40*(95°C/0:15sec, 58°C/0:30sec, 72°C/0:30sec), 1*(95°C/0:15sec, 60°C/1:00, 95°C/0:15sec). Primer information is located in **Table 3**.

	Target	Sequence (5' to 3')
Gene Expression Analysis	mPGC-1α-f	CCCTGCCATTGTTAAGACC
	mPGC-1α-r	TGCTGCTGTTCCTGTTTTC
	mGAPDH-f	AGTTCAACGGCACAGTCAAG
	mGAPDH-r	GTGGTGAAGACGCCAGTAGA

Table 3. Primer information for gene expression analysis.

3.3.6 Western Blotting

Snap frozen tissue (liver, skeletal muscle, adipose tissue) were homogenized in PLC lysis buffer with protease inhibitor cocktail (Roche), 1 mM PMSF, 10 μM TSA (Trichostatin A, Selleckchem) and 5 mM Nicotinamide (Alfa Aesar) using a Bullet Blender® (Next Advance, Inc.). Total protein concentrations were determined using the DC protein assay (Bio-Rad). Antibody catalog numbers and vendors are as follows: GAPDH antibody (MA5-15738) from Pierce; Complex 1 (C1- A21344) and Complex 3 (C3- A21362) antibody from Invitrogen; PGC-1α antibody (ab54481) from abcam.

3.3.7 Statistical Analysis

Pearson's correlation and linear regression analysis were applied for the examination of correlative properties amongst diabetes related risk factors, metabolic indices, mitochondrial DNA levels, and promoter methylation. In instances of skewed variables, logarithm transformations were conducted. Unless otherwise specified, data are presented

as mean ± SE. Differences were examined by a two-tailed t-test (for 2-group comparison) or one-way ANOVA with the least significant difference post hoc test to detect statistical differences (for more than 2-group comparison). A value of $p < 0.05$ was considered statistically significant.

3.4 Results

3.4.1 mtDNA Content & Physiological Markers

At day 0, relative mtDNA content in the leukocytes was similar between chow and HFD groups (3.70 ± 0.82 vs. 3.21 ± 0.003; p=0.71) (**Figure 1**). Significant differences in leukocyte mtDNA content between the chow and HFD groups was apparent as early as day 3 (8.24 ± 1.66 vs. 2.97 ± 0.33, respectively; p<0.01). This difference were not statistically significant at day 6 (6.85 ± 1.14 vs. 4.07 ± 0.54, p=0.14), but were again at the day 14 timepoint (6.00 ± 0.87 vs. 3.69 ± 0.54, p<0.05).

Correlation analysis between mtDNA content and physiological markers revealed a significant negative association between mtDNA content and fasting glucose (r= -0.43, p < 0.05) and body fat % (r=-0.37, p<0.001), but not fasting insulin (r= 0.20, p=0.29) (**Figure 2**). Further, there was no significant association between mtDNA content and clinical indices utilized to diagnose diabetes, which included HOMA-IR (r= 0.056, p=0.77), HOMA-β (r= 0.24, p=0.21), and QUICKI (r= -0.17, p=0.38) (**Figure 3**).

3.4.2 PGC-1α Methylation & mtDNA Content

In order to evaluate whether PGC-1α methylation influences mtDNA content, correlation analyses were conducted and showed that PGC-1α methylation indeed was significantly and negatively associated with mtDNA content at both day 3 ($r= -0.70$, $p<0.05$) and day 6 ($r= -0.75$, $p<0.05$) (**Figure 4**). Significant correlation between PGC-1α methylation and mtDNA content was not maintained at day 84 ($r= 0.02$, $p=0.21$).

3.4.3 PGC-1α Methylation Correlation in Leukocytes and Metabolically Active Tissue

In order to confirm that patterns of PGC-1α methylation were mirrored in metabolically active tissue as compared to those observed in leukocytes, correlation and linear regression analyses were conducted and showed that patterns of leukocyte PGC-1α methylation are mirrored across tissues, including the liver ($r= 0.53$, $p<0.05$), skeletal muscle ($r= 0.81$, $p<0.001$), subcutaneous adipose tissue ($r= 0.73$, $p<0.05$), and visceral adipose tissue ($r= 0.54$, $p<0.05$) (**Figure 5**).

3.4.4 PGC-1α Gene Expression

At day 6, PGC-1α gene expression was significantly different between chow and HFD groups in the in the (4.16 ± 0.01 vs. 1.78 ± 0.21, $p<0.05$). This trend was not observed in the visceral adipose tissue (1.06 ± 0.21 vs. 1.06 ± 0.05, $p=0.99$) and subcutaneous adipose tissue (1.47 ± 0.73 vs. 1.30 ± 0.64, $p=0.86$) (**Figure 6**). At the 56 day timepoint, we examined PGC-1α gene expression in chow and HFD groups, as well as a third group (INT) which had been placed on a HFD for 2 months, and then switched back to chow 2 months. Interestingly, there were no significant differences between the chow and HFD groups in the visceral adipose tissue (4.12 ± 0.09 vs. 2.45 ± 0.07, $p=0.08$), subcutaneous adipose

tissue (5.30 ± 0.99 vs. 3.55 ± 0.18, p=0.45), but was significantly different in the liver (4.16 ± 0.09 vs. 1.78 ± 0.02, $p<0.05$). However, the PGC-1α gene expression was significantly different between HFD and INT groups at day 56 in the subcutaneous adipose tissue (3.55 ± 0.18 vs. 0.54 ± 0.001, $p<0.05$) and significantly different between both chow and INT (4.07 ± 0.04 vs. 6.64 ± 0.20, $p<0.01$) as well as HFD and INT in the liver (3.94 ± 0.05 vs. 6.64 ± 0.20, $p<0.01$) (**Figure 7**). PGC-1α gene expression was not significantly different at day 56 in the visceral adipose tissue between HFD and INT groups (2.45 ± 0.07 vs. 1.92 ± 0.24, p=0.30) or between chow and INT group (4.12 ± 0.09 vs. 1.92 ± 0.24, p=0.83). Further analysis on leukocytes was not conducted due to limited samples, making it difficult to draw parallels between correlative properties between tissue samples and leukocytes.

3.4.5 PGC-1α Methylation & Markers of Insulin Sensitivity

At day 3, PGC-1α promoter methylation was significantly correlated with HOMA-IR (r= 0.65, $p<0.05$), and QUICKI (r= - 0.63, $p<0.05$), indices. PGC-1α promoter methylation was not significantly correlated with body weight (r= 0.06, p= 0.87), fat % (r= 0.15, p= 0.67), fasting glucose (r= 0.43, p= 0.22), fasting insulin (r= 0.45, p= 0.15), or HOMA-β (r= -0.25, p= 0.48), (**Figure 8**). At day 6, PGC-1α promoter methylation was significantly positively correlated with body weight only (r= 0.86, $p<0.01$). No significant correlation existed between PGC-1α promoter methylation and fat % (r= 0.15, p= 0.70), fasting glucose (r= 0.59, p= 0.10), fasting insulin (r= -0.48, p= 0.19), HOMA-IR (r= 0.46, p= 0.21), HOMA-β (r= -0.45, p= 0.23), or QUICKI (r= 0.51, p= 0.16) (Figure 9).

3.4.6 Western Blot Analysis of Mitochondrial Proteins and PGC-1α

At day 6, the liver, skeletal muscle, and adipose tissue were analyzed to compare mitochondrial protein expression (complex I and complex III) and PGC-1α protein expression between the chow and HFD groups (**Figure 11**). These values were normalized to GAPDH protein expression. Interestingly, there was an observed significant reduction in PGC-1α protein expression in subcutaneous adipose tissue ($p=0.005$) and in visceral adipose tissue ($p=0.037$). There was no significant difference in in PGC-1α protein expression in the liver ($p= 0.21$) or in the skeletal muscle ($p= 0.43$). At day 6, there were no significant differences in complex I protein expression between groups (liver and skeletal muscle; $p= 0.17$ and 0.18, respectively). Further, there were no significant differences in complex I protein expression between groups (liver, skeletal muscle, subcutaneous adipose tissue, and visceral adipose tissue; $p= 0.39$, 0.19, 0.30, and 0.66, respectively).

3.5 Discussion

Despite the growing number of humans affected by insulin resistance, along with the evidence of the effectiveness of behavioral interventions (i.e. diet and exercise) in ameliorating the disease, little is known about the molecular mechanisms underlying disease progression. The present study simultaneously examined the DNA methylation and mRNA expression of PGC-1α in liver, skeletal muscle, adipose tissue, and white blood cells, as well as the mitochondrial copy number in these respective tissues. Mitochondrial DNA copy number, in conjunction with mitochondrial protein content, were analyzed to provide functional data to support the notion that hypermethylation of the promoter of PGC-1α leads to decreased mitochondrial biogenesis by

repressing transcription of PGC-1α, which is considered a master regulator of mitochondrial biogenesis. In this study, we revealed a relationship between acute high fat diet feeding and alterations in methylation levels, which coincided with reductions in mitochondrial DNA content following three days of HFD. Indeed, HFD induced glucose dysregulation coincided with increases in methylation levels at the promoter region of PGC-1α as early as day 3 in leukocytes, a pattern which persisted at day 6 and corresponded to increased PGC-1α methylation in metabolically active tissues, indicating a mechanism whereby epigenetic modifications may precede overt disease onset.

Our results, too, confirm the positive correlation between body weight and body fat with fasting glucose levels (**Figure 9**), which is anticipated given a plethora of previous evidence illustrating a strong positive correlation between adiposity and lipid overload with glucose dysregulation(*31-41*). Interestingly, correlation analysis also showed a negative and significant correlation between body fat% and fasting glucose levels with mitochondrial DNA content, suggesting that mitochondrial dysfunction may precede the onset of insulin resistance and coincide with changes in anthropometric measures, which have been shown previously to manifest prior to significant metabolic alterations(*42-48*). We did not, however, observe a significant association between fasting insulin and mitochondrial DNA content at day 3, suggesting that an acute relationship between insulin levels and mitochondrial dysfunction may not exist.

Our results indicated that promoter hypermethylation in PGC-1α coincided with decreased mitochondrial content, as quantified by mtDNA analyses, as early as 3 days following initiation of a high fat diet in C57/BL6 male mice. Though we did see significant changes in PGC-1α

methylation levels at day 6, this did not coincide with decreased PGC-1α gene expression in subcutaneous and visceral adipose tissue. Interestingly, protein expression analysis revealed a significant reduction in PGC-1α protein expression in both the subcutaneous and adipose tissue, which would have been predicted given the hypermethylation seen in the promoter region, however these results contrast with the gene expression analysis, which showed no significant change. There was, however, a marked and significant decrease in PGC-1α gene expression in the liver in mice fed a HFD versus those on chow, potentially indicating an early marker for disease risk, as the liver is one of the primary organs responsible for maintaining blood glucose homeostasis, however PGC-1α protein expression analysis showed no significant change between groups (*49-52*). Surprisingly, there were no observed significant differences in complex 1, complex III, or PGC-1α protein expression in skeletal muscle at day 6, perhaps indicating a less acute reaction to HFD on PGC-1α protein expression versus adipose tissue.

Interestingly, the intervention group (INT), which were placed on a 2 month HFD follow by a 2 month chow diet, saw markedly significant increases in PGC-1α gene expression in the liver. In obese individuals, PGC-1α gene expression tends to be lower in the liver than in obese subjects(*53*). Additionally, and unlike other metabolically active tissues, increased PGC-1α expression has been shown to correlate with lowered triacylglycerols in the liver, further confirming that reversion to a regular chow diet and normoglycemia could potentially lead to increased expression of PGC-1α in lean mice compared to obese(*53*). Interestingly, we were not able to identify a correlation between PGC-1α promoter methylation and early markers of insulin resistance, with the exception of HOMA-IR and QUICKI at day 3 and body weight at day 6. This

may be due to the potential phenomenon whereby acute changes occurring at the genomic level may precede the phenotypical changes seen in the bloodstream (**Figure 10**).

A clear causal mechanism whereby PGC-1α DNA methylation in metabolically active tissues during the progression of insulin resistance has not been established. Indeed, PGC-1α DNA methylation has not conclusively been shown to be a primary or secondary effect of insulin resistance, however, our results indicate these genomic alterations may precede overt disease onset, indicating a potential mechanism whereby transcriptional regulation of mitochondrial biogenesis may manifest in altered glucose handling and insulin signaling.

T2DM is denoted by marked deficits in mitochondrial function, leading to impaired glucose and lipid metabolism(*54-60*). The known relationship that exists between OXPHOS function and diabetes has been confirmed by numerous prior studies(*4, 7, 17, 23, 61-63*). Indeed, altered dynamics in mitochondria function lead to lipid accumulation, oxidative stress, and inflammation, each of which contribute to the activation of the serine and threonine kinases, subsequently inhibiting insulin signaling through phosphorylation effects on the insulin receptor substrates(*64-69*). It would stand to reason, therefore, that the early stages of HFD initiation cause increases in methylation of PGC-1α, leading to decreases in mitochondrial biogenesis, thereby causing insulin resistance in the hepatocytes and myocytes. Our study confirmed an association between both PGC-1α methylation with mitochondrial DNA content and mitochondrial DNA content with fasting glucose and insulin at day 3, indicating a potential role of DNA methylation in regulating the body's response to increased caloric intake via control of mitochondrial biogenesis and alterations in oxidative phosphorylation pathways.

Importantly, the fluid mechanistic nature of DNA methylation/de-methylation adds credence to the theory that behavioral intervention may ameliorate molecular alterations that occur as a consequence of the metabolic complications associated with diabetes(*70-75*). A multiplicity of tissue and cell types are involved in the pathogenesis of diabetes, therefore caution should be taken in over-extrapolating the epigenetic changes seen in one tissue versus those seen in another. Further, the association between DNA methylation and subsequent gene expression is not entirely understood. Interestingly, our study revealed no significant correlation between PGC-1α promoter methylation and PGC-1α gene expression, warranting further investigation into downstream protein expression alterations.

Taken together, effectual approaches for T2DM prevention include early identification of high risk populations for the disease. Methods to identify acute and chronic molecular-level alterations during disease progression, such as methylation analysis, may be effective in coordination with physiological measure analysis to identify high risk populations for T2DM, as overweight and obesity alone are not accurate identifiers for risk. The results of the present study indicate that further analyses examining the role of DNA methylation as a modifier of mitochondrial function in glucose regulation, and that DNA methylation profiling may be effective in identifying high risk populations for downstream metabolic dysregulation during progressive insulin resistance.

Limitations of this study include the lack of protein expression data in the leukocytes, which would have shed clearer light on the functional effects of reduced PGC-1α expression and how those changes parallel with those seen in the analyzed metabolically active tissues. In order to paint a

full picture of the downstream effects of PGC-1α hypermethylation, it is of interest to examine how mitochondrial protein expression is impacted by an increase in methylation, or whether they are at all, therefore a more extensive examination of mitochondrial protein alteration is warranted. Further, given the fact that our study revealed acute changes in the methylation status of PGC-1α in leukocytes as early as day 3, it would be beneficial to have tissue samples analyzed to determine if these changes are mirrored at day 3. Examining even shorter exposures to a HFD (ex. hours) may help us to understand the plasticity of methylation from a very acute standpoint. Finally, due to limited tissue availability, some of the analyses included smaller sample sizes, lessening the statistical power of the correlation analyses, specifically in the gene expression studies.

3.6 Figures

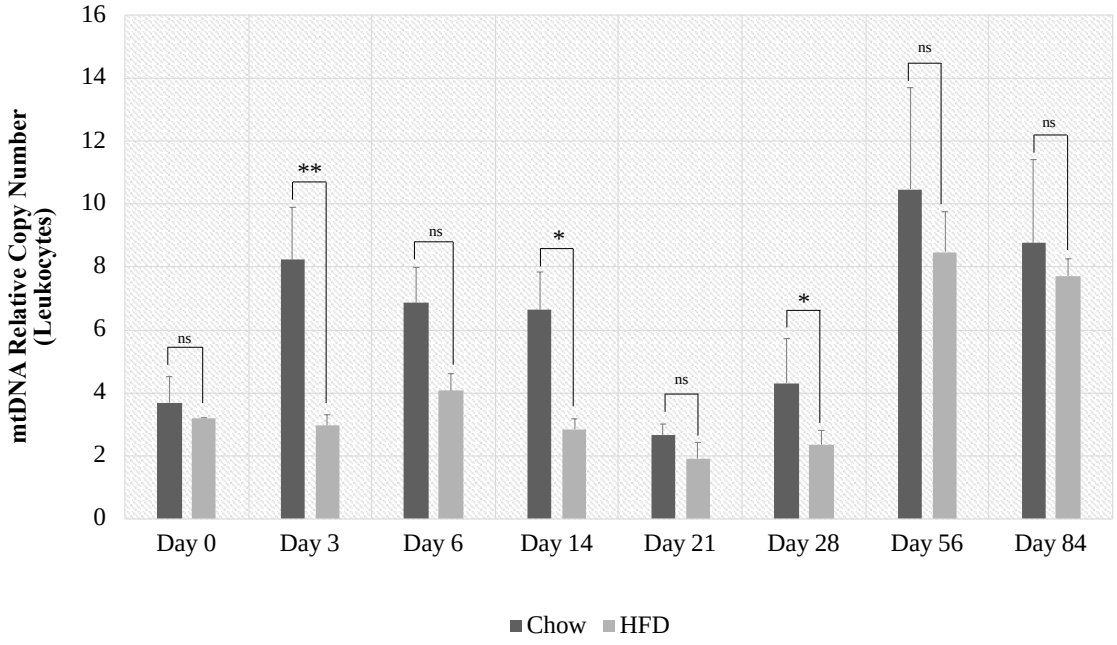
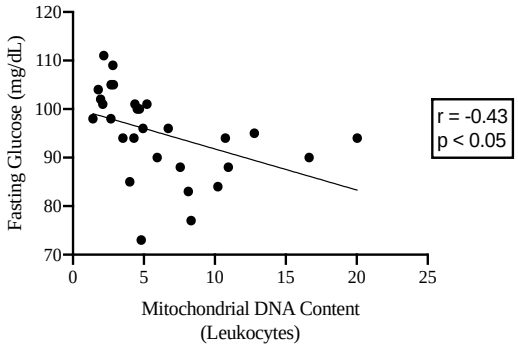


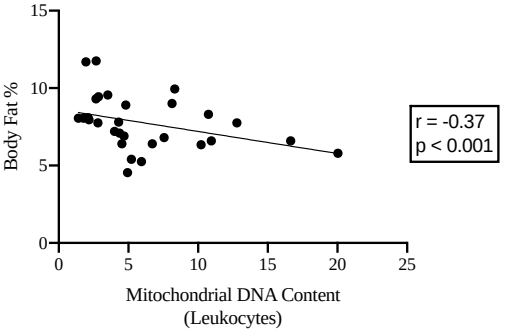
Figure 1. The changes in relative mitochondrial DNA copy number in C57BL/6J mice fed on control (chow) and high fat diet (HFD). The mice were 4-5 week old at week 0 (n=4-48).

* $p<0.05$; ** $p<0.01$.

a.)



b.)



c.)

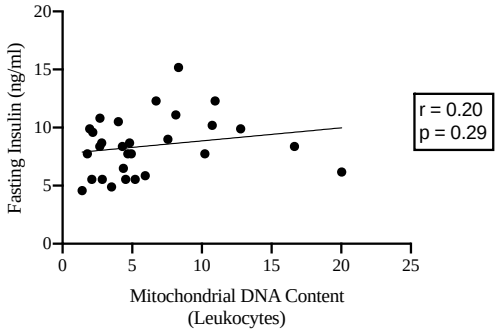
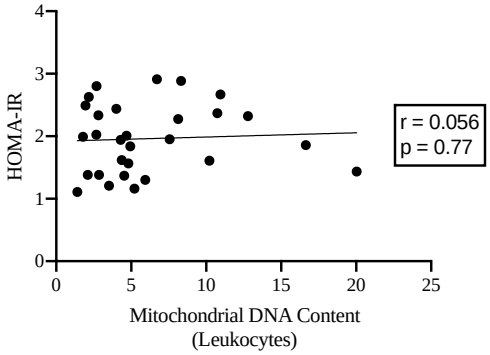
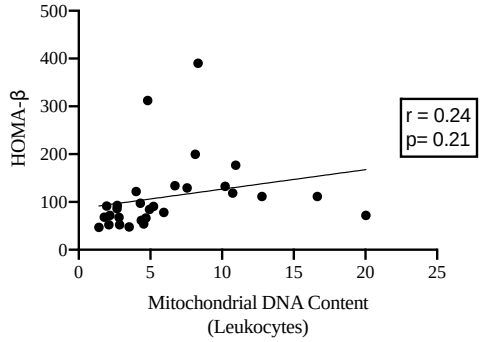


Figure 2 (a, b, & c). The association between relative leukocyte mitochondrial DNA copy number and markers of glucose dysregulation in C57BL/6J mice fed on control (chow) and high fat diet (HFD) on day 3. Fasting glucose and body fat % were significantly and negatively associated with mitochondrial DNA content (a,b). Fasting insulin was not significantly associated with mitochondrial DNA content (c) (n=29).

a.)



b.)



c.)

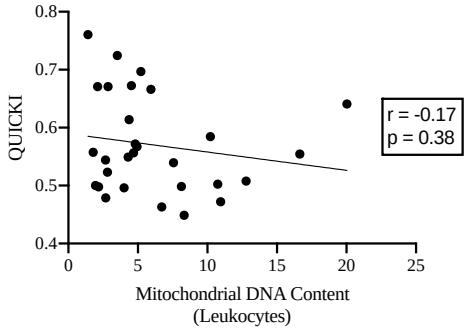
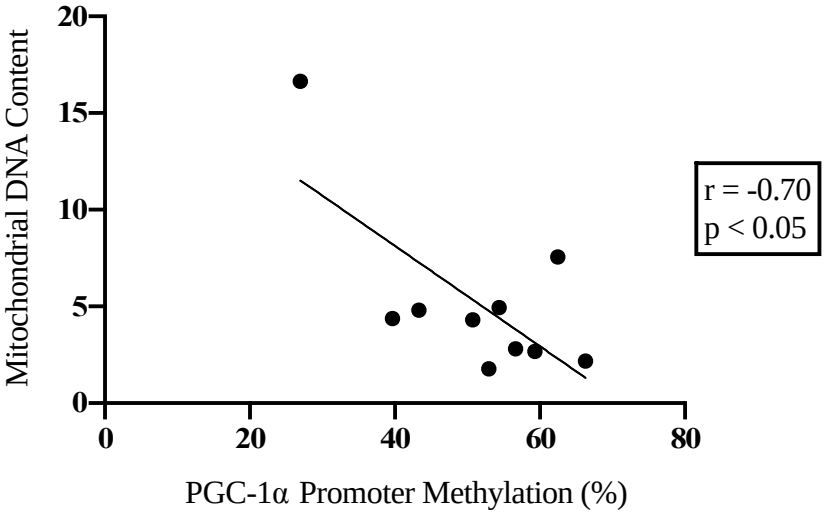


Figure 3 (a, b & c). The association between relative leukocyte mitochondrial DNA copy number and clinically utilized indices representing glucose dysregulation in C57BL/6J mice fed on control (chow) and high fat diet (HFD) on day 3. There was no significant association between HOMA-IR(a), HOMA-β(b), or QUICKI (c) measurements with mitochondrial DNA content (n=29).

a.)



b.)

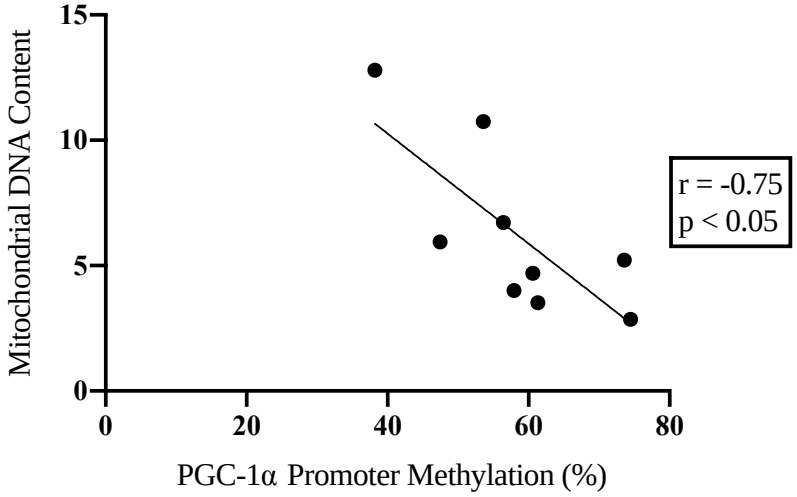


Figure 4 (a & b). The association between relative leukocyte mitochondrial DNA copy number and PGC-1α promoter methylation in C57BL/6J mice fed on control (chow) and high fat diet (HFD) on day 3 (a) and day 6 (b). There were significant negative associations between mitochondrial DNA copy number and PGC-1α promoter methylation at both time points (n=10).

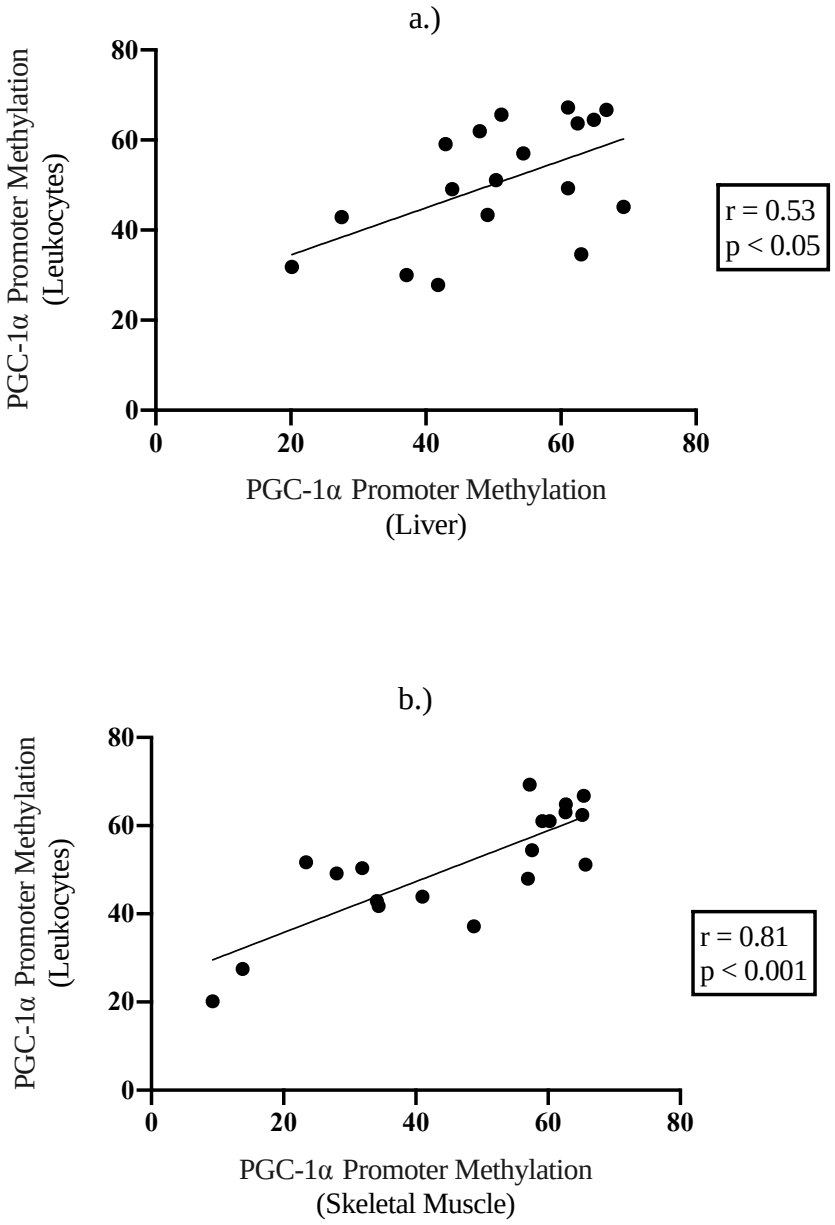
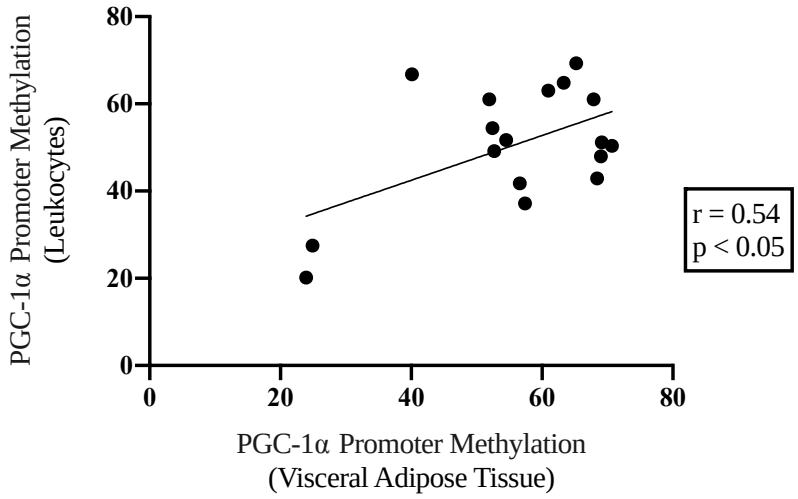


Figure 5 (a & b). The association between PGC-1α promoter methylation in leukocytes as compared with metabolically active tissues in C57BL/6J mice fed on control (chow) and high fat diet (HFD). There were significant positive associations between PGC-1α promoter methylation in leukocytes as compared with PGC-1α promoter methylation in the liver (a) and skeletal muscle (b) (n=18).

c.)



d.)

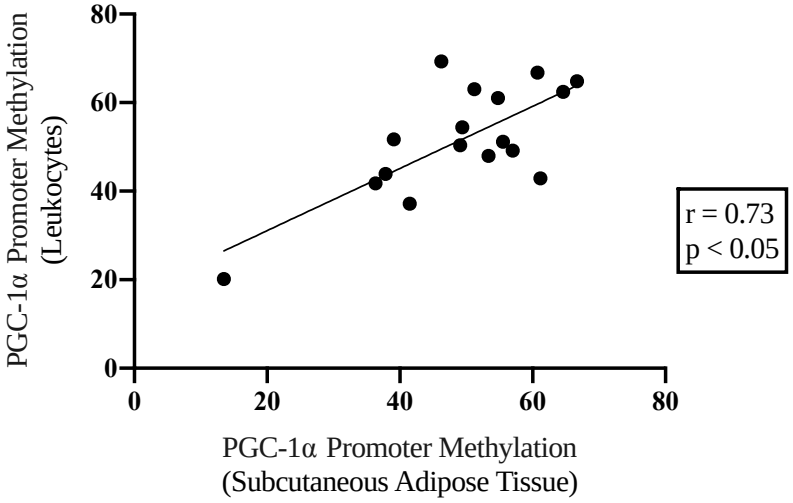
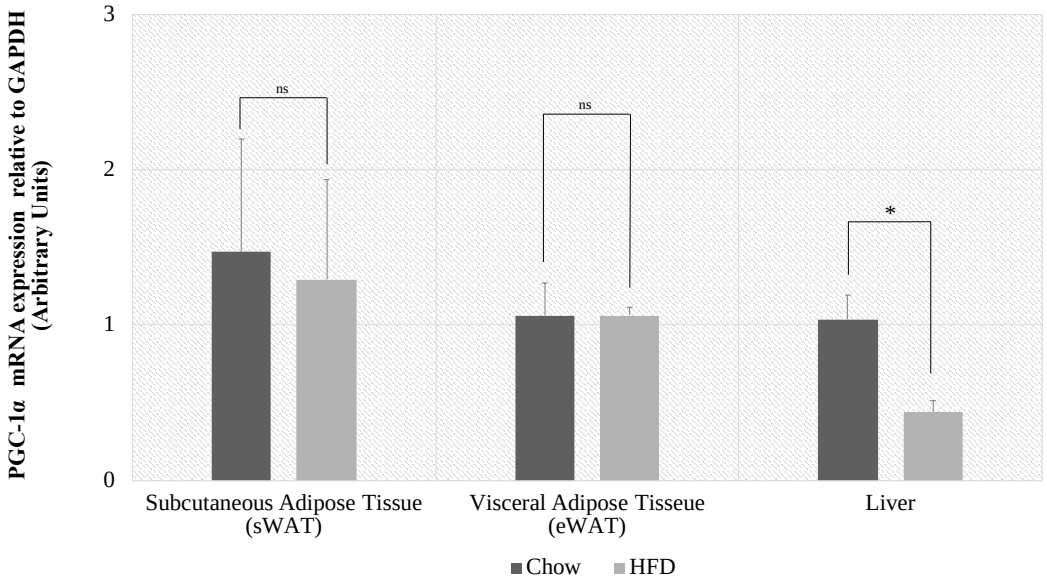


Figure 5 (c & d). The association between PGC-1α promoter methylation in leukocytes as compared with metabolically active tissues in C57BL/6J mice fed on control (chow) and high fat diet (HFD). There were significant positive associations between PGC-1α promoter methylation in leukocytes as compared with PGC-1α promoter methylation in the visceral adipose tissue (c) and subcutaneous adipose tissue (d)(n=17).

a.)



b.)

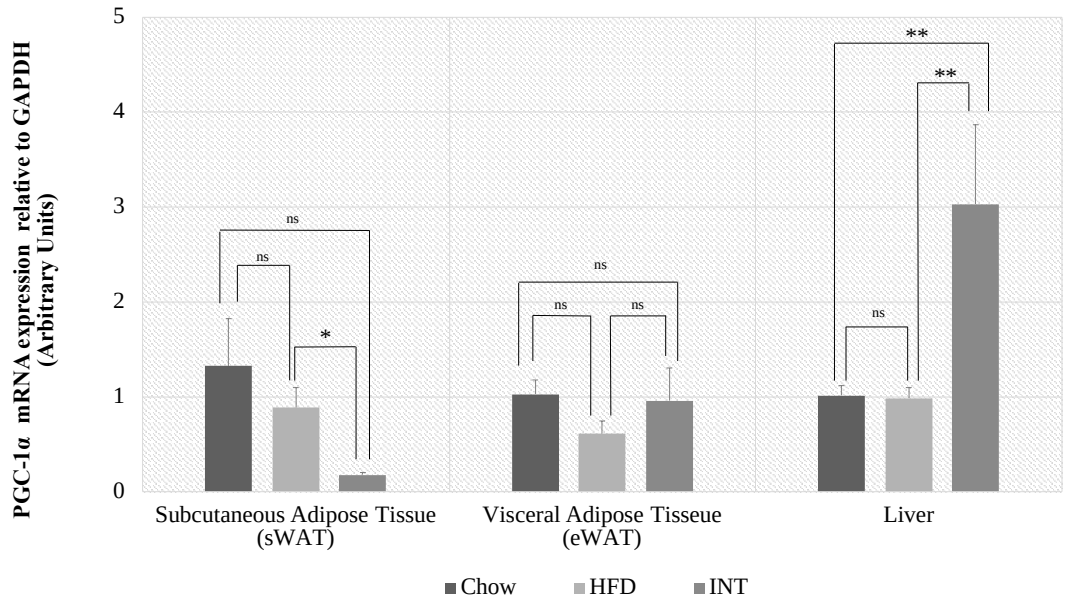
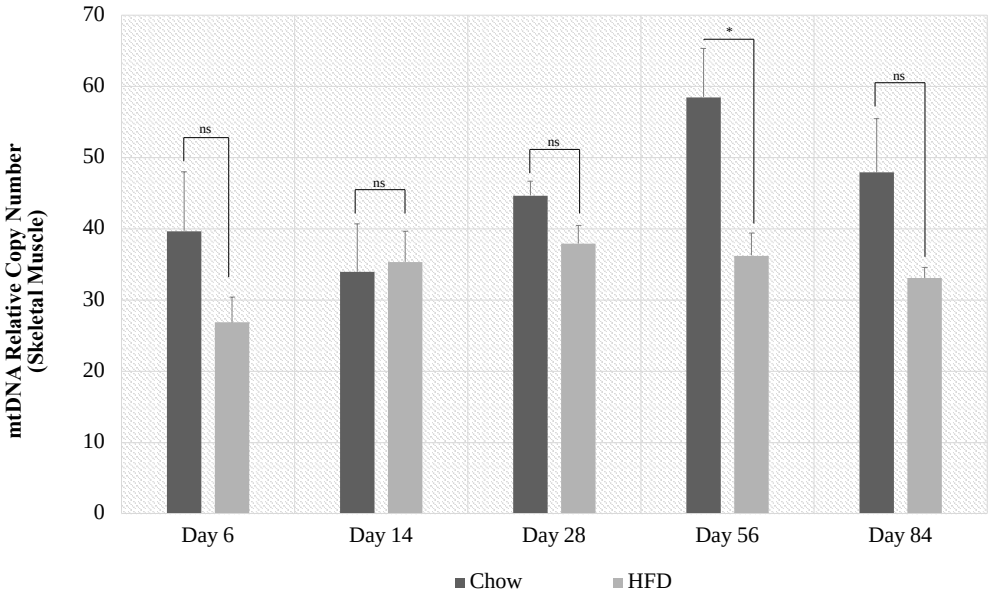


Figure 6 (a & b). The relative PGC-1α mRNA expression in C57BL/6J mice fed on control (chow) and high fat diet (HFD) at day 6 (a) and those fed on control (chow) and high fat diet (HFD) and the intervention group (INT), which consumed HFD for 2 months, followed by a 2 month chow diet. * $p<0.05$; ** $p<0.01$.

a.)



b.)

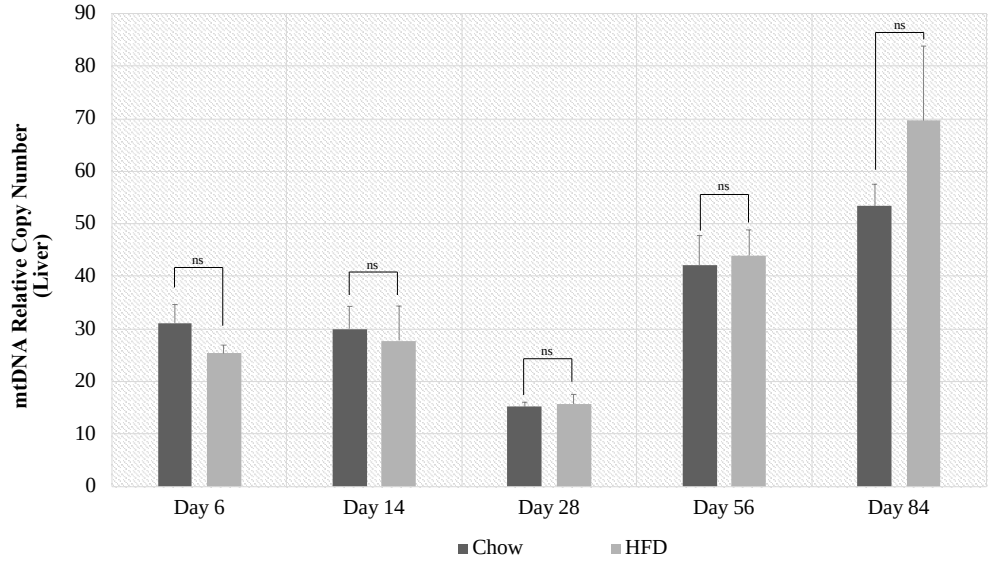
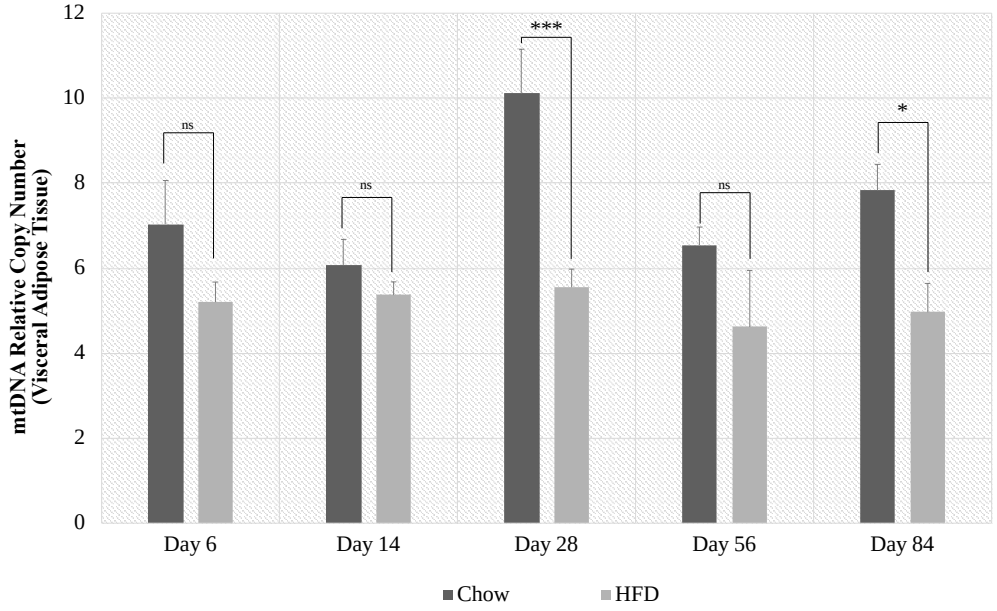


Figure 7 (a & b). The changes in relative mitochondrial DNA copy number in C57BL/6J mice fed on control (chow) and high fat diet (HFD) seen in skeletal muscle (a) and liver (b). The mice were 4-5 week old at week 0 (n=4-12). * $p<0.05$

c.)



d.)

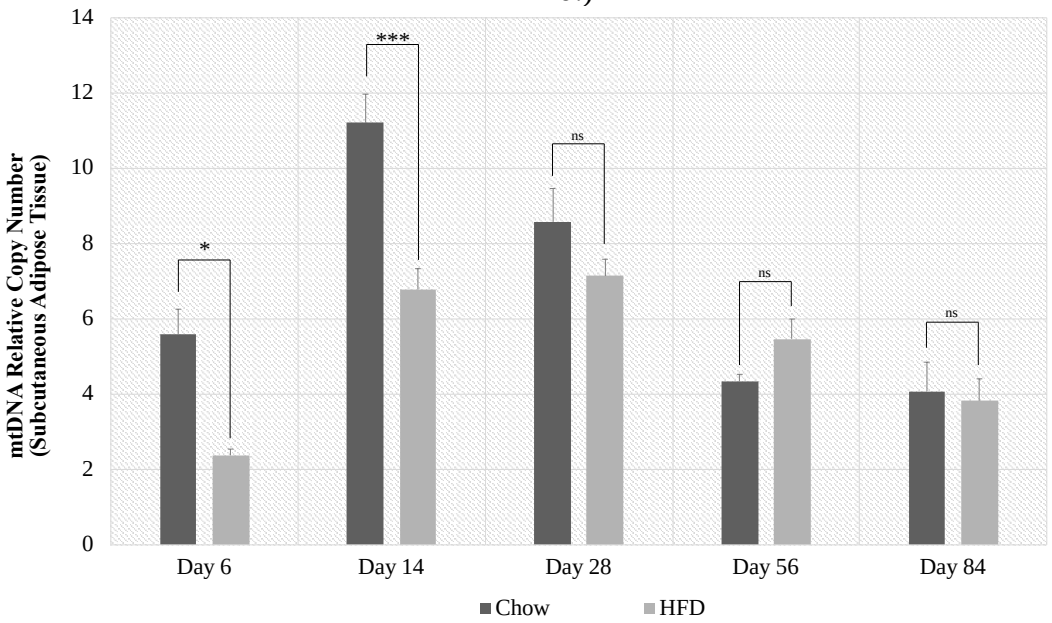


Figure 7 (c & d). The changes in relative mitochondrial DNA copy number in C57BL/6J mice fed on control (chow) and high fat diet (HFD) seen in visceral adipose tissue (c) and subcutaneous adipose tissue (d). * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

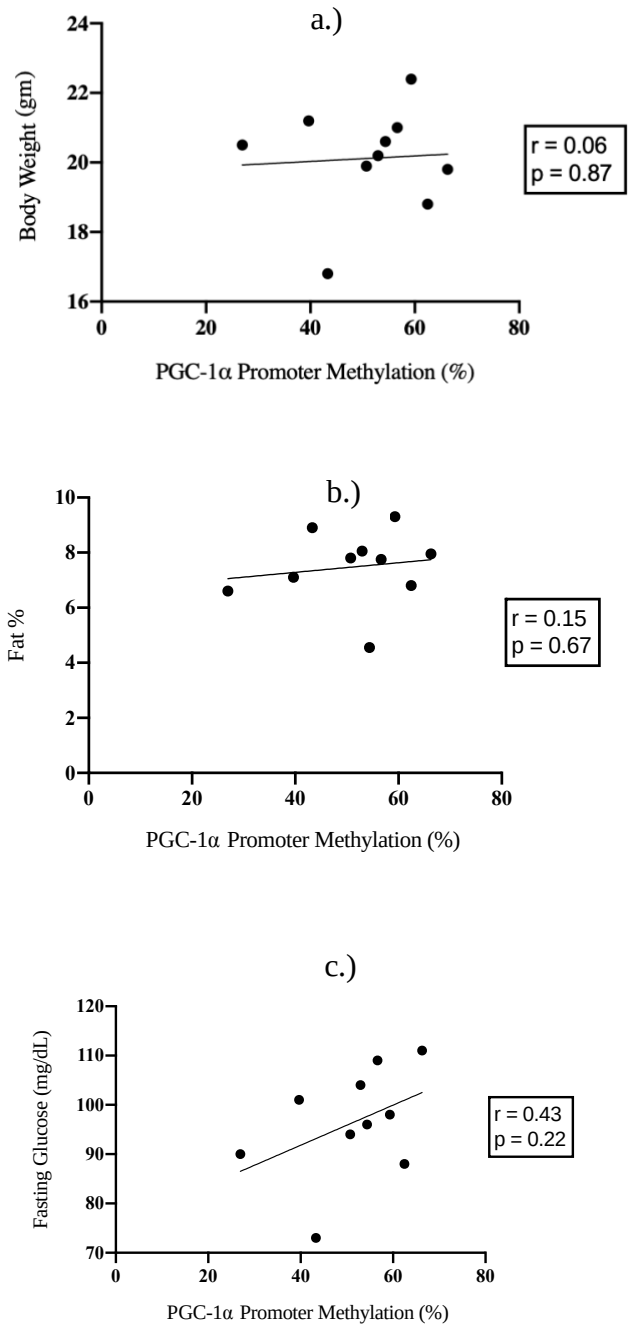
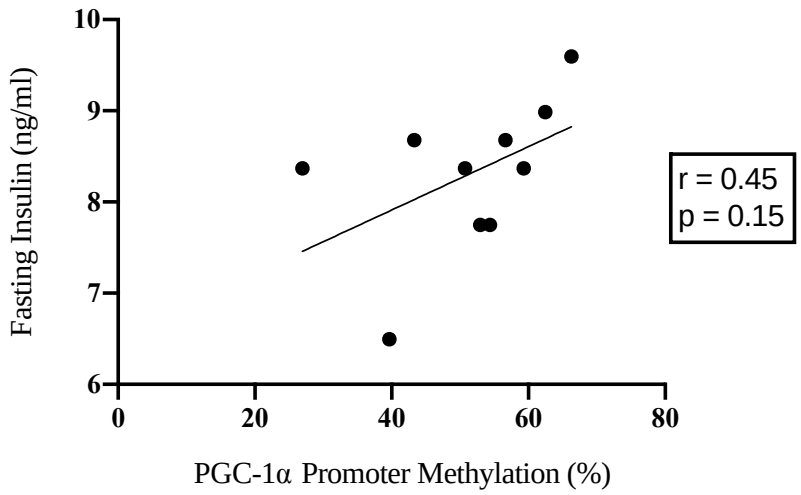


Figure 8 (a, b & c). The association between PGC-1α promoter methylation in leukocytes as compared with body weight (a), body fat % (b) and fasting glucose (c) in C57BL/6J mice following 3 days of a high fat diet (n=10).

d.)



e.)

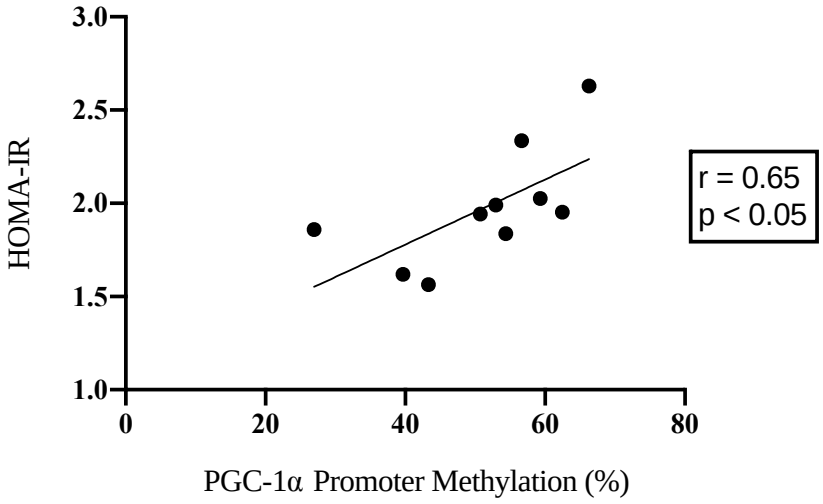
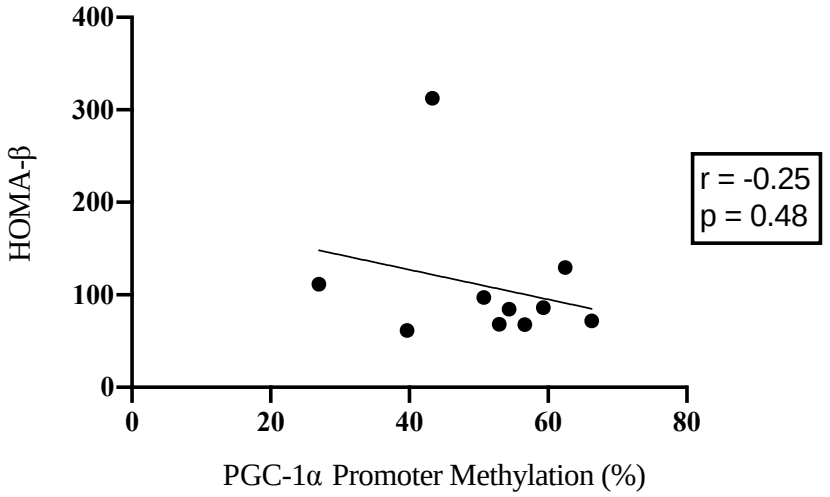


Figure 8 (d & e). The association between PGC-1α promoter methylation in leukocytes as compared with fasting insulin (d) and HOMA-IR (e) values in C57BL/6J mice following 3 days of a high fat diet (n=10).

f.)



g.)

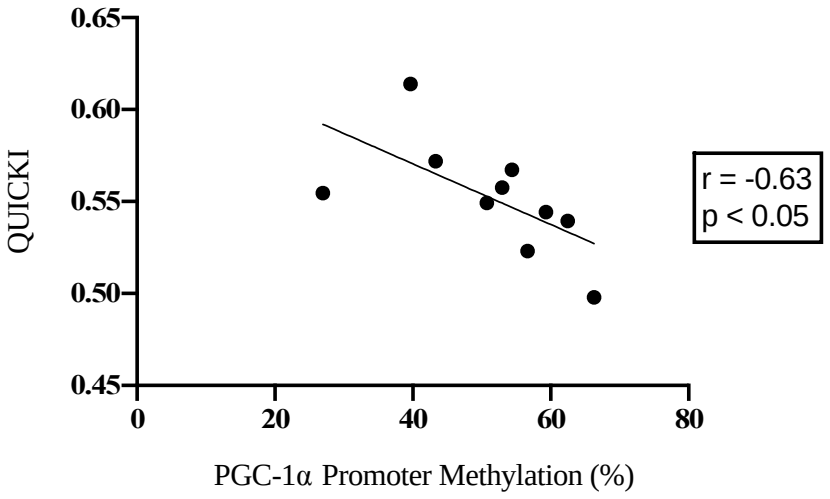


Figure 8 (f & g). The association between PGC-1α promoter methylation in leukocytes as compared with HOMA- β (d) and QUICKI (e) values in C57BL/6J mice following 3 days of a high fat diet (n=10).

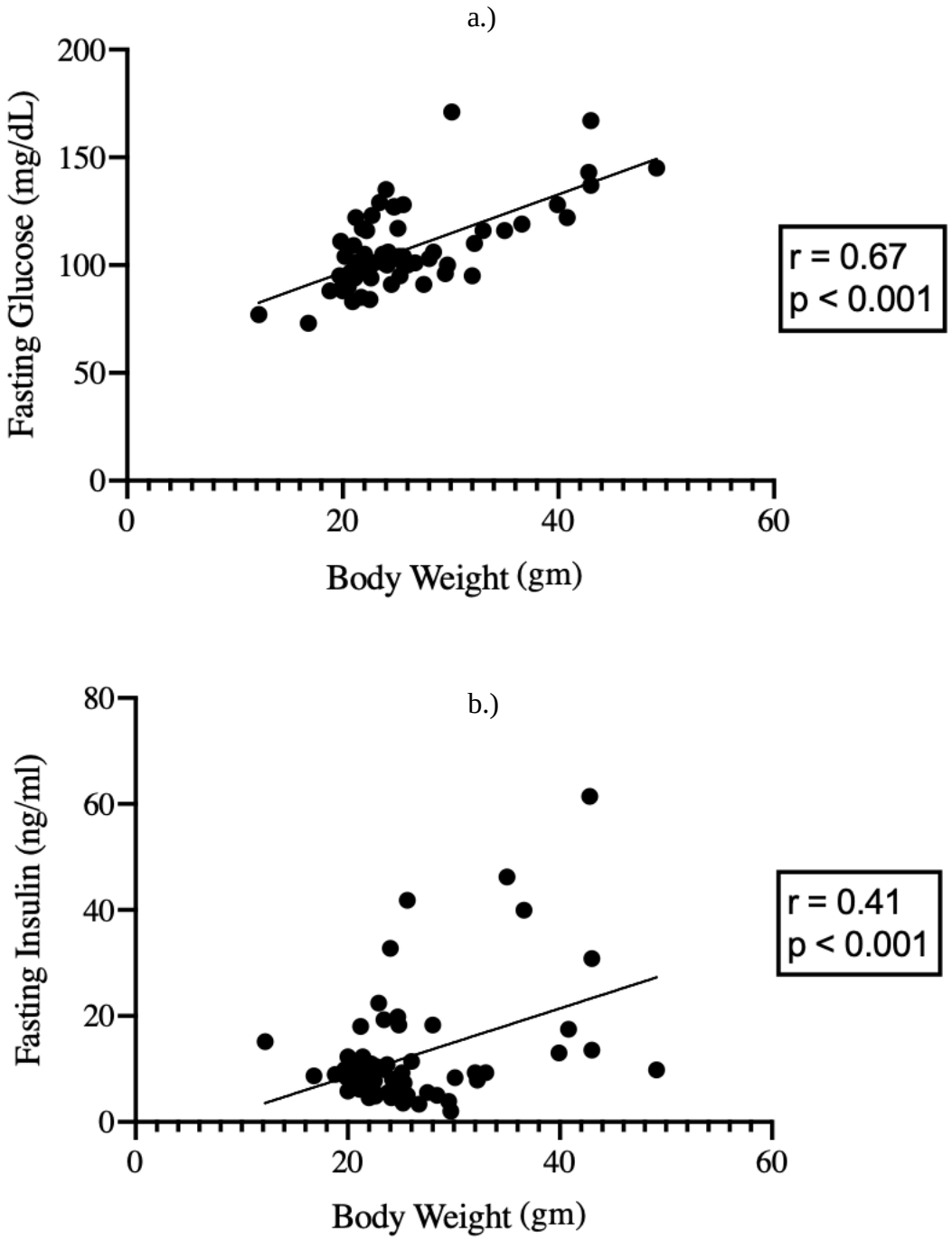
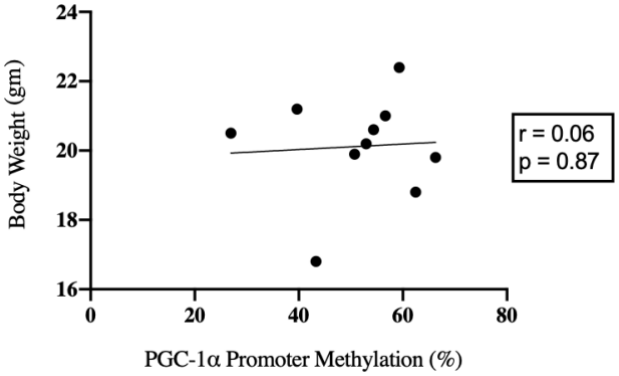
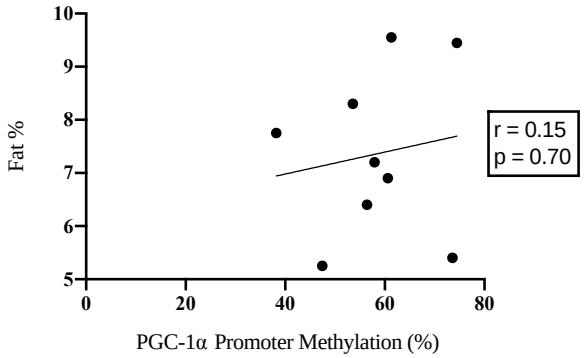


Figure 9 (a & b). The association between body weight and fasting glucose (a) and fasting insulin (b) in C57BL/6J mice following 6 days of a high fat diet (n=48).

a.)



b.)



c.)

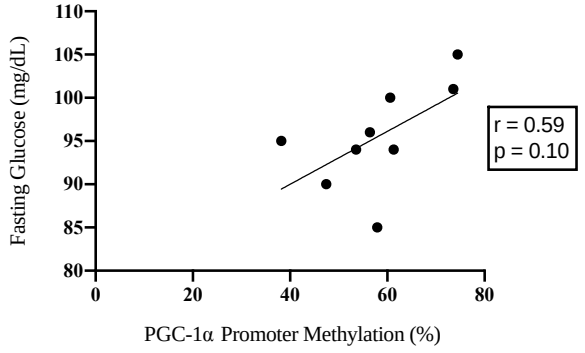
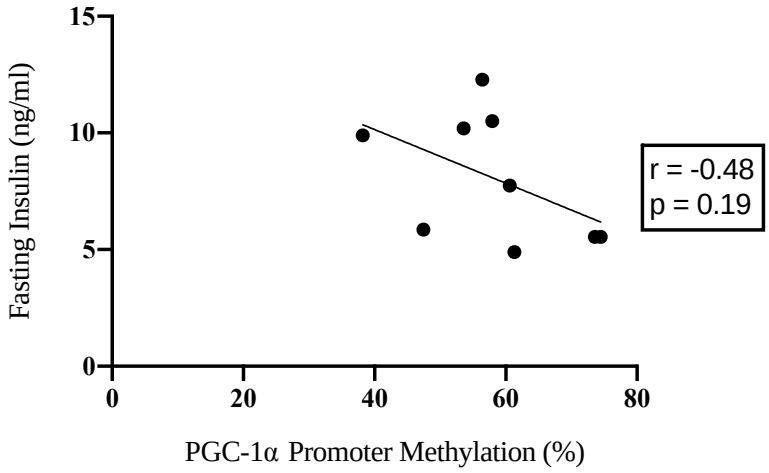


Figure 10 (a, b & c). The association between PGC-1α promoter methylation in leukocytes as compared with body weight (a), body fat % (b) and fasting glucose (c) in C57BL/6J mice following 6 days of a high fat diet (n=9).

d.)



e.)

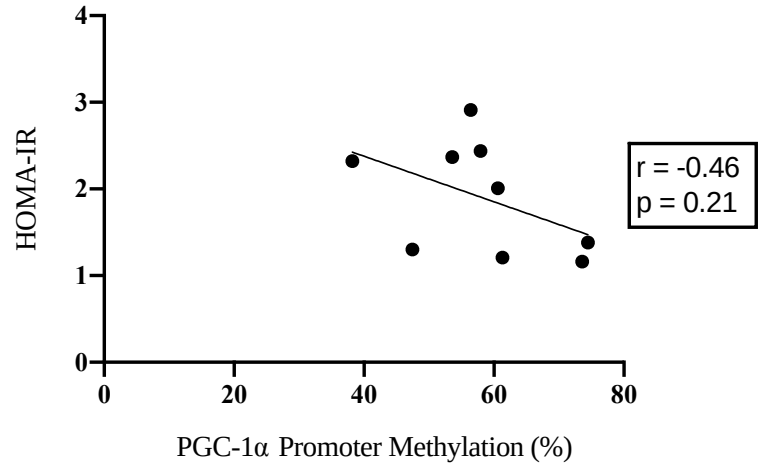
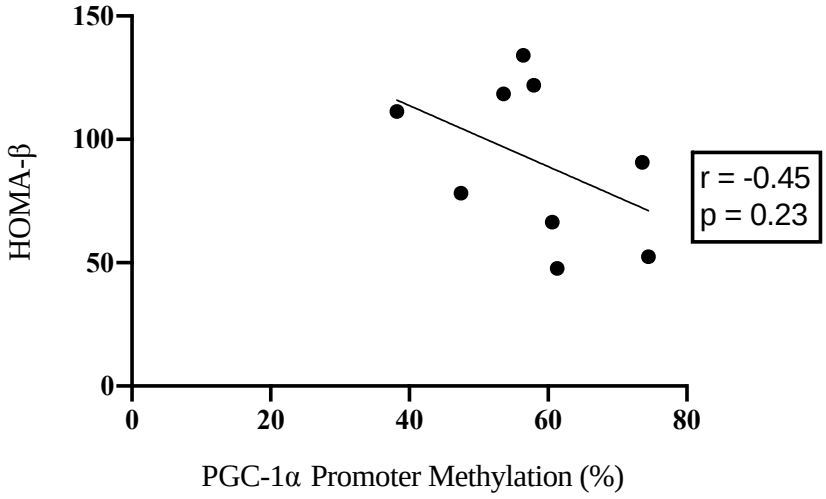


Figure 10 (d & e). The association between PGC-1α promoter methylation in leukocytes as compared with fasting insulin (d) and HOMA-IR (e) values in C57BL/6J mice following 6 days of a high fat diet (n=9).

f.)



g.)

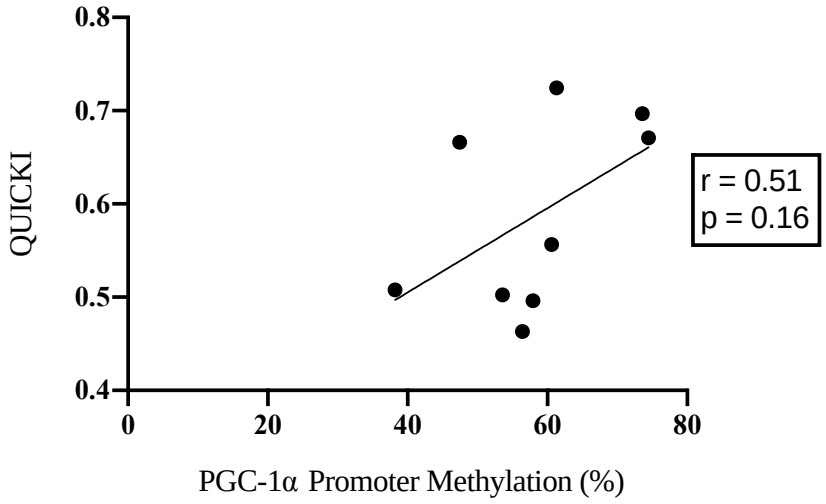


Figure 10 (f & g). The association between PGC-1α promoter methylation in leukocytes as compared with HOMA- β (d) and QUICKI (e) values in C57BL/6J mice following 6 days of a high fat diet (n=9).

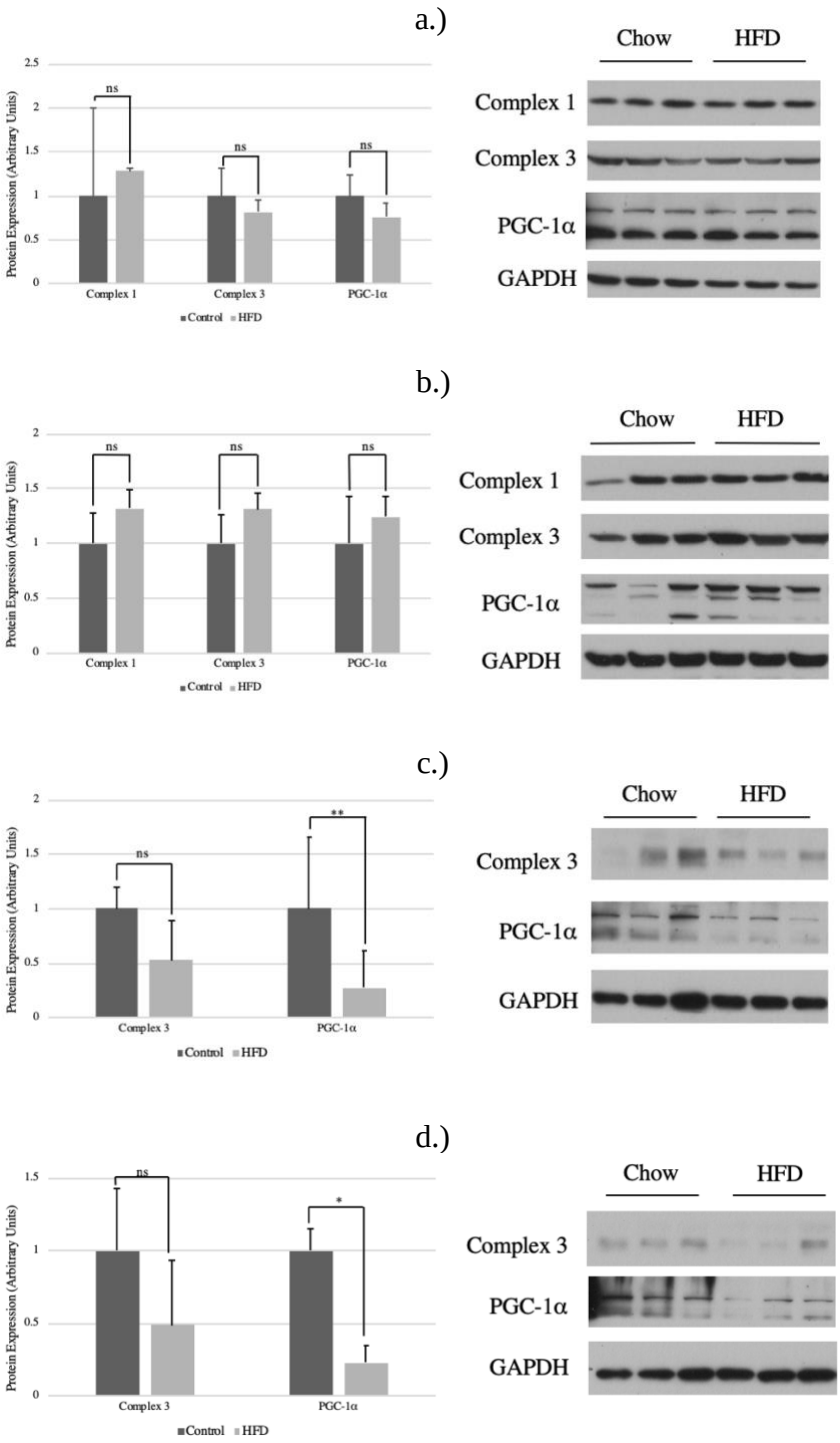


Figure 11 (a-d). Western blot analysis for protein expression of mitochondrial proteins (complex 1 and complex 3 and PGC-1α on day 6 in liver (a), skeletal muscle (b), visceral adipose tissue (c), and subcutaneous adipose tissue (d) (n=6). * $p<0.05$; ** $p<0.01$.

3.7 References

1. L. D. Zheng *et al.*, Mitochondrial Epigenetic Changes Link to Increased Diabetes Risk and Early-Stage Prediabetes Indicator. *Oxid Med Cell Longev* **2016**, 5290638 (2016).
2. L. D. Zheng *et al.*, Insulin resistance is associated with epigenetic and genetic regulation of mitochondrial DNA in obese humans. *Clin Epigenetics* **7**, 60 (2015).
3. Z. Cheng, F. A. Almeida, Mitochondrial alteration in type 2 diabetes and obesity: an epigenetic link. *Cell Cycle* **13**, 890-897 (2014).
4. Z. Cheng, E. M. Schmelz, D. Liu, M. W. Hulver, Targeting mitochondrial alterations to prevent type 2 diabetes--evidence from studies of dietary redox-active compounds. *Mol Nutr Food Res* **58**, 1739-1749 (2014).
5. Z. Cheng, Y. Tseng, M. F. White, Insulin signaling meets mitochondria in metabolism. *Trends Endocrinol Metab* **21**, 589-598 (2010).
6. Z. Cheng, L. Zheng, F. A. Almeida, Epigenetic reprogramming in metabolic disorders: nutritional factors and beyond. *J Nutr Biochem* **54**, 1-10 (2018).
7. A. D. Attie, C. M. Kendziorski, PGC-1alpha at the crossroads of type 2 diabetes. *Nat Genet* **34**, 244-245 (2003).
8. R. Clarke-Harris *et al.*, PGC1alpha promoter methylation in blood at 5-7 years predicts adiposity from 9 to 14 years (EarlyBird 50). *Diabetes* **63**, 2528-2537 (2014).
9. P. S. Eisele, R. Furrer, M. Beer, C. Handschin, The PGC-1 coactivators promote an anti-inflammatory environment in skeletal muscle in vivo. *Biochem Biophys Res Commun* **464**, 692-697 (2015).
10. S. H. Koo *et al.*, PGC-1 promotes insulin resistance in liver through PPAR-alpha-dependent induction of TRB-3. *Nat Med* **10**, 530-534 (2004).

11. V. K. Mootha *et al.*, PGC-1alpha-responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes. *Nat Genet* **34**, 267-273 (2003).

12. R. Ventura-Clapier, A. Garnier, V. Veksler, Transcriptional control of mitochondrial biogenesis: the central role of PGC-1alpha. *Cardiovasc Res* **79**, 208-217 (2008).

13. B. N. Finck, D. P. Kelly, Peroxisome proliferator-activated receptor gamma coactivator-1 (PGC-1) regulatory cascade in cardiac physiology and disease. *Circulation* **115**, 2540-2548 (2007).

14. D. P. Kelly, R. C. Scarpulla, Transcriptional regulatory circuits controlling mitochondrial biogenesis and function. *Genes Dev* **18**, 357-368 (2004).

15. R. Garesse, C. G. Vallejo, Animal mitochondrial biogenesis and function: a regulatory cross-talk between two genomes. *Gene* **263**, 1-16 (2001).

16. M. T. Ryan, N. J. Hoogenraad, Mitochondrial-nuclear communications. *Annu Rev Biochem* **76**, 701-722 (2007).

17. B. B. Lowell, G. I. Shulman, Mitochondrial dysfunction and type 2 diabetes. *Science* **307**, 384-387 (2005).

18. P. H. Albers *et al.*, Human muscle fiber type-specific insulin signaling: impact of obesity and type 2 diabetes. *Diabetes* **64**, 485-497 (2015).

19. C. A. Stuart *et al.*, Slow-twitch fiber proportion in skeletal muscle correlates with insulin responsiveness. *J Clin Endocrinol Metab* **98**, 2027-2036 (2013).

20. A. Oberbach *et al.*, Altered fiber distribution and fiber-specific glycolytic and oxidative enzyme activity in skeletal muscle of patients with type 2 diabetes. *Diabetes Care* **29**, 895-900 (2006).

21. J. R. Zierath *et al.*, Insulin action on glucose transport and plasma membrane GLUT4 content in skeletal muscle from patients with NIDDM. *Diabetologia* **39**, 1180-1189 (1996).

22. P. Marin, B. Andersson, M. Krotkiewski, P. Bjorntorp, Muscle fiber composition and capillary density in women and men with NIDDM. *Diabetes Care* **17**, 382-386 (1994).

23. J. He, S. Watkins, D. E. Kelley, Skeletal muscle lipid content and oxidative enzyme activity in relation to muscle fiber type in type 2 diabetes and obesity. *Diabetes* **50**, 817-823 (2001).

24. J. M. Kristensen *et al.*, A PGC-1alpha- and muscle fibre type-related decrease in markers of mitochondrial oxidative metabolism in skeletal muscle of humans with inherited insulin resistance. *Diabetologia* **57**, 1006-1015 (2014).

25. P. Maechler, Mitochondrial function and insulin secretion. *Mol Cell Endocrinol* **379**, 12-18 (2013).

26. P. Maechler, C. B. Wollheim, Mitochondrial function in normal and diabetic beta-cells. *Nature* **414**, 807-812 (2001).

27. F. X. Xu, X. Zhou, F. Shen, R. Pang, S. M. Liu, Decreased peripheral blood mitochondrial DNA content is related to HbA1c, fasting plasma glucose level and age of onset in type 2 diabetes mellitus. *Diabet Med* **29**, e47-54 (2012).

28. F. J. Miller, F. L. Rosenfeldt, C. Zhang, A. W. Linnane, P. Nagley, Precise determination of mitochondrial DNA copy number in human skeletal and cardiac muscle by a PCR-based assay: lack of change of copy number with age. *Nucleic Acids Res* **31**, e61 (2003).

29. Y. M. Feng *et al.*, Decreased mitochondrial DNA copy number in the hippocampus and peripheral blood during opiate addiction is mediated by autophagy and can be salvaged by melatonin. *Autophagy* **9**, 1395-1406 (2013).

30. C. J. Pirola *et al.*, Epigenetic modification of liver mitochondrial DNA is associated with histological severity of nonalcoholic fatty liver disease. *Gut* **62**, 1356-1363 (2013).

31. I. J. Bujalska, S. Kumar, P. M. Stewart, Does central obesity reflect "Cushing's disease of the omentum"? *Lancet* **349**, 1210-1213 (1997).

32. E. Rask *et al.*, Tissue-specific changes in peripheral cortisol metabolism in obese women: increased adipose 11beta-hydroxysteroid dehydrogenase type 1 activity. *J Clin Endocrinol Metab* **87**, 3330-3336 (2002).

33. M. A. Crook, P. Tutt, J. C. Pickup, Elevated serum sialic acid concentration in NIDDM and its relationship to blood pressure and retinopathy. *Diabetes Care* **16**, 57-60 (1993).

34. J. C. Pickup, M. A. Crook, Is type II diabetes mellitus a disease of the innate immune system? *Diabetologia* **41**, 1241-1248 (1998).

35. A. Festa, R. D'Agostino, Jr., R. P. Tracy, S. M. Haffner, S. Insulin Resistance Atherosclerosis, Elevated levels of acute-phase proteins and plasminogen activator inhibitor-1 predict the development of type 2 diabetes: the insulin resistance atherosclerosis study. *Diabetes* **51**, 1131-1137 (2002).

36. M. W. Mansfield, D. M. Heywood, P. J. Grant, Circulating levels of factor VII, fibrinogen, and von Willebrand factor and features of insulin resistance in first-degree relatives of patients with NIDDM. *Circulation* **94**, 2171-2176 (1996).

37. J. Nilsson, S. Jovinge, A. Niemann, R. Reneland, H. Lithell, Relation between plasma tumor necrosis factor-alpha and insulin sensitivity in elderly men with non-insulin-dependent diabetes mellitus. *Arterioscler Thromb Vasc Biol* **18**, 1199-1202 (1998).

38. E. S. Ford, M. E. Cogswell, Diabetes and serum ferritin concentration among U.S. adults. *Diabetes Care* **22**, 1978-1983 (1999).

39. A. J. Hanley *et al.*, Factor analysis of metabolic syndrome using directly measured insulin sensitivity: The Insulin Resistance Atherosclerosis Study. *Diabetes* **51**, 2642-2647 (2002).

40. J. M. Chan, E. B. Rimm, G. A. Colditz, M. J. Stampfer, W. C. Willett, Obesity, fat distribution, and weight gain as risk factors for clinical diabetes in men. *Diabetes Care* **17**, 961-969 (1994).

41. V. J. Carey *et al.*, Body fat distribution and risk of non-insulin-dependent diabetes mellitus in women. The Nurses' Health Study. *Am J Epidemiol* **145**, 614-619 (1997).

42. S. B. Votruba, M. D. Jensen, Insulin sensitivity and regional fat gain in response to overfeeding. *Obesity (Silver Spring)* **19**, 269-275 (2011).

43. B. H. Goodpaster, D. E. Kelley, R. R. Wing, A. Meier, F. L. Thaete, Effects of weight loss on regional fat distribution and insulin sensitivity in obesity. *Diabetes* **48**, 839-847 (1999).

44. B. H. Goodpaster, F. L. Thaete, J. A. Simoneau, D. E. Kelley, Subcutaneous abdominal fat and thigh muscle composition predict insulin sensitivity independently of visceral fat. *Diabetes* **46**, 1579-1585 (1997).

45. B. Tousignant *et al.*, Body fat distribution modulates insulin sensitivity in post-menopausal overweight and obese women: a MONET study. *Int J Obes (Lond)* **32**, 1626-1632 (2008).

46. M. J. Muller *et al.*, Beyond the body mass index: tracking body composition in the pathogenesis of obesity and the metabolic syndrome. *Obes Rev* **13 Suppl 2**, 6-13 (2012).

47. H. Yaghootkar *et al.*, Genetic Evidence for a Link Between Favorable Adiposity and Lower Risk of Type 2 Diabetes, Hypertension, and Heart Disease. *Diabetes* **65**, 2448-2460 (2016).

48. L. A. Lotta *et al.*, Integrative genomic analysis implicates limited peripheral adipose storage capacity in the pathogenesis of human insulin resistance. *Nat Genet* **49**, 17-26 (2017).

49. H. S. Han, G. Kang, J. S. Kim, B. H. Choi, S. H. Koo, Regulation of glucose metabolism from a liver-centric perspective. *Exp Mol Med* **48**, e218 (2016).

50. M. Konig, S. Bulik, H. G. Holzhutter, Quantifying the contribution of the liver to glucose homeostasis: a detailed kinetic model of human hepatic glucose metabolism. *PLoS Comput Biol* **8**, e1002577 (2012).

51. R. S. Sherwin, Role of the liver in glucose homeostasis. *Diabetes Care* **3**, 261-265 (1980).

52. K. Aoki *et al.*, Role of the liver in glucose homeostasis in PI 3-kinase p85alpha-deficient mice. *Am J Physiol Endocrinol Metab* **296**, E842-853 (2009).

53. E. M. Morris *et al.*, PGC-1alpha overexpression results in increased hepatic fatty acid oxidation with reduced triacylglycerol accumulation and secretion. *Am J Physiol Gastrointest Liver Physiol* **303**, G979-992 (2012).

54. Z. Cheng, M. Ristow, Mitochondria and metabolic homeostasis. *Antioxid Redox Signal* **19**, 240-242 (2013).

55. M. E. Patti *et al.*, Coordinated reduction of genes of oxidative metabolism in humans with insulin resistance and diabetes: Potential role of PGC1 and NRF1. *Proc Natl Acad Sci U S A* **100**, 8466-8471 (2003).

56. M. E. Patti, S. Corvera, The role of mitochondria in the pathogenesis of type 2 diabetes. *Endocr Rev* **31**, 364-395 (2010).

57. J. Szendroedi, E. Phielix, M. Roden, The role of mitochondria in insulin resistance and type 2 diabetes mellitus. *Nat Rev Endocrinol* **8**, 92-103 (2011).

58. E. Cersosimo, C. Triplitt, C. Solis-Herrera, L. J. Mandarino, R. A. DeFronzo, in *Endotext,* K. R. Feingold *et al.*, Eds. (South Dartmouth (MA), 2000).

59. R. A. DeFronzo, Pathogenesis of type 2 diabetes mellitus. *Med Clin North Am* **88**, 787-835, ix (2004).

60. I. Pagel-Langenickel, J. Bao, L. Pang, M. N. Sack, The role of mitochondria in the pathophysiology of skeletal muscle insulin resistance. *Endocr Rev* **31**, 25-51 (2010).

61. B. Halliwell, Oxidative stress, nutrition and health. Experimental strategies for optimization of nutritional antioxidant intake in humans. *Free Radic Res* **25**, 57-74 (1996).

62. S. D. Hursting *et al.*, Reducing the weight of cancer: mechanistic targets for breaking the obesity-carcinogenesis link. *Best Pract Res Clin Endocrinol Metab* **22**, 659-669 (2008).

63. A. Martin-Montalvo, R. de Cabo, Mitochondrial metabolic reprogramming induced by calorie restriction. *Antioxid Redox Signal* **19**, 310-320 (2013).

64. C. M. Taniguchi, B. Emanuelli, C. R. Kahn, Critical nodes in signalling pathways: insights into insulin action. *Nat Rev Mol Cell Biol* **7**, 85-96 (2006).

65. D. Thiebaud *et al.*, The effect of graded doses of insulin on total glucose uptake, glucose oxidation, and glucose storage in man. *Diabetes* **31**, 957-963 (1982).

66. P. D. Home, G. Pacini, Hepatic dysfunction and insulin insensitivity in type 2 diabetes mellitus: a critical target for insulin-sensitizing agents. *Diabetes Obes Metab* **10**, 699-718 (2008).

67. K. D. Copps, M. F. White, Regulation of insulin sensitivity by serine/threonine phosphorylation of insulin receptor substrate proteins IRS1 and IRS2. *Diabetologia* **55**, 2565-2582 (2012).

68. I. Mothe, E. Van Obberghen, Phosphorylation of insulin receptor substrate-1 on multiple serine residues, 612, 632, 662, and 731, modulates insulin action. *J Biol Chem* **271**, 11222-11227 (1996).

69. K. Paz *et al.*, A molecular basis for insulin resistance. Elevated serine/threonine phosphorylation of IRS-1 and IRS-2 inhibits their binding to the juxtamembrane region of the insulin receptor and impairs their ability to undergo insulin-induced tyrosine phosphorylation. *J Biol Chem* **272**, 29911-29918 (1997).

70. T. Dayeh *et al.*, Genome-wide DNA methylation analysis of human pancreatic islets from type 2 diabetic and non-diabetic donors identifies candidate genes that influence insulin secretion. *PLoS Genet* **10**, e1004160 (2014).

71. M. D. Nitert *et al.*, Impact of an exercise intervention on DNA methylation in skeletal muscle from first-degree relatives of patients with type 2 diabetes. *Diabetes* **61**, 3322-3332 (2012).

72. K. J. Dick *et al.*, DNA methylation and body-mass index: a genome-wide analysis. *Lancet* **383**, 1990-1998 (2014).

73. C. Ling *et al.*, Genetic and epigenetic factors are associated with expression of respiratory chain component NDUFB6 in human skeletal muscle. *J Clin Invest* **117**, 3427-3435 (2007).

74. T. Ronn *et al.*, Age influences DNA methylation and gene expression of COX7A1 in human skeletal muscle. *Diabetologia* **51**, 1159-1168 (2008).

75. T. Ronn *et al.*, Impact of age, BMI and HbA1c levels on the genome-wide DNA methylation and mRNA expression patterns in human adipose tissue and identification of epigenetic biomarkers in blood. *Hum Mol Genet* **24**, 3792-3813 (2015).

Chapter 4: Conclusion and Future Directions

4.1 Conclusion

T2DM is increasing in pervasiveness worldwide, with nearly 422 million affected in 2014, as compared to 108 million in 1980(*1*). Even more concerning, pre-diabetes is on the rise in the United States and around the world, signaling an impending inflation in future cases of clinically diagnosable T2DM(*2*). Numerous previous studies have indicated the importance of behavioral interventions in ameliorating insulin resistance, especially in its earliest stages of onset(*3-11*). The utilization of epigenetic markers as biosignatures for disease status and disease risk is expanding in prevalence in the scientific and medical communities due to the plethora of novel techniques to identify molecular alterations at the level of the genome. As such, DNA methylation has been examined in disease states to identify it's potential use in detection, diagnosis, prognosis, and monitoring of disease status(*12-19*).

Defects in mitochondrial function are a hallmark feature of metabolic disease(*20-25*). In the present work, we examined the DNA methylation profile of the promoter region of PGC-1α, a gene considered to be a master regulator of mitochondrial biogenesis. We identified a marked and significant increase in PGC-1α promoter methylation which coincided with a significant reduction in mitochondrial DNA content following acute HFD consumption, indicating a potential mechanism whereby nutrient surplus impedes homeostatic mitochondrial biogenesis, thereby leading to impairments in oxidative phosphorylation, lipid handling, and insulin sensitivity.

Future studies should aim to define the mechanism whereby hypermethylation and subsequent downregulation of PGC-1α expression leads to insulin resistance. As mitochondrial function, biogenesis, and dynamics have been shown to strongly correlate with T2DM, functional analysis of oxidative phosphorylation detriments will be needed in the future. It will also be of interest to differences in age, gender, and physiological alterations and how these factors contribute to the severity of hypermethylation and insulin resistance. As the SAM cycle is so closely tied to the mechanism of methylation, dietary nutrient manipulation will be of interest to further elucidate the mechanism whereby PGC-1α becomes hypermethylated during the time course of disease progression.

In summary, the data presented in this study reveal a potential early-stage marker of progressing insulin resistance following the initiation of a high fat diet. Future studies are warranted to confirm the functional relevance of the methylation alterations seen at the promoter region of PGC-1α, and to examine how these changes manifest into phenotypical modifications. Importantly, the present work supports ongoing work examining the fluidity of DNA methylation as a molecular response to external stimuli, adding substantiation to the theory that such markers may be responsible for the metabolic dysregulation commonly associated with behavioral traits such as excess nutrient intake and minimal physical activity.

4.2 References

1. Y. Zheng, S. H. Ley, F. B. Hu, Global aetiology and epidemiology of type 2 diabetes mellitus and its complications. *Nat Rev Endocrinol* **14**, 88-98 (2018).
2. K. I. Galaviz *et al.*, Global Diabetes Prevention Interventions: A Systematic Review and Network Meta-analysis of the Real-World Impact on Incidence, Weight, and Glucose. *Diabetes Care* **41**, 1526-1534 (2018).
3. T. L. Gary, J. M. Genkinger, E. Guallar, M. Peyrot, F. L. Brancati, Meta-analysis of randomized educational and behavioral interventions in type 2 diabetes. *Diabetes Educ* **29**, 488-501 (2003).
4. P. Gaede *et al.*, Multifactorial intervention and cardiovascular disease in patients with type 2 diabetes. *N Engl J Med* **348**, 383-393 (2003).
5. K. A. Cradock *et al.*, Diet Behavior Change Techniques in Type 2 Diabetes: A Systematic Review and Meta-analysis. *Diabetes Care* **40**, 1800-1810 (2017).
6. J. Pillay *et al.*, Behavioral Programs for Type 2 Diabetes Mellitus: A Systematic Review and Network Meta-analysis. *Ann Intern Med* **163**, 848-860 (2015).
7. V. Silfee, R. Petosa, D. Laurent, T. Schaub, B. Focht, Effect of a behavioral intervention on dimensions of self-regulation and physical activity among overweight and obese adults with type 2 diabetes: a pilot study. *Psychol Health Med* **21**, 715-723 (2016).
8. A. Mottalib, M. Sakr, M. Shehabeldin, O. Hamdy, Diabetes Remission after Nonsurgical Intensive Lifestyle Intervention in Obese Patients with Type 2 Diabetes. *J Diabetes Res* **2015**, 468704 (2015).
9. J. W. Stevens, R. C. Harvey, M. Johnson, K. Khunti, Preventing the Progression to Type 2 Diabetes Mellitus in Adults at High Risk: A Systematic Review And Network Meta-

Analysis of Lifestyle, Pharmacological and Surgical Interventions. *Value Health* **17**, A335 (2014).

10. B. V. Reamy, Preventing the progression of diabetes mellitus. *Am Fam Physician* **71**, 425 (2005).
11. N. R. den Braver *et al.*, Determinants of lifestyle behavior change to prevent type 2 diabetes in high-risk individuals. *Int J Behav Nutr Phys Act* **14**, 78 (2017).
12. P. Costa-Pinheiro, D. Montezuma, R. Henrique, C. Jeronimo, Diagnostic and prognostic epigenetic biomarkers in cancer. *Epigenomics* **7**, 1003-1015 (2015).
13. K. Otani, X. Li, T. Arakawa, F. K. Chan, J. Yu, Epigenetic-mediated tumor suppressor genes as diagnostic or prognostic biomarkers in gastric cancer. *Expert Rev Mol Diagn* **13**, 445-455 (2013).
14. D. Deng, Z. Liu, Y. Du, Epigenetic alterations as cancer diagnostic, prognostic, and predictive biomarkers. *Adv Genet* **71**, 125-176 (2010).
15. M. P. Boks *et al.*, The relationship of DNA methylation with age, gender and genotype in twins and healthy controls. *PLoS One* **4**, e6767 (2009).
16. K. Yamashita, K. Hosoda, N. Nishizawa, H. Katoh, M. Watanabe, Epigenetic biomarkers of promoter DNA methylation in the new era of cancer treatment. *Cancer Sci* **109**, 3695-3706 (2018).
17. T. Nieto *et al.*, A systematic review of epigenetic biomarkers in progression from non-dysplastic Barrett's oesophagus to oesophageal adenocarcinoma. *BMJ Open* **8**, e020427 (2018).
18. S. Horvath, K. Raj, DNA methylation-based biomarkers and the epigenetic clock theory of ageing. *Nat Rev Genet* **19**, 371-384 (2018).

19. A. J. Stevenson *et al.*, Trajectories of inflammatory biomarkers over the eighth decade and their associations with immune cell profiles and epigenetic ageing. *Clin Epigenetics* **10**, 159 (2018).

20. J. A. Kim, Y. Wei, J. R. Sowers, Role of mitochondrial dysfunction in insulin resistance. *Circ Res* **102**, 401-414 (2008).

21. K. F. Petersen *et al.*, Mitochondrial dysfunction in the elderly: possible role in insulin resistance. *Science* **300**, 1140-1142 (2003).

22. N. Turner, R. L. Robker, Developmental programming of obesity and insulin resistance: does mitochondrial dysfunction in oocytes play a role? *Mol Hum Reprod* **21**, 23-30 (2015).

23. E. M. Jeong *et al.*, Role of Mitochondrial Oxidative Stress in Glucose Tolerance, Insulin Resistance, and Cardiac Diastolic Dysfunction. *J Am Heart Assoc* **5**, (2016).

24. H. Nojiri *et al.*, Oxidative stress causes heart failure with impaired mitochondrial respiration. *J Biol Chem* **281**, 33789-33801 (2006).

25. R. Blake, I. A. Trounce, Mitochondrial dysfunction and complications associated with diabetes. *Biochim Biophys Acta* **1840**, 1404-1412 (2014).