EPIGENETICS OF OBESITY: DNA METHYLATION IN SINGLE WHITE BLOOD CELL TYPES

by

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(Under the Direction of Mary Ann Johnson and Richard B Meagher)

ABSTRACT

Obesity is a complex disease involving interactions between genetics and the environment. Epigenetic modifications are able to integrate changes in the environment (i.e. diet) into the genomes of cells. This dissertation identifies distinct leukocyte type specific changes in DNA methylation that are associated with obesity. Three studies were performed to (1) identify which peripheral leukocyte types may be the best to assess DNA methylation in response to a phenotype, (2) examine the DNA methylation profile of CD4+ T cells, CD8+ T cells, and CD16+ neutrophils in obese and normal weight women, and (3) examine the DNA methylation profile in CD4+ T cells in overweight and obese women before and after a weight loss intervention. In all studies of this dissertation, cell type specific differences in regards to their DNA methylome were observed. The first study (Chapter 2) identified that CD4+ T cells, CD8+ T cells, and CD14+ monocytes are the most potentiated to respond to physiological cues via their methylomes. Thus, CD4+ T cells and CD8+ T cells were selected for analysis in obese women, as well as a third leukocyte type, CD16+ neutrophils, which were found to be less potentiated to respond, but are the majority leukocyte type. In the obese women DNA methylation was found to be altered in 19 sites in CD4+ T cells and 16 sites in CD8+ T cells, while no alterations were

identified in neutrophils ($q<0.05$) (Chapter 3). Additionally, in the CD4+ T cells, 79 sites were identified to have methylation levels correlated with the amount of visceral adipose tissue ($q<0.05$). When DNA methylation was examined in relation to weight loss in CD4+ T cells (Chapter 4), 448 sites were identified to have methylation levels post-intervention that were associated with the amount of android fat lost over the intervention ($q<0.05$). Changes in DNA methylation associated with weight loss were only observed in the women who began the intervention with the lowest amount of android fat. Collectively the studies of this dissertation provide evidence that there are leukocyte type specific alterations in DNA methylation that are associated with obesity.

INDEX WORDS: DNA methylation, obesity, weight loss, leukocytes, CD4+ T cells, CD8+ T cells, adiposity, VAT

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW: LEUKOCYTE SPECIFIC ANALYSIS OF THE DNA CYTOSINE METHYLOME AS A VIEW TO UNDERSTANDING OBESTIY RELATED HEALTH RISK

Introduction

Obesity is a complex disease that develops through interactions between both the environment and genetics (Ling and Groop, 2009). Despite widespread knowledge of the comorbidities associated with obesity and their resulting health burden, the prevalence of obesity remains high (CDC, 2015; WHO, 2015). Furthermore, only 20% of individuals who are able to lose ≥10% body weight are able to keep the weight off for at least a year (Kraschnewski et al., 2010). It is thought that the body 'reprograms' itself in the obese state resulting in metabolic adaptations that favor higher adiposity, even when weight is lost. To gain insight into the molecular alterations which may be facilitating this 'reprograming' that occurs in obesity, I chose to investigate the epigenetic modification of DNA methylation. Understanding the epigenetic profile of obese individuals will give insight into the molecular mechanisms which may be facilitating the 'reprograming' and negative health related consequences of obesity.

It is believed that through epigenetic modifications (i.e., changes in chromatin structure) cells can integrate environmental (i.e. diet) and physiological cues (i.e. changes in energy stores), and hence, transiently modify expression of the genome. This view has become a particularly popular area of obesity research with numerous studies examining the relationship between DNA cytosine methylation and obesity. Yet the majority of studies examining DNA methylation and

obesity have used samples derived from whole tissue or mixed cell types. Different cell types have been shown to have distinct DNA methylation profiles (Reinius et al., 2012; Gu et al., 2016), and thus the data derived from mixed cell types is the weighted average of the methylation profile of all included cells. Therefore, the cell type specific DNA methylation signatures in obesity are likely lost in the average. Studying individual cell types instead of a mixture might reveal critical information to understanding the relationship between DNA methylation and obesity.

Blood has been a common tissue source to study the relationship between DNA methylation and obesity. However, blood is made up of a variety of cell types, each which have their own distinct biological role. These cell type specific differences are likely reflected in their methylomes, and may be why conflicting data has been observed when assessing DNA methylation from blood samples. One study has examined the global DNA methylation levels in PBMCs (containing B cells, T cells, monocytes and natural killer cells), lymphocytes (B cells, T cells and natural killer cells), monocytes, CD4+ T cells, CD8+ T cells, B cells, and natural killer cells and found that the global methylation levels were only altered in the B cells in obesity (Simar et al., 2014). This B cell specific difference was not observed in the PBMCs or lymphocytes, which both contain this cell type (Simar et al., 2014). This study provides initial evidence that cell type specific methylation associated with obesity can be lost in the average when examining multiple cell types. Furthermore, peripheral leukocytes are altered in their levels, activation and differentiation capacity in obesity. Thus, it is reasonable to suspect that DNA methylation may be facilitating some of these changes in the obese state. In order to discover more about the cell type specific epigenetic changes that are associated with obesity, I

focused my dissertation work on examining DNA methylation of distinct peripheral leukocyte cell types in relation to obesity.

I began the first study of my dissertation to determine the potential of the seven main leukocyte types in peripheral blood to respond via changes in DNA methylation (Chapter 2). Additionally in this chapter I present a rapid reiterative isolation protocol to isolate a few to seven peripheral leukocyte types from one small sample of fresh or frozen blood. This work facilitated the selection of the best leukocyte types to study the environmentally responsive phenotypes of obesity, and how to enrich for them starting with whole fresh or frozen blood for the remainder of my dissertation. The next chapter of my dissertation evaluates genome wide methylation of three leukocyte types in a population of obese women with age and sex matched controls of a healthy weight (Chapter 3). The leukocyte types were selected for this analysis based on their potential to respond to physiological cues via changes in methylation as determined in chapter 2. In the third study of my dissertation, I examined genome wide DNA methylation in CD4+ T cells in response to a specific weight loss program consisting of both dietary and exercise components in a group of overweight and obese women (Chapter 4). Collectively, these studies provide insight into the molecular basis of changes that occur with obesity at the level of DNA cytosine methylation in peripheral leukocyte types.

To provide a basis for this dissertation, my literature review will focus on providing a relevant background on obesity, different adipose tissue depots in obesity, adipokines, the inflammatory state in obesity, treatment and management of obesity through weight loss, epigenetics, DNA methylation, the relationship between DNA methylation, obesity and diet, and the use of leukocytes to study epigenetics.

Obesity

Obesity is a global health problem and was officially recognized by the American Medical Association (AMA) as a disease in June of 2013 (Frellick, 2013). The World Health Organization defines overweight and obesity as the accumulation of excess body fat which may impair health (WHO, 2015). Overweight and obese adults make up the majority of the US population, and 34.9% of US adults are classified as obese (Ogden et al., 2014; CDC, 2015). Overweight and obesity are defined in adults by the body mass index (BMI), where a BMI of 25 to 29.9 kg/m^2 is considered overweight, and a BMI $\geq$30 kg/m^2 is considered obese (1998). Obesity is further classified by BMI into three classes of obesity defined as, class I (BMI 30-34.9 kg/m^2), class II (BMI 35-40 kg/m^2), and class III (BMI $\geq$40 kg/m^2) (2000).

BMI is commonly used to assess weight status, as it is easy to use in clinical and community settings (Shah and Braverman, 2012). However, BMI does not provide any measure of body composition, as it is just a weight to height ratio (Gallagher et al., 1996; Shah and Braverman, 2012). Thus, classifying obesity by BMI can lead to misclassification due to its inability to distinguish free fat mass (FFM) from fat mass (FM) (Gallagher et al., 1996; Shah and Braverman, 2012). In additional to classifying weight status by BMI, obesity can be classified by percent body fat. According to the American Society of Bariatric Physicians and the AMA specialty board in their guidelines in 2009, $\geq$25% body fat for men and $\geq$30% body fat for women is considered obese, and these have been commonly used in research settings (Okorodudu et al., 2010; Shah and Braverman, 2012). Body fat levels can be determined by an estimation or direct measurement by many techniques including measurements of skin fold thickness, waist circumference, waist to hip ratio, bioelectrical impedance, DXA (duel energy x-ray absorptiometry), MRI (magnetic resonance imaging), densitometry (underwater weighing

and air displacement plethysmography), and CT (computed tomography) imaging (Ellis, 2000; Wells and Fewtrell, 2006; Shuster et al., 2012). The gold standards for determining body composition are DXA, MRI, and CT (Shuster et al., 2012). DXA, MRI, and CT can provide not only measures of total body fat, but also provide information on the different adipose depots, such as VAT (visceral adipose tissue) (Ellis, 2000; Wells and Fewtrell, 2006; Micklesfield et al., 2012; Shuster et al., 2012). To fully characterize the state of obesity in the studies of this dissertation, both calculations of BMI and body fat as determined by DXA were used.

Obesity increases the risk for many comorbidities (i.e. type 2 diabetes, coronary heart disease, stroke, sleep apnea, and some cancers) and is associated with the development of metabolic syndrome (Finkelstein et al., 2009; CDC, 2015). The medical costs for obesity are estimated at $147 billion a year (Finkelstein et al., 2009; CDC, 2015). Additionally, life expectancy has been shown to decrease with increasing BMI (up to the age of 75), especially among younger adults (Stevens et al., 1998). Different distributions of body fat are associated with worse metabolic phenotypes, with abdominal obesity (accumulation in the trunk region) associated with poorer metabolic outcomes, including insulin resistance, impaired glucose metabolism, and impaired lipid metabolism as well as a worse prognosis (Fox et al., 2007; Ritchie and Connell, 2007; Shuster et al., 2012). The accumulation of adipose tissue in the lower extremities, or the development of peripheral obesity, it thought to be more protective, and the associations with metabolic disturbances are lower (Evans et al., 1984; Bjorntorp, 1991; Jensen, 2008; Grundy, 2015). Although the health consequences of obesity are well established, the prevalence of this disease remains high, with rates more than doubled worldwide since 1980 (WHO, 2015). This high occurrence of obesity emphasizes the need to further understand this complex disease, define biomarkers, and develop therapeutics to help reduce this burden.

Adipose tissue

Adipose tissue accumulates when energy intake chronically exceeds energy expenditure, caused by the storage of the excess energy as triglycerides in adipocytes (Martinez, 2000; Wright and Aronne, 2012; Jung and Choi, 2014). The balance between these two forces is at will to a complex pathological process including both genetic and environmental interactions (Martinez, 2000; Wright and Aronne, 2012). At the simplest level these environmental influences consist of unhealthy lifestyle choices. For example, poor dietary habits and a sedentary lifestyle are known to contribute to increased adipose mass (Wright and Aronne, 2012). Additional factors including the food environment, social environment, sleep disturbances, some drugs, mental health, and other illness can contribute to the development of obesity at the environmental level (Martinez, 2000; Haslam and James, 2005; Keith et al., 2006). Genetic factors and predisposition can influence different aspects of metabolism that can lead to weight gain including lower resting metabolic rates, macronutrient utilization, rates of oxidation, and altered hormonal signaling (Martinez, 2000).

The distribution of adipose tissue has been shown to be an important determinant in the development of the metabolic abnormalities associated with obesity (Abate et al., 1995; Sites et al., 2000; Fox et al., 2007). Accumulation of adiposity in the abdomen is commonly called android adiposity or central adiposity (Lee et al., 2013), and is associated with worse metabolic outcomes than the accumulation of fat in the lower extremities (Evans et al., 1984; Jensen et al., 1989; Vega et al., 2006; Azuma et al., 2007; Pinnick and Karpe, 2011). White adipose tissue (WAT) depots are distributed across the body and are broadly classified as subcutaneous adipose tissue (SAT, under the skin) and VAT (intraperitoneal, associated with organs), which have different characteristics (Shuster et al., 2012; Lee et al., 2013). SAT depots store over 80% of

total body fat while VAT contains 10-20% and 5-10% of body fat stores in men and women respectively (Lee et al., 2013). In obesity, there is increased lipolysis from VAT, which is thought to contribute to the development of metabolic disturbances (Lee et al., 2013). VAT also produces higher levels of pro-inflammatory cytokines in obesity, which is also thought to be involved in the development of metabolic disturbances (Lee et al., 2013). In response to modest weight loss, which improves metabolic dysfunction, there is a preferential loss of VAT over SAT, which suggests that even small losses in VAT are due to the improvement of metabolic parameters (Chaston and Dixon, 2008).

Adipose tissue functions not only as a storage organ for energy, but also as a major endocrine and metabolic organ (Apostolopoulos et al., 2016). The accumulation of excess adiposity leads to dysfunction of its endocrine roles, and increased free fatty acid release into circulation, which both play a role in the development of metabolic abnormalities (Jung and Choi, 2014). The free fatty acids that are released in obesity increase insulin secretion, decrease insulin sensitivity in the liver and muscle, induce endothelial dysfunction, and increase VLDL secretion from the liver (Lee et al., 2013). Additionally, in obese adipose tissue there is an increased release of pro-inflammatory cytokines, which can lead to systemic inflammation (Lee et al., 2013). Further, as the adipocytes expand (hypertrophy) in response to increased energy intake, macrophage infiltration increases which then secrete more pro-inflammatory factors (Lee et al., 2013). As the adipose tissue grows, there can be localized hypoxia if there is inadequate vasculature, which can further exacerbate localized inflammation (Lee et al., 2013). Regardless of the cause of obesity, the increased adipose mass that develops, specifically in the trunk region, is independently associated with the development of metabolic abnormalities which can develop further into chronic disease (Evans et al., 1984; Goodpaster et al., 1997; Carr et al., 2004).

Adipokines influence peripheral organs including blood

As an endocrine organ adipose tissue releases adipokines, lipids, and metabolites which provide information of the functional status of the adipose tissue to other organs (Fasshauer and Bluher, 2015). Adipokines are peptides released from the adipocytes to mediate an effect, but have also been defined to include all peptides secreted from the adipose tissue (Lehr et al., 2012; Fasshauer and Bluher, 2015). In the adipose tissue adipokines can influence adipogenesis, the migration of immune cells into the tissue, and other functions of adipocytes (Fasshauer and Bluher, 2015). Adipokines can also affect other organs including the brain, liver, muscle, vasculature, heart, pancreas, and the immune system (Fasshauer and Bluher, 2015).

There are many different adipokines with over 600 secreted peptides released from adipose tissue (Lehr et al., 2012; Aguilar-Valles et al., 2015; Fasshauer and Bluher, 2015) which are involved in lipid metabolism, insulin sensitivity, vascular hemostasis, alternative complement system, blood pressure, angiogenesis, inflammation, appetite, and energy balance (Trayhurn and Wood, 2004; Aguilar-Valles et al., 2015). The most well-known adipokines include leptin and adiponectin. There are also pro-inflammatory adipokines (i.e. TNFα, IL-6, adipsin, resistin), anti-inflammatory adipokines (i.e. Omentin, IL-10), adipokines involved in glucose homeostasis (i.e. DPP-4, FGF21), and adipokines involved in regulating food intake (i.e. BMP-7, Vaspin) (Trayhurn and Wood, 2004; Fasshauer and Bluher, 2015). Leptin is mainly known for its role as a satiety signal (regulated food intake and appetite), but also is involved in the regulation of energy expenditure, reproductive function, activity, and atherogenesis (Fasshauer and Bluher, 2015). Adiponectin is secreted from adipocytes, and is present in human plasma (Ohashi et al., 2014). Adiponectin is involved in both improving insulin sensitivity and carrying out anti-inflammatory roles (Ohashi et al., 2014; Fasshauer and Bluher, 2015). In example, adiponectin

increases macrophages ability to remove apoptotic bodies, which is important in preventing inflammation (Ohashi et al., 2014). In adipose tissue of healthy weight individuals, the adipokines secreted work to maintain homeostasis in the body (Fasshauer and Bluher, 2015). As the adipose tissue expands in obesity, the secreted adipokine profile is altered and can contribute to metabolic and inflammatory disease (Fasshauer and Bluher, 2015).

In obesity, the adipose tissue expands which affects the biological function of the adipocytes (Fasshauer and Bluher, 2015). The resulting hypertrophy of the adipocytes is thought to begin the dysfunction of the adipose tissue leading to both the altered secretion of adipokines and the recruitment of immune cells (Fasshauer and Bluher, 2015). In obesity, there is increased secretion of more harmful adipokines including angiotensin, TNFα, IL-6, PAI-1, and leptin (Wu et al., 2007; Trayhurn et al., 2008; Wozniak et al., 2009; Karastergiou and Mohamed-Ali, 2010; Paz-Filho et al., 2011; Wu et al., 2012; Cohen et al., 2014; Rosenwald and Wolfrum, 2014). There is also a corresponding decrease in the secretion of the more helpful adipokines in obesity including IL-10, adiponectin, apelin, and omentin (Sethi and Vidal-Puig, 2005; Howard et al., 2010; Boydens et al., 2012; Van de Voorde et al., 2013; Matsuda and Shimomura, 2014). Leptin has been a well-studied example of the changing adipokines in obesity. Levels of circulating leptin are proportional to body fat, and are thus increased in obesity (Fasshauer and Bluher, 2015). The chronically high circulating levels of leptin lead to leptin resistance, diminishing the beneficial effects of leptin of appetite suppression and food intake regulation (Fasshauer and Bluher, 2015). Humans and mice genetically deficient in leptin are characterized by hyperphagia, obesity, and insulin resistance (Nakamura et al., 2014). Both leptin and adiponectin (among other adipokines) have been shown to interact with different leukocytes to promote pro-inflammatory and anti-inflammatory roles respectively (Nakamura et al., 2014; Aguilar-Valles et al., 2015).

The increased pro-inflammatory adipokines released in obesity promotes both metabolic and cardiovascular disease (Nakamura et al., 2014). For example, the increased levels of TNFα in obesity (a pro-inflammatory adipokine) contributes to insulin resistance by inhibiting the phosphorylation of the insulin receptor and insulin receptor substrate 1 in muscle and adipose tissue, which is required for their activation (Hotamisligil et al., 1994; Nakamura et al., 2014). Further, the altered adipokine profile secreted from obese adipose tissue impacts inflammatory cells, contributing to the localized and systemic inflammation in obesity (Nakamura et al., 2014).

Inflammation and obesity

Many of the metabolic abnormalities stemming from obesity are related to the development of an inflammatory state in adipose tissue (specifically in the VAT), which results in chronic systemic inflammation (Lee and Pratley, 2005; Anderson et al., 2010). This chronic low-grade inflammation is the link between obesity (increased adipose mass) and the development of metabolic abnormalities (Mathis, 2013). In obese adipose tissue there are dynamic changes including increased release of free fatty acids (FFA), hormones, and adipokines, as well as an increase in the presence of inflammatory cells (Apostolopoulos et al., 2016). These dynamic changes disturb the balance of the adipose tissue that is maintained in those of a healthy weight, and results in the activation of the inflammatory response (Apostolopoulos et al., 2016).

Adipose tissue of normal weight individuals consists of adipocytes, precursor cells, endothelial cells, and immune cells (Huh et al., 2014). The immune cells of adipose tissue consist of both innate (macrophages, neutrophils, eosinophils, and mast cells) and adaptive (various T cells, and B cells) cell types (Huh et al., 2014). Adipocytes are able to communicate with these immune cells and are involved in their activation and proliferation in adipose tissue

(Huh et al., 2014). For example, obese adipocytes secrete TNFα and IL-6, pro-inflammatory cytokines, which are involved in the development of adipose tissue inflammation (Huh et al., 2014). Additionally adipocytes secrete leptin (in proportion to adipose mass), which activates CD4+ T cells to secrete pro-inflammatory cytokines and increases activation of monocytes (Apostolopoulos et al., 2016). Adiponectin, also secreted by adipocytes, works to inhibit TNFα expression in adipocytes and macrophages and its expression is decreased in obesity (Ohashi et al., 2015). Obese adipocytes can also activate CD4+ T cells by acting as MHC class II antigen presenting cells (Deng et al., 2013).

As adipose mass increases, there is a shift in the immune cells present and their activity to that of a pro-inflammatory nature, which is thought to be involved in the development of insulin resistance (Huh et al., 2014). There are mainly anti-inflammatory immune cells (M2 macrophages, regulatory T cells (Tregs), and eosinophils) in the adipose tissue of lean individuals which helps maintain insulin sensitivity (Huh et al., 2014). Macrophages are a major immune cell involved in the establishment of a pro-inflammatory state in obese adipose tissue. Monocytes are differentiated into either M1 macrophages (classically activated) or M2 macrophages (alternatively activated) depending on different stimuli (Lumeng et al., 2007). M2 macrophages are present in lean adipose tissue and produce anti-inflammatory cytokines (Lumeng et al., 2007a), which promote local and systemic insulin sensitivity (Chawla et al., 2011; Osborn and Olefsky, 2012). The eosinophils present in lean adipose tissue are though to promote the presence of M2 macrophages, as they release IL-4 (major contributor of this cytokine in adipose tissue) in VAT promoting this lineage, and are reduced in obesity (Wu et al., 2011). In obesity, the pro-inflammatory cytokines LPS and IFN-γ promote M1 macrophages which secrete pro-inflammatory cytokines including TNFα (Lumeng et al., 2007) which

promotes insulin resistance (Chawla et al., 2011; Osborn and Olefsky, 2012). Neutrophils are implicated in the early development of the inflammatory state in adipose tissue and have been shown to be recruited into adipose tissue just three days after mice are fed a high fat diet (Elgazar-Carmon et al., 2008). Neutrophils further promote the inflammatory response in obesity by their anti-microbial activities as well as recruit circulating monocytes to the tissue (which can be differentiated into macrophages) (Mathis, 2013). CD8+ T cells are also involved in the early stages, and have been shown to be recruited into adipose tissue within two weeks of a high fat diet, prior to the recruitment of macrophages (Nishimura et al., 2009). When CD8+ T cells are inhibited in high fat diet induced obesity, there is both improved inflammation in VAT and systemic insulin sensitivity independent of changes in body weight (Nishimura et al., 2009). Further, CD8+ T cells have been shown to be activated by only obese adipocytes and are involved with the recruitment and differentiation of macrophages (Nishimura et al., 2009). Additionally there is a decrease in the anti-inflammatory CD4+ T helper 2 (Th2) and Treg populations in obese adipose tissue (Nishimura et al., 2009; Zuniga et al., 2010; Huh et al., 2014). Th1 CD4+ T cells, a pro-inflammatory T cell, increase in obese adipose tissue and produce IFN-γ (Winer et al., 2009; Zuniga et al., 2010), further promoting the presence of M2 macrophages. B cells also appear to have a pro-inflammatory effect in obese adipose tissue, and are increased in the obese state (Winer et al., 2011; DeFuria et al., 2013).

The increased inflammatory state in adipose tissue with obesity contributes to the development of chronic systemic inflammation (Huh et al., 2014). There is evidence that the cytokines released from obese adipose tissue are reflected in the blood as elevated levels of TNFα have been observed both in obese adipose tissue and systemically (Hotamisligil et al., 1993). When TNFα is neutralized, systemic insulin resistance is improved (Hotamisligil et al.,

1993). Furthermore, total white blood cell (WBC) counts have been shown to correlate with obesity and measures of adiposity (Fisch and Freedman, 1975; Pratley et al., 1995; Dixon and O'Brien, 2006). Peripheral leukocytes in obese individuals have higher activations marks on neutrophils, monocytes, T lymphocytes, and Th1 cells (Nijhuis et al., 2009; Viardot et al., 2012). Finally, circulating leukocytes have been shown to be altered in obesity. Circulating monocytes from obese individuals have different gene expression and surface molecules than the circulating monocytes in normal weight individuals (Satoh et al., 2010; Viardot et al., 2012). Circulating neutrophils are increased with obesity, and are higher in people with metabolic syndrome (Dixon and O'Brien, 2006; Kaur et al., 2013). CD4+ T cells (naïve, memory, Treg, and Th2) increase in circulation with obesity and have been shown to correlate with insulin sensitivity (van der Weerd et al., 2012). It also appears that the proliferation of both CD4+ and CD8+ T cells is increased with obesity (Womack et al., 2007; van der Weerd et al., 2012). Leptin negatively affects proliferation of Tregs, and in obese individuals, higher leptin levels correlate with lower Treg levels (De Rosa et al., 2007; Wagner et al., 2013; Apostolopoulos et al., 2016). B cell counts are also increased in the periphery of obese individuals (Nieman et al., 1999; Ilavska et al., 2012). Further details on the development of the inflammatory state in adipose tissue has been previously well reviewed (Chawla et al., 2011; Osborn and Olefsky, 2012; Mathis, 2013; Huh et al., 2014; Apostolopoulos et al., 2016).

Treatment and management of obesity: weight loss

Weight loss is the primary focus of the guidelines for the management of obesity from the Academy of Nutrition and Dietetics, the American Heart Association (AHA)/American College of Cardiology (ACC)/The Obesity Society (TOS) and Endocrine society (Jensen et al., 2014; Apovian et al., 2015; Raynor and Champagne, 2016). The guidelines state that even

modest weight loss of 3-5% body weight can produce benefits of reductions of triglycerides, blood glucose, hemoglobin A1c, and the risk of developing type 2 diabetes (Jensen et al., 2014; Raynor and Champagne, 2016). Higher levels of weight loss lead to even more beneficial changes to metabolic parameters including reductions in blood pressure and LDL-cholesterol, increases in HDL-cholesterol, and may eliminate the need for medications to control blood pressure, blood glucose, and serum lipids (Jensen et al., 2014; Raynor and Champagne, 2016). Furthermore, many studies have shown that reductions in body weight of approximately 10% improved metabolic parameters and the prevalence of metabolic syndrome (Goldstein, 1992; Case et al., 2002; Grundy et al., 2005; Phelan et al., 2007). Thus, the recommended weight loss goal for overweight and obese individuals is 5 to 10% of body weight in a six month period (Raynor and Champagne, 2016). Weight loss is primarily recommended to be implemented through lifestyle interventions targeting diet and physical activity, however individuals with comorbidities or who are morbidly obese (BMI $\geq$40 kg/m^2) may need additional pharmacological treatment or bariatric surgical treatment (Jensen et al., 2014; Apovian et al., 2015).

Lifestyle interventions to promote weight loss should focus both on dietary and physical activity aspects, facilitated through the use of behavioral strategies, or a comprehensive lifestyle program (Jensen et al., 2014). The AHA, ACC, and TOS conclude in their guideline for the treatment and management of overweight and obesity, that there is strong evidence that a calorically restricted diet will promote weight loss (Jensen et al., 2014). They also conclude that there is strong evidence that the use of comprehensive lifestyle programs for at least 6 months will promote weight loss (Jensen et al., 2014). This was confirmed in the 2016 guidelines published by the Academy of Nutrition and Dietetics (Raynor and Champagne, 2016). Thus, it is well accepted that an energy restricted diet alone or in combination with a comprehensive

lifestyle intervention promotes weight loss. Yet, most weight loss intervention studies still observe large individual variation in the response to weight loss interventions, suggesting individual factors play a role in weight loss success (Dansinger et al., 2005; King et al., 2008; Napolitano et al., 2012; O'Neil et al., 2012; Kullgren et al., 2013).

To make caloric intake recommendations to promote weight loss (up to 2 lbs. per week) the resting metabolic rate (RMR) needs to be determined (Raynor and Champagne, 2016). The RMR should ideally be determined though indirect calorimetry, but if not available the Mifflin-St. Jeor equation using the actual body weight of the overweight or obese individual can be used (Raynor and Champagne, 2016). The RMR must then be multiplied by an activity factor (sedentary 1-1.4, low activity 1.4-1.6, active 1.6-1.9, very active 1.9-2.5) to determine the daily energy expenditure (DEE) of the overweight or obese individual (Raynor and Champagne, 2016). Once the DEE is determined, a weight loss plan can be developed. In order to produce an energy deficit to facilitate weight loss, dietary intake should be 500 kcal to 750 kcal per day less than the DEE (Raynor and Champagne, 2016). It is important that the calorically restricted diet still provides adequate nutrition and meets the reconditions of the Dietary Guidelines for Americans (Raynor and Champagne, 2016). As long as the caloric deficit is achieved, the macronutrient distribution of the diet (i.e. low carbohydrate, low fat) produce similar weight loss (Tobias et al., 2015; Raynor and Champagne, 2016).

Increased moderate to vigorous physical activity alone is not as effective in weight loss as caloric restriction alone (Johns et al., 2014; Raynor and Champagne, 2016). Yet the combination of both physical activity and caloric restriction produces the most significant amounts of weight loss (Johns et al., 2014; Raynor and Champagne, 2016). Although not the most important factor for weight loss, moderate to vigorous physical activity is crucial for weight loss maintenance

(Raynor and Champagne, 2016). This may be related to the ability to offset the reduced energy expenditure that is experienced in those who lose significant amounts of weight (Maclean et al., 2011). By increasing physical activity, daily energy expenditure is increased, and maintaining a non-excessive dietary intake become more manageable. Indeed, moderate to vigorous physical activity needs are increased to more than 250 min per week for weight loss maintenance, while the general recommendations for a healthy individual are 105 min per week (Donnelly et al., 2009; Raynor and Champagne, 2016).

Finally, cognitive behavioral therapy is recommended to complete a comprehensive lifestyle intervention for weight loss. This type of behavioral intervention focuses on relating thoughts and behaviors to their impact on health outcomes, and how change can facilitate improvements in these outcomes (i.e. obesity) (Raynor and Champagne, 2016). This type of behavior intervention should focus on self-monitoring to facilitate change in thought and behaviors (Raynor and Champagne, 2016). Motivational interviewing is a technique RDNs should use when counseling overweight and obese patients on weight loss, leading patients to thought and behavior changes (Raynor and Champagne, 2016). Thus, comprehensive interventions are likely to produce better weight loss as lifestyle factors which need to be maintained for weight maintenance are introduced to an individual.

Another aspect of obesity management is the ability to keep weight off once it is lost. However, this has proven to be quite the challenge for most individuals with successful weight loss. In a large systemic analysis of 33 weight loss interventions based on lifestyle modifications, after 1 year approximately half of the weight lost was regained (Stevens et al., 2001). It has been found that only approximately 20% of adults in the US are able to maintain weight loss of at least 10% initial body weight for at least a year (Kraschnewski et al., 2010). A higher portion of

adults may be able to keep the weight off (39.3% of participants able to maintain a 10% weight loss for one year) when comprehensive lifestyle intervention is used (focused on diet, physical activity, and cognitive behavioral intervention) (Look, 2014). The ability to maintain lifestyle changes after weight loss are crucial to weight maintenance success (Raynor and Champagne, 2016), as once weight loss is achieved, there are metabolic adaptations which favor weight regain (Rosenbaum et al., 2008). The metabolic adaptations that persist with weight loss have been well reviewed (Maclean et al., 2011) and include a decreased resting metabolic rate, altered endocrine signaling, increased appetite, and overall suppressed energy expenditure (Rosenbaum et al., 2008; Maclean et al., 2011). The persistence of these metabolic adaptations makes maintaining weight loss difficult. These adaptations are in part a result of the remembrance of the obese state which has 'reprogramed' the body (Leung et al., 2016).

Dietary interventions for weight loss, and a role for personalized interventions?

Despite the strong evidence that caloric restriction (alone or in part of a comprehensive weight loss program) results in weight loss, there is inter-individual differences in weight lost from dietary changes (Moreno-Aliaga et al., 2005; Wu H, 2013). It has been suggested that weight loss should be viewed as a complex trait with environmental, behavioral, and genetic determinants (Moreno-Aliaga et al., 2005; Wu H, 2013). Thus, in addition to the environmental and behavioral aspects that are targeting by comprehensive weight loss programs, individual genetic background can also influence their weight loss success and corresponding improvements in metabolic parameters (Moreno-Aliaga et al., 2005). An example of how individual genetic disposition can effect weight loss involves the leptin receptor. Overweight women with the 343T/C SNP in the leptin receptor lost more weight following a low calorie diet than those without this variant (Mammes et al., 2001). As leptin is an appetite suppressant

(Moreno-Aliaga et al., 2005), this polymorphism links individual genetic profiles to diet, which affects weight loss success. Additionally, the Pro12Ala polymorphisms in PPARγ2 (adipose tissue specific) is associated with better weight loss over three years (diet and exercise intervention) than those with other genotypes (Lindi et al., 2002). As PPARGγ2 is a transcription factor involved in adipogenesis and glucose and lipid homeostasis (Moreno-Aliaga et al., 2005), this polymorphism in another link between weight loss and diet. Furthermore, people who have the rs1558902 risk allele in the *FTO* (fat mass and obesity associated gene) have larger response (weight reductions, positive body composition changes) to a high protein weight loss diet than on a low protein weight loss diet for 2 years (Zhang et al., 2012), suggesting that for those with this variant the type of weight loss diet is crucial for success. Thus, understanding gene-diet interactions may provide the framework needed for personalized weight management (Wu H, 2013). Epigenetics, specifically DNA methylation may be one modality though which these gene-diet interactions occur and warrants further investigation.

Epigenetics

Epigenetics is commonly defined as the heritable changes in gene expression that occur without changing the DNA sequence (Campion et al., 2009c; Dupont et al., 2009; Perez-Cornago et al., 2014). Others have defined epigenetics as "any long-term change in gene function that persists even when the initial trigger is long gone that does not involve a change in gene sequence or structure" (McGowan and Szyf, 2010). It has been proposed that the epigenome is responsive to different environmental cues such as inflammation, oxidative stress, smoking, physical activity, stress, and nutrition (Campion et al., 2010; Martin-Nunez et al., 2014; Samblas et al., 2016). Thus, epigenetics can act as a link between genotype and phenotype, which is responsive to different physiological cues (Goldberg et al., 2007). The most commonly studied

epigenetic modifications are DNA methylation and covalent histone modifications. The sum of the epigenetic information in a cell is called the 'epigenome', and the epigenome differs between cells (Suzuki and Bird, 2008). Thus, there are at least as many epigenomes as there are cell types in the body, but likely more due to the dynamic nature of epigenetic modifications (Suzuki and Bird, 2008). As the studies included in this dissertation focus on DNA methylation in relation to obesity and adiposity, the remaining review on epigenetics will focus on DNA methylation.

DNA methylation

DNA methylation occurs at cytosine residues by the addition of a methyl group in the 5th carbon position (5mC) (Newell-Price et al., 2000; Gibney and Nolan, 2010). 5mC typically occurs in the context of a CG dinucleotide (Newell-Price et al., 2000). CG dinucleotides are found at high densities in the genome in regions termed CG islands (CGIs) which cover ~60% of genes promoters (Antequera and Bird, 1993). Immediately upstream and downstream of the CGI are the shores and shelves (Bibikova et al., 2011). The shores are the 2kb regions immediately upstream and downstream of the CGI, while the shelves are the 2kb regions upstream and downstream of the shores (Bibikova et al., 2011). Most CG dinucleotides are methylated in the genome, with the exception that most CGIs are demethylated (Newell-Price et al., 2000). Typically DNA methylation is associated with a heterochromatin state, resulting in the repression of gene expression (Siegfried and Simon, 2010). The data is strong supporting this relationship in CG sites located in the promoter region and CGIs of genes (Goldberg et al., 2007; Pinnick and Karpe, 2011; Paluch et al., 2016). However, gene body methylation has a less clear relationship with gene expression (Bird and Wolffe, 1999; Klose and Bird, 2006; Suzuki and Bird, 2008).

DNA is methylated by DNA methyltransferase enzymes (DNMTs), which add the methyl group to the 5' position of cytosine residues (Newell-Price et al., 2000; Kim et al., 2009; Gibney and Nolan, 2010). S-adenosylmethionine (SAM) is the methyl donor for this methylation reaction (Remely et al., 2015). There are three common DNMTs: DNMT1, which is responsible for maintenance of DNA methylation, able to methylated hemi-methylate CG sites, and DNMT3a and DNMT3b, which are the de novo methyltransferases able to methylate both un-methylated and hemi-methylated CG sites (Newell-Price et al., 2000; Gibney and Nolan, 2010).

It has been suggested that 20% of CG sites are tissue specific and may be involved with gene regulation (Lister et al., 2013; Meagher, 2014; Wu and Zhang, 2014; Gu et al., 2016). DNA methylation is thought to be involved in regulating gene expression in two main ways, altering transcription factor binding and the binding of methyl CG binding proteins (Tate and Bird, 1993; Newell-Price et al., 2000). Methylated CG sites can interfere with the ability of some transcription factors to bind to a DNA sequence (Tate and Bird, 1993; Newell-Price et al., 2000). Additionally, the DNMTs can interact with transcription factors to methylate specific CG sites, which then can affect the recruitment of transcriptional machinery or alter the chromatin structure to affect gene regulation (Hervouet et al., 2009; Gibney and Nolan, 2010). The proteins that bind to 5mCG are the methyl-CG binding proteins (MBD1, MBD2, MBD3, MBD4, MeCP2) (Du et al., 2015), and can affect gene transcription (Gibney and Nolan, 2010). MBD1-3 recruit various histone deacetylases and co-repressors, which results in transcriptional repression. Another of the methyl-CG binding proteins, MBD4 has a glycosylase domain (Otani et al., 2013), like TDG (thymine DNA glycosylase, involved in the de-methylation of 5mC), and thus may contribute to the modification of 5mC, but has not been associated with repressing gene transcription (Newell-Price et al., 2000; Gibney and Nolan, 2010). MeCP2 can be affected by

changes in external stimuli (cocaine and ethanol) (Grigera et al., 2013; Pol Bodetto et al., 2013). Additionally, MeCP2 can bind to both 5mCG and 5-hydroxymethylated CG sites, being associated with repressed and active transcription respectively (Mellen et al., 2012). The repression domain of MeCP2 can repress transcription at locations a few hundred base pairs away (Bird and Wolffe, 1999) and can also recruit the Sin3 complex which is a co-repressor containing histone deacetylase 1 and 2, as well as other co-repressors to further facilitate repression (Newell-Price et al., 2000; Gibney and Nolan, 2010). Finally, MeCP2 can also bind to the linker DNA and to nucleosomes, altering chromatin compaction and thus can form physical barriers to the transcriptional machinery (Gibney and Nolan, 2010). Thus, the complex interactions between DNA methylation, the binding of cellular machinery, and chromatin structure work together to regulate gene transcription.

There is evidence that the turnover of the 5mC at sites that are relevant to gene expression is rapid (Meagher, 2014) with approximately 10% of 5mC sites in the genome able to be demethylated in as little as two hours (Yamagata et al., 2012). Recently the process of demethylation has been determined. The ability of DNA to not only be methylated, but demethylated as well, shows the ability of this epigenetic modification to have a dynamic response to different physiological cues. The demethylation of 5mC begins by oxidation by one of three ten eleven translocase methyl-cytosine dioxygenase enzymes (TET1, TET2, TET3) to 5hmC (5-hydroxymethylcytosine), and is followed by repair back to cytosine by thymine-DNA glycosylase (TDG) and growth and arrest DNA damage 45 (GADD45 A, B, and G) enzymes (Calabrese et al., 2014). 5hmC has been proposed to be not only an intermediate of the demethylation of 5mC, but is associated with gene expression changes. The prevalence of 5hmC is much lower than 5mC, but is correlated with differential gene regulation (Mellen et al., 2012;

Lister et al., 2013; Tsagaratou et al., 2014). In contrast to 5mC, 5hmC is associated with euchromatin (Ficz, 2015), suggested to be poising genes for transcription (Pastor et al., 2011), or involved in 'on demand' gene regulation (Irier et al., 2014). As with 5mC, 5hmC seems to be tissue specific and involved in differentiation (Nestor et al., 2012; Taylor et al., 2016). For example, in TET1 and TET2 known down embryonic stem cells, methylation of pluripotency related genes is increased with corresponding changes in gene expression, resulting in an altered differentiation potential (Ficz et al., 2011). There can be changes in the levels of 5mC and 5hmC independent of each other (Ruzov et al., 2011; Salvaing et al., 2012; Hahn et al., 2013). In addition to this active demethylation, sites can become demethylated passively, if methylation is not maintained with replication (Gibney and Nolan, 2010).

As was described when introducing the concept of the epigenome, there are at least as many epigenomes as there are cells in the body (Suzuki and Bird, 2008). Thus, it is critically important to examine epigenetic modifications, like DNA methylation, in single cell types. An example of the vast differences in the DNA methylome between different cell types has been shown in peripheral blood leukocytes. Reinius et al. (2012) performed a genome wide analysis of the DNA methylation profile in seven main leukocyte types. They found that the two closely related PBMCs, the CD4+ T cells and the CD8+ T cells differed in ~45,000 sites of the ~485,000 sites assayed (9%) in methylation levels (Reinius et al., 2012). The distantly related PBMC CD8+ T cells and the granulocytic eosinophils differed in a remarkable ~193,000 sites of the ~485,000 sites assayed (40%) (Reinius et al., 2012). Additionally, the granulocytes (neutrophils and eosinophils) are hypomethylated, with five to ten times less methylation across the genome than the hypermethylated PBMC lymphoid cell types (T cells, B cells, NK cells, and monocytes) (Reinius et al., 2012). Differences in DNA methylation between different cell and tissue types

other to leukocytes has also been demonstrated (Gu et al., 2016). Thus, when mixed leukocytes DNA methylomes are analyzed together, the resulting data is a weighted average of all cell types included. This can lead to the loss of many biologically relevant differences, which are obscured in the weighted average.

DNA methylation, obesity, and dietary intake

Worldwide the rate of obesity has more than doubled in the last 35 years (WHO, 2015). Epigenetics has been suggested to be a major player in this development and persistence of obesity (Campion et al., 2010). It has been suggested that epigenetic factors facilitating the interaction between gene regulation and the environment may explain the inter-individual differences that are observed in complex diseases, like obesity (Ling and Groop, 2009). There are many factors in obesity that may influence DNA methylation and the epigenome including diet, oxidative stress, inflammation, and hypoxia (Milagro et al., 2011).

As obesity in part develops from the chronic disturbed energy balance where intake exceeds total energy expenditure, diet may play a role in the proposed altered DNA methylome in obesity (Milagro et al., 2011). The methyl groups available for DNA methylation are affected by intake of dietary methyl donors (de Mello et al., 2014). The main dietary methyl donors include methionine, betaine, folate, and choline (Niculescu and Zeisel, 2002; Anderson et al., 2012). The utilization of methyl groups can also be affected by diet, as the enzymes involved in the DNA methylation process require different nutrients and their metabolites for proper functionality (Remely et al., 2015). S-adenosylmethionine (SAM) is the enzyme that provides the methyl group to be used for DNA methylation (Niculescu and Zeisel, 2002; Remely et al., 2015), and folate, cobalamin, pyridoxine, and riboflavin can influence the availability of SAM (Ross, 2003; Anderson et al., 2012; de Mello et al., 2014). DNMT activity can be influenced by

curcumin, genistein, EGCG, and equol (Remely et al., 2015). TETs activity can be affected by vitamin C, which can affect the formation of 5hmC, 5fC, and 5caC as well as the de-methylation process (Blaschke et al., 2013).

Additionally, changes in diet have been shown to be associated with changes in DNA methylation. For example a high fat diet (HFD) has been shown to alter the methylation status of adiponectin, which was associated with changes in gene expression in the adipocytes of mice (Kim et al., 2015). The altered methylation status of adiponectin was found to be due to changes in DNMT1 activity. This activity can be induced in vitro by treating differentiated adipocytes with TNFα, suggesting an involvement of the inflammatory state in obesity in which TNFα levels are elevated (Kim et al., 2015). In humans, the methylation of adiponectin and its expression level have been shown to correlate with BMI, suggesting that this gene's responsive to the obesogenic diet in humans as well (Kim et al., 2015). Berry intake, in addition to a high fat diet in mice, altered DNA methylation profiles in liver compared to the mice on a high fat diet alone (Heyman-Linden et al., 2016). In mice, a high fat diet decreases PPARγ promoter methylation and increases its expression in muscle, which can be prevented by the supplementation of fish oil (Amaral et al., 2014). Additionally, short term overfeeding in humans has altered DNA methylation at over 7000 sites corresponding to over 6500 genes in skeletal muscle (Jacobsen et al., 2012). These changes in DNA methylation were only partially reversed after six weeks with no sites significantly restored to baseline levels (Jacobsen et al., 2012). Thus, cumulating evidence is suggesting that alterations in diet, which occur in obesity, are associated with altered DNA methylation.

Medications, age and DNA methylation

In addition to obesity and dietary intake, DNA methylation levels can be impacted by different medications and by age. The effect of medications of DNA methylation warrants discussion as many obese patients develop complications that require medication use. Additionally, if older participants are included in studies of DNA methylation and obesity, the likely hood for pharmaceutical use is increased, representing a potential confounding effect of DNA methylation and obesity. It is also important to consider the impact of age on DNA methylation when considering studies that have been conducted in populations with different ages or with large age ranges.

Some of the earliest drugs known to affect DNA methylation are 5-azacytidine and 5-deoxyazacytidine, both of which are hypo-methylating drugs and are used for cancer treatment (Issa and Kantarjian, 2009). These drugs are nucleoside analogs which trap DNMT onto the DNA where this drug is incorporated (Szyf, 2009). Another nucleoside analog, Zebularine is also used for cancer treatment and has a similar effect (Szyf, 2009). Hydralazine which is a vasodilator used to treat hypertension inhibits the DNMTs, resulting in a drug induced lupus like autoimmune disease (Csoka and Szyf, 2009). The anti-arrhythmic sodium channel blocker procainamide induces the same affect (Csoka and Szyf, 2009). Methotrexate, which is an anticancer drug, affects methionine synthesis which alters DNA methylation (Csoka and Szyf, 2009). The teratogens thalidomide and isotretinoin have been shown to affect DNA methylation in the offspring, yet the mechanism of action in unknown (Csoka and Szyf, 2009). Tamoxifen, a non-steroidal anti-estrogen which is used for breast cancer prevention and treatment has also been suggested to affect DNA methylation (Csoka and Szyf, 2009). Valproic acid, which is used to treat epilepsy and as a mood stabilizer, has been shown to induce demethylation (Szyf, 2009).

Additionally, the histone deacetylate inhibitors, TSA, valproate, and benzamide have been shown to cause demethylation in the brain (Szyf, 2009). The use or past use of these drugs in participants in DNA methylation studies may confound the DNA methylation data. It is also likely that other medications lead to changes in DNA methylation, albeit indirect, that may also confound data. It is thus important to report or consider the medications used by participants in these DNA methylation studies.

DNA methylation changes throughout the lifespan. Following birth, DNA methylation levels are elevated for the first year (Jones et al., 2015). Once adulthood is reached, DNA methylation decreases as measured by LINE-1, Alu, and microarray with the ageing process (van Otterdijk et al., 2013; Jones et al., 2015). In individuals whose DNA methylation profile were measured over eight years showed that DNA methylation decreased over time (Bollati et al., 2009). In addition to decreases in methylation with aging, site specific DNA methylation becomes more variable (van Otterdijk et al., 2013; Jones et al., 2015). So with aging, there is not only a global reduction in DNA methylation, but increased differences in methylation between individuals (Jones et al., 2015). Not all regions in the genome decrease methylation with age, there are some regions that have increased methylation with age, usually occurring within CGIs (van Otterdijk et al., 2013; Jones et al., 2015). Specific sites have been shown to have highly associated methylation levels with age, and have been used to predict age (Bocklandt et al., 2011; Johnson et al., 2012; Jones et al., 2015). Thus, it is important to consider the effect of age when comparing DNA methylation studies in relation to obesity as well as other phenotypes.

Prior studies of DNA methylation and obesity in blood

There has been much interest in studying the relationship between obesity and DNA methylation in the recent years. According to PubMed, prior to 2006 there were less than 20

manuscripts published a year on DNA methylation and obesity, yet since 2006 the topic has skyrocketed in interest with 152 articles published on the topic in 2015 alone (2016). As there are many aspect of DNA methylation and obesity, there have been numerous areas of research on the topic including the maternal and paternal influences, manipulation of DNA methylation by numerous sources in animal models as well as in humans, exploratory studies on DNA methylation in obese, and DNA methylation and weight loss. As the studies included in this dissertation focus on the DNA methylation profile in obese adults and in response to weight loss in adults in blood cells, this will be the focus of the studies reviewed. The other aspect of DNA methylation and obesity, as well as the relationship between DNA methylation and obesity in other tissue types, have been previously reviewed (de Mello et al., 2014; Martinez et al., 2014; van Dijk et al., 2015).

Global DNA methylation levels (**Table 1.1**) have been assessed in blood of both adults and children. There have been mixed results in adults with one study showing no relationship of global methylation with obesity (Wang et al., 2010; Keller et al., 2014), while others have shown associations of global methylation with BMI (Perng et al., 2013; Na et al., 2014; Simar et al., 2014). Interestingly, the one study examining single leukocyte types found that there are changes in global methylation in obesity in only the B cells while PBMCs and the other leukocyte types are unaltered, emphasizing the importance of single cell type analysis (Simar et al., 2014). In the one study examining global DNA methylation in adolescents, an association with both BMI and skinfold thickness was identified (Perng et al., 2013).

Genome wide studies of DNA methylation and obesity in adults have also been conducted in blood cells (**Table 1.2**). Many sites with differential methylation associated with obesity have been identified, many of which are unique to its own study (**Table 1.2**). In children,

similar results have been observed in blood cells (**Table 1.2**). Only one study assessing genome wide DNA methylation in blood has been performed in a single leukocyte type, CD4+ T cells (Aslibekyan et al., 2015). Site-specific methylation levels in CD4+ T cells were found to be associated with both BMI and waist circumference (Aslibekyan et al., 2015). Finally, studies examining methylation levels of candidate genes in obesity have also been performed in blood cells (**Table 1.3**). All of the candidate genes studies conducted in blood have been performed on samples derived from multiple cell types (**Table 1.3**). Many different genes have been shown to have associations between DNA methylation and obesity, yet many differences between studies have been observed.

In addition to examining the DNA methylation profile that is associated with the obese state, researchers have begun to examine the response of the DNA methylome to weight loss (**Table 1.4**). These studies can answer many questions about DNA methylation in obesity. First, does DNA methylation change with weight loss, or is the reprograming to the methylome that occurs with obesity remembered even once the weight is lost? Second, can baseline methylation predict the amount of weight loss success an individual will have? And third, are the corresponding changes in DNA methylation with weight loss involved in the metabolic adaptations that are associated with weight loss? Studies examining DNA methylation and weight loss have been performed in many tissues including skeletal muscle, adipose tissue and blood cells, yet the majority have been conducted in blood which are described in **Table 1.4**. Again these studies have produced conflicting results, but have identified potential biomarkers for weight loss and changes in methylation that occur with weight loss. No studies have examined this relationship in single cell types.

The conflicting results obtained from studies on DNA methylation and obesity and/or weight loss may be due to a variety of factors. First, they have been conducted in many different study populations, with different ages, genders, and ethnicities, all of which have been shown to be independently associated with differences in DNA methylation (Boks et al., 2009; Liu et al., 2010; Adkins et al., 2011; Fraser et al., 2012; Heyn et al., 2012; Florath et al., 2014; Xia et al., 2014; Jung and Pfeifer, 2015). A few studies have examined DNA methylation profiles in association to their phenotype in multiple tissues or in multiple study populations (Dick et al., 2014; Su et al., 2014; Aslibekyan et al., 2015; Demerath et al., 2015; Gomez-Uriz et al., 2015; Houde et al., 2015; Huang et al., 2015a; Al Muftah et al., 2016; Mansego et al., 2016). These studies have only found a small number of methylation differences that have been consistent across both tissues and populations. For example, Al Muftah et al (2016) examined 39 sites with methylation levels previously associated with obesity in Caucasians in an Arab population. Only seven of these sites had methylation levels associated with obesity in the Arab population (Al Muftah et al., 2016). Additionally, all but two studies (Simar et al., 2014; Aslibekyan et al., 2015; Maples et al., 2015) examining these relationships have been performed on mixed cell types. DNA methylation is cell type specific, and the DNA methylation profile of the different cell types in a tissue is unique (Reinius et al., 2012; Gu et al., 2016). Thus, the data obtained from these studies is the weighted average of the methylation profiles of all the included cell types. While this may be informative, there may be meaningful data that is missed when individual cell types are not examined. For examples, Aslibekyan et al (2015) only identified global methylation differences in B cells in obesity, which were not observed in the PBMC fraction which contains B cells among the other mononuclear leukocytes. Thus, there is a larger

gap in our knowledge of single cell type DNA methylation and obesity, which needs to be further examined.

Another aspect that may be related to the conflicting results that have been identified in relation to DNA methylation and obesity are dietary differences. The previous studies examining the relationship between obesity and DNA methylation in blood cells have been conducted in different areas across the world, all of which will have their own culturally different diet. Even in different regions of the United States the diets can vary considerably representing a confounding factor when comparing these studies. Many different aspects of diet can influence DNA methylation. For example, the western diet (high fat, high calories, low in calcium, vitamin D, fiber, methionine, and choline) has been shown to decrease DNA methylation in mice (Choi et al., 2015). The bioactive compound epigallocatechin 3-gallate in green tea and genestein in soybeans, which are consumed in higher degrees in differing populations have also been shown to affect DNA methylation (Fang et al., 2007). Dietary differences in folate, a dietary methyl donor, have also been shown to affect DNA methylation levels (Niculescu and Zeisel, 2002). Many countries including the United States have folate fortification in the food supply, yet others do not. The difference in folate intake from countries who have fortification and those who do not may also impact DNA methylation levels. The maternal intake of different nutrients can also alter offspring's DNA methylation, representing another cultural dietary difference that could impact DNA methylation (Lillycrop and Burdge, 2015). Collectively these examples as well as many other dietary differences may provide a potential explanation for the conflicting results obtained from previous studies of DNA methylation and obesity.

Benefits of examining DNA methylation and obesity in blood cells

Examining DNA methylation in blood cells in relation to obesity has advantages. Blood is a minimally invasive tissue source, of which the different leukocytes can be easily isolated from (Hohos et al., 2016). Leukocytes are involved in the development and potentiation of the chronic low-grade inflammatory state in obesity, which is thought to be the link to the development of metabolic abnormalities (Mathis, 2013; Apostolopoulos et al., 2016). Thus, many of the studies examining DNA methylation in obesity have been performed in blood cells (**Tables 1.1-1.4**), yet only two studies have examined DNA methylation in individual leukocyte types (Simar et al., 2014; Aslibekyan et al., 2015).

There are seven major leukocyte types in blood including CD4+ T cells (helper cells), CD8+ T cells (cytoxic T cells), monocytes, natural killer (NK) cells, and B cells which are involved in the innate immune system, and the eosinophils and neutrophils which are involved in the adaptive immune system. Each of these cell types has a unique role in the immune response and in the development of inflammation in obesity (Elgazar-Carmon et al., 2008; Nishimura et al., 2009; Winer et al., 2009; Mathis, 2013; Huh et al., 2014). Additionally, DNA methylation has been shown to be involved in the regulation of leukocyte type specific genes. In example, hydroxymethylcytosine has been shown to be involved in the T cell development and differentiation (Tsagaratou et al., 2014). Further, methylation of *Foxp3* regulates the stimulation and development of Treg cells (Wang et al., 2013), which are decreased in obesity (Nishimura et al., 2009; Zuniga et al., 2010; Huh et al., 2014). CD4+ T cells have also been shown to involve DNA methylation in the activation of genes involved in the immune response which may be involved in the 'priming' of the memory T cells to carry out a response upon stimulation (Komori et al., 2015). Moreover, bacterial infection of neutrophils leads to changes in DNA

methylation and gene expression following infection (Sinclair et al., 2015). The seven peripheral leukocytes have also been shown to each have distinct DNA methylation profiles (Reinius et al., 2012). These features of leukocytes suggest that they will each have a unique DNA methylome in obesity, making them relevant cell types to assess the relationship of DNA methylation and obesity in. Furthermore, these leukocyte type specific differences in both function and DNA methylation make it critical to examine gene sequence specific DNA methylation in each cell type, yet this has only been performed in CD4+ T cells (Aslibekyan et al., 2015).

Rationale, hypotheses, and specific aims

Studying the DNA methylation profile in obesity represents an opportunity to learn more about the interaction between the environment and gene regulation in the obese and post obese state. DNA methylation is responsive to changes in the environment and has been shown to be altered with changes in diet and with measures of adiposity. Despite a surge in the number of studies on DNA methylation and obesity, there is still much to be learned from studying this relationship. The vast majority of the studies examining the DNA methylome in obesity have been performed in samples derived from mixed cell types, and in populations with both males and females of different ethnicities and ages. To help fill the gap in knowledge of single cell DNA methylation in obesity in a more focused manner, this dissertation has focused on determining the best leukocyte types to utilize for studies of DNA methylation, and then utilizing this knowledge to assess the DNA methylome of different peripheral leukocytes associated with obesity.

The overall hypothesis are that (1) each leukocyte type will have a different potential to respond via their methylome to physiological cues, (2) there will be DNA methylation differences in the obese women that are specific to each of the three leukocyte types assayed, and

(3) there will be changes in DNA methylation with weight loss in overweight and obese women. These hypotheses were testing in different study populations recruited from the Athens, GA area. Chapter 2 of this dissertation addresses the first specific aim which was to determine which leukocyte types are best to utilize for DNA methylation and obesity studies. Chapter 3 addresses the second specific aim, which was to measure the genome wide DNA methylation profile in CD4+ T cells, CD8+ T cells, and CD16+ neutrophils in a group of obese and healthy weight women to determine cell type specific methylation differences in obesity. Finally, chapter 4 addresses the last specific aim, which was to examine the genome wide DNA methylation profile in CD4+ T cells before and after a six-month weight loss intervention consisting of dietary and exercise components.

Two (Chapter 3 and 4) of the included dissertation studies were parts of larger projects. Chapter 3 was part of a folic acid supplementation study examining the effects supplementation on leukocyte DNA methylation in obese and healthy weight women (Principle Investigator: Lynn B. Bailey). Chapter 4 was part of a funded project by a grant from the National Cattleman's Beef Association entitled, Effects of a Higher Protein Weight Loss Diet and Exercise on Body Composition, Physical Function, and Fatigue in Overweight Older women (Principle Investigator: Ellen M. Evans).

Table 1.1. Global DNA methylation and obesity in blood cells

Study	Design	Study Population	Tissue/ Cell type	Methylation Methodology	Main Outcome
Adults					
1. (Keller et al., 2014)	Global DNA methylation levels were tested for associations with percent body fat and measures of glucose homeostasis in a population of Sorb individuals with a wide range of BMIs	N=559 (217 men) consisting of: N= 231 lean (38±14 yrs.) N=218 overweight (49±14 yrs.) N=108 obese (53±13 yrs.) From Germany, all white	Leukocytes	LUMA	Global methylation in the leukocytes was not associated with percent body fat or glucose homeostasis after correction for multiple testing
2. (Simar et al., 2014)	Global DNA methylation levels in obese with or without T2D were assessed in eight leukocyte populations	N=14 healthy obese males (35±6.7 yrs.) N=11 healthy lean male controls for health obese (34.8±3 yrs.) N=12 T2D obese (44.1±6.5 yrs.) N=7 healthy lean controls for T2D obese (44.1±6 yrs.) From Denmark	T cells (helper and cytotoxic), mononuclear cells, monocytes, lymphocytes, B cells, NK cells, PBMCs	Flow cytometry	Increased global methylation levels in B cells in obese and T2D. Increased global methylation in NK cells in T2D DNA methylation in B and NK cells was also correlated with Insulin resistance
3. (Na et al., 2014)	Examine global methylation of peripheral blood cells in a group of healthy Korean women with a	N=244 healthy women with a range of BMIs from 20 to 51(32±7.9 yrs.) From Korea	Peripheral blood	Pyrosequencing of Alu elements	A U-shaped association between BMI and Alu methylation was observed with the lowest methylation levels occurring at BMIs of 23 and 30

	range of BMIs				
Adolescents (<18 yrs.)					
4. (Perng et al., 2013)	Examine global DNA methylation in school aged children, with 30 month follow up data on anthropometrics.	N=533 (8.8±1.7 yrs.), 45.9% boys From Colombia	Peripheral leukocytes	Pyrosequencing of *LINE-1* DNA methylation	There was a non-linear inverse relation between LINE1 methylation and BMI and skinfold thickness ratio Boys with the lowest quartile of LINE 1 methylation had higher annual gains in BMI and skinfold thickness than the other quartiles.

Studies assessing global DNA methylation in blood cells which included an obese group and a comparison group, or a range of BMIs were included in the table. Only manuscripts with the full text available were included. Only data from the included studies relevant to global methylation and obesity in blood cells is provided. Studies are separated by those conducted in adults and those in adolescents.

Table 1.2. Genome wide analysis of DNA methylation and obesity in blood cells

Study	Design	Study Population	Tissue/ Cell type	Methylation Methodology	Main Outcome
Adults					
1. (Aslibekyan et al., 2015)	Examine DNA methylation in a population with a large range of BMIs and validate findings in separate cohorts.	N=991 (49±16 yrs.), 52% female All European Americans	CD4 T cells	HumanMethylation 450 BeadChip by Illumina	Eight loci had methylation levels associated with BMI and five loci had methylation levels associated with WC. Two genes *CPT1A* and *PHGDH* were validated in two large cohorts of similar age.
2. (Feinberg et al., 2010)	Examine DNA methylation in a population with a range of BMIs. Samples collected at two study visits of the Reykjavik Study. Included study visits were ~11 yrs. apart.	N=74 consisting of: Visit 6: N=48 (74.1±3.5 yrs.), 33% male Visit 7: N=64 (82.8±3.5 yrs.), 31% male With N=38 at both time points From Iceland	Lymphocytes	CHARM analysis examining 4.5 million CG sites	At visit 7, 13 regions had methylation levels associated with BMI. Four of these regions were also associated with BMI at visit 6 (*PM20D1, MMP9, PRKG1, RFC5)*. In *PM20D1* there was a 20% increase in methylation in the obese compared to the normal BMI groups. Three of the sites identified at visit 7 had conflicting associations with BMI between the two visits, suggesting an interaction of BMI and age.
3. (Dick et al., 2014)	Genome wide analysis of methylated CG sites in relation to BMI. Sites identified in discovery cohort were validated in	Discovery cohort: N=479 Replication cohort: N=339, 22% male Second replication cohort:	Whole blood	HumanMethylation 450 BeadChip by Illumina	In the discovery cohort. Five sites associated with three genes had methylation levels associated with BMI. Three of these sites were in *HIF3A*, and were all confirmed in the two replication cohorts.

	replication cohort. The sites replicated in the replication cohort were validated in the second replication cohort.	N=1789, 49% men All cohorts of European decent			
4. (Mansego et al., 2015)	To examine DNA methylation differences between two BMI categories: Low HRO (overweight and class 1 obesity) and high HRO (class 2 and 3 obesity).	N=73 (45±10 yrs.), 35.6% men All from Spain	WBC	HumanMethylation 450 BeadChip by Illumina	No differences in methylation were identified in relation to HRO Associations between DNA methylation and BMI and methylation of five sites were identified, with correlations to the expression levels of two genes (*GPR133, ITGB5*)
5. (Al Muftah et al., 2016)	Analyzed the association of DNA methylation and BMI or T2D in an Arab population.	N=72 females (39±16.9 yrs.) N=51 males (36.3±17.2 yrs.) All of Arabic decent from Qatar Validation cohort: N=810 twins (age range from 16 to 98 yrs.), >80% female All Caucasians from the UK	Whole blood	HumanMethylation 450 BeadChip by Illumina, with a correction applied for blood cell types in whole blood	In their Arab population, no sites had methylation levels associated with BMI, but one site in DQX1 was associated with T2D, which was replicated in the validation cohort.
6. (Demerath	Examine	N=2097	Leukocytes	HumanMethylaiton	Methylation was associated with

et al., 2015)	associations of DNA methylation and BMI or WC in a population of African Americans.	(56.2±5.7 yrs.), 36.4% male All African American		450 BeadChip by Illumina	BMI in 76 sites and WC in 164 sites. These identified sites included associations with *HIF3A, CPT1A,* and *ABCG1,* which have been identified in previous studies. 37 of the sites correlating with BMI and 1 correlated with WC were replicated in previous data sets of DNA methylation and blood in Caucasian adults. Additionally, 16 sites were replicated in a previous data set of DNA methylation in adipose tissue of women.
7. (Ollikainen et al., 2015)	Examine DNA methylation differences in clinically healthy young adult MZ twins discordant for BMI	N=40 twin pairs (27±3.3 yrs.), 42.5% male, 30 twin pairs discordant for BMI All Finish	Leukocytes	HumanMethylation 450 BeadChip by Illumina	No differences in methylation between twins with different BMIs. However, if the heavier twin had higher liver fat (N=13 twins), there were 1236 sites identified with differential methylation between the twins. 23 of the associated genes are known to be associated with obesity.
8. (Gomez-Uriz et al., 2015)	Examine DNA methylation in obese individuals, and obese individuals with stroke.	N=6 non-obese-non-stroke (69.8±6.9 yrs.) N=6 non-obese-stroke (74.6±3 yrs.) N=6 obese-non-stroke (67.5±5.8 yrs.) N=6 obese-stroke (66.5±6.7	Blood	HumanMethylation 27 BeadChip by Illumina Validation with Massarray Epityper	96 sites were differentially methylated between obese and lean and 59 sites had an interaction between stroke and obesity In the validation cohort of the subset of genes validated, only two sites in *KCNQ1* were replicated

		yrs.) All half male and from Spain Validation cohort: N=115 with a range of BMIs (age range of 50 to 80 yrs.) From Spain			
9. (Almen et al., 2014)	Examine effect of obesity and aging on DNA methylation	N=24 obese (57 yrs. ranging from 42-70) N=22 lean (55 yrs. ranging from 41-69) All females, from Latvia	Peripheral blood	HumanMethylation 27 BeadChip by Illumina	10 CG sites had methylation differences between the lean and obese groups, with one region, LINC00304, associated with obesity independent of age
Adolescents (<18 yrs.)					
10. (Wang et al., 2010)	Examine DNA methylation associated with obesity in African American male children	N=7 lean (15.9±1.4 yrs.) N=7 obese (15.8±1 yrs.) All African American males Validation cohort: N=46 obese (20.3±5 yrs.) N=46 lean (17.6±3.1 yrs.) All African American males	Peripheral blood leukocytes	HumanMethylation 27 BeadChip, validation with pyrosequencing	No sites were associated with obesity after correcting for multiple testing. Yet, six sites that were with associated with obesity at lower significance levels were validated with pyrosequencing (*UBASH3A*, *TRIM3*, *CTSZ*, *HIPK2*, *CDH5*, *CREB3L3*). Pyrosequencing confirmed methylation of *UBASH3A* was higher in obese, and *TRIM3*, *HIPK3*, and *CDH5* were lower in obese.

11. (Huang et al., 2015a)	Examine DNA methylation in obese children	N=78 obese (12.6 yrs. with range of 9.4 to 13.7 yrs.) N= 71 lean (12.9 yrs., with range of 10.7 to 14.1 yrs.) All from western Australia	Whole blood	HumanMethylation 450 BeadChip by Illumina, validation via pyrosequencing For initial analysis, obese and lean samples were pooled into four samples for each group. Pyrosequencing was performed in individuals	129 differentially methylated sites were identified in the obese children, with 80 unique genes having greater than 10% difference in methylation. Validation of results were conducted for three genes (*FYN, PIWIL4* and *TAOK3*), all three of which were replicated
12. (Ding et al., 2015)	Examine DNA methylation an obesity in preschool aged children	N=32 obese (5±0.73 yrs.) N=32 controls (5±0.69 yrs.), 19 females in both groups. From China	Peripheral blood	NimbleGen Human DNA methylation 385K Promoter Plus CG Island Microarray, validation with bisulfite sequencing	Compared to lean children, 251 promoters and 575 CGIs were demethylated in the obese children, while 141 promoters and 277 CGI were hypermethylated in the obese children. The correlation of methylation and obesity was validated in four genes (*FZD7, PRLHR, EXOSC4, EIF6*)

Studies assessing genome wide DNA methylation in blood cells which included an obese group and a comparison group or a range of BMIs were included in the table. Only manuscripts with the full text available were included. Only data from the included studies relevant to global methylation and obesity in blood cells is provided. Studies are separated by those conducted in adults and those in adolescents.

Table 1.3. Candidate gene analysis of DNA methylation and obesity in blood cells

Study	Design	Study Population	Tissue/ Cell type	Methylation Methodology	Main Outcome
Adults					
1. (Houde et al., 2015)	Examined DNA methylation levels of *LEP* and *ADIPOQ*, which they had shown to be associated with BMI, WC, and LDL-cholesterol in adipose tissue, to see if methylation differences were similar.	N=73 severely obese (34.7±7.1 yrs.), 33 men All from Canada	Blood	Bisulfite pyrosequencing	*LEP* methylation levels in blood cells were negatively associated with BMI, the opposite relationship of what was observed in SAT in their study population. Fasting LDL levels were positively correlated with methylation of two sites in *LEP* in blood, which was also observed in SAT.
2. (Remely et al., 2014)	Examined the DNA methylation of five CG sites associated with *FFAR3* in obese, T2D, and lean controls at baseline and after a four month intervention of nutrition counselling and GLP-1 agonist treatment for T2D.	N=14 obese (39±15 yrs.), 7 males N=24 T2D (58±9), 10 males N=18 lean controls (25±3), 15 males All from Austria	Blood	Bisulfite conversion followed by PCR	There were lower methylation levels in *FFAR3* in the obese and T2D groups than the lean controls. The methylation levels were slightly increased over the intervention. There were also significant inverse correlations between BMI and methylation of FFAR3.
3. (Bollati et al., 2014)	Examine the DNA methylation profile of *CD14*, *ET-1*,	N=165 (mean age of 50 yrs. with interquartile range	Blood	Bisulfite conversion followed by PCR	No associations between BMI and DNA methylation was found in any of the genes. Positive

	iNOS, HERV-w, and *TNFα* in overweight and obese adults to test associations between BMI and measures of serum cholesterol.	of 43 to 58 yrs.), 20% male All from Italy			associations between DNA methylation of *TNFα* and LDL, TC/HDL, and LDL/HDL cholesterol.
4. (Stepanow et al., 2011)	Examined DNA methylation in a 315 bp region of *MCHR1*, which contains 2 SNPs associated with obesity.	N=49 (21 to 77 yrs.), 11 males All from Germany	Blood	Bisulfite sequencing	The GT allele of one of the SNPs had decreased methylation status with increasing BMI, where the methylation of the AC allele is not associated with this phenotype. In cell culture they show that this gene has SNP dependent transcription which is abolished by treatment with the DNA methylase inhibitor 5-aza-2′-deoxycytidine.
5. (Al Muftah et al., 2016)	Examined DNA methylation levels of 39 sites previously associated with BMI and eight sites previously associated with T2D in Caucasians in their Arab population.	N=72 females (39±16.9 yrs.) N=51 males (36.3±17.2 yrs.) All of Arabic decent from Qatar Validation cohort: N=810 twins (age range from 16 to 98 yrs.), >80% female All Caucasians from the UK	Blood	HumanMethylation 450 BeadChip by Illumina, with a correction applied for blood cell types in whole blood	Seven of the sites previously associated with BMI were replicated (*SOCS3, SREBF1, SBNO2, CPT1A, PRR5L, LY6G6E* and cg03078551*)* and one site previously associated with T2D (*TCNIP*).
6. (Hermsdorf f et al.,	Examined the relationship	N=40 high and low trunk fat women	WBCs	Sequome EpiTyper (promoter region -	Women with higher trunk fat had lower methylation in two

2013)	between *TNFα* methylation and central adiposity in young women.	(21±3 yrs.), all normal weight All in Spain		170 to 359)	sites of *TNFα*. The methylation levels of one of these sites correlated with circulating TNFα levels.
7. (Na et al., 2015)	Examined the methylation of *IL6, TNFα, TFAM,* and *GLUT4* in a group of Korean women with a range of BMIs.	N= (31.9±7.8 yrs.), all women All from Korea	Blood	Methylation specific PCR	Obese women had increased methylation of IL6 compared to normal weight women and the overweight women.
Adolescents (<18 yrs.)					
8. (Mansego et al., 2016)	Examined DNA methylation of miRNA coding regions in obese and lean children.	N=12 obese (10.6±.4 yrs.) N=12 lean (10.8±.3 yrs.) 50% males All Caucasian, from Spain Validation cohort: N=95 (10-16 yrs.)	Blood	HumanMethylation 450 BeadChip Validation with mass array	In miRNA coding regions, 16 sites were differentially methylated in the obese children. DNA methylation levels at miR-1203, miR-412 and miR-216A significantly correlated with BMI-SDS score and explained up to 40% of the variation in BMI-SDS. Validation of the three miRNAs was found between the obese and lean children.
9. (Wang et al., 2015)	Determine if DNA methylation in *HIF3A*, which has previously been shown to have altered methylation in obesity, is altered in obese Chinese children.	N=110 severely obese (11.1±2.6 yrs.), males N=110 normal weight controls (1.02±2.6 yrs.) All Chinese	Blood	Sequome MassArray	There was significantly higher methylation n two sites of *HIF3A* in the obese children. Positive associations between DNA methylation od *HIF3A* and ALT levels found a positive correlation for methylation and ALT levels after adjusting for age, gender, and BMI.

10. (Carobin et al., 2015)	Examined the methylation profile of *SNRPN* in obese and lean adults and children.	N=10 obese children (12±2.7 yrs.), 5 male N=10 lean children (10±2.6 yrs.) N=15 obese adults (40±10.3 yrs.) 7 male N=15 lean adults (35±12.8 yrs. 7 male All from Brazil	Blood	Bisulfite conversion followed by PCR	No differences in DNA methylation of *SNRPN* and obesity were identified. There were correlations between methylation and age.
11. (Wu et al., 2015a)	Examined the DNA methylation levels of the promoter of *FAIM2* in obese and lean children with different levels of activity.	N=59 obese (8-18 yrs.) N=39 lean (8-18 yrs.), mixed males and females All from Beijing	Blood	Sequome MassArray	7 sites had differences in DNA methylation between the obese and lean children with low levels of sedentary behavior. Only two differences between the obese and lean children who had high levels of sedentary behavior. One site had methylation differences between the two groups with low levels of high activity and two sites in those with high levels of high activity. There were four sites identified with differences in methylation between the obese and lean children who met physical activity guidelines.
12. (Wu et al., 2015b)	Examined DNA methylation of the *FAIM2* promoter in obese and lean	N=59 obese (8-18 yrs.) N=39 lean (8-18 yrs.), mixed males	Leukocytes	Sequome MassArray	Methylation at eight sites were differentially methylated between the obese and lean children and several sites with

	Chinese children.	and females All Chinese			dyslipidemia after adjusting for age, gender and BMI
13. (Dave et al., 2015)	First they identified differences in gene expression between the obese and lean children in blood. They then examined the DNA methylation levels of the differentially expressed genes in the obese and lean children.	N=69 (9.1±.1 yrs.), 50% obese, 50% male All Mexican American	Blood clots	HumanMethylation 450 BeadChip by Illumina	In the differentially expressed genes, *ADIPOR1* and *PPARγ,* DNA methylation. Identified sites whose methylation levels correlated with gene expression, but there were no association with BMI or WC.
14. (Su et al., 2014)	Examined the methylation levels of 117 previously identified genes from GWAS for obesity and its related traits in there population of African American youths. Their results were validated in four different cohorts of youth.	N=7 obese (15.8±1 yrs.) N=7 controls (15.9±1.4 yrs.) All African American males Validation cohorts: all three validation cohorts contained only adolescents	Blood	HumanMethylation 27 BeadChip by Illumina Validation cohort 1 used HumanMethylation 450 BeadChip by Illumina, Remaining validation cohorts with pyrosequencing	In the first population, 89 of the 117 sites were included on the array and only four sites in *LEPR*, *SNRPN*, *KREMEN1*, and *LY86*, were differentially methylated in the obese children. In the first validation group, only *LY86* had differences in methylation in the obese children, and was validated in the remaining cohorts.
15. (Garcia-Cardona et al., 2014)	Examine correlations between DNA methylation of *LEP*	N=102 (10 to 16 yrs.), 66 males All from Mexico	Blood	Methylation specific PCR	Obese adolescents without insulin resistance had the same methylation levels in two sites of *LEP* as the lean adolescents. The

	and *ADIPOQ* and obesity and insulin resistance in a group of adolescents with a range of BMIs.				methylation levels of these sites in the obese with insulin resistance children were decreased. The same relationship was identified in one site of *ADIPOQ.* One site in *LEP* was methylated in all the normal weight children, but only in 79% of overweight and 56% of obese and 36% in morbid obese, with a similar response in another site as well. Similarly, one site in the *ADIPOQ* promoter had 3% methylation in normal weight, 0% in overweight, 51% in obese and 46% in morbid obese adolescents. *No differences were observed between sexes, so they were combined.*
16. (Deodati et al., 2013)	Examine DNA methylation of *IGF2* with the body composition indices and metabolic status is obese adolescents.	N=84 obese (11.6±2.1 yrs.), 44 males All from Italy	Lymphocytes	DNA methylation by restriction enzyme digest	There were no correlations between body composition measures and methylation. Percentage of methylated sites was related to TG levels, TG/HDL and C-peptide levels.
17. (Milagro et al., 2012)	Examined the association of DNA methylation of *CLOCK*, *BMAL1*, and *PER2* in	N=60 (yrs.) women From	WBCs	Sequome MassArray	Differences in methylation between normal weight and the overweight and obese groups were observed in *CLOCK* and *BMAL1*.

	normal weight, overweight and obese women				

Studies assessing DNA methylation and obesity in blood cells in a candidate gene approach were included if the full text was available. Additionally, studies that examined methylation in an obese group and control group, or a range of BMIs were included. Only information relevant to candidate gene methylation status in obesity is included from the studies in the table. Studies are separated by those conducted in adults and those conducted in adolescents.

Table 1.4. DNA methylation and weight loss in blood cells

Study	Design	Study Population	Tissue/ Cell type	Methylation Methodology	Main Outcomes
Adults					
1. (Crujeiras et al., 2013)	Examined the methylation level of *LEPR, POMC, GHSR, NPY*¸ six months after an 8 week 30% energy restricted diet by weight regain. Participants who lost ≥5% BW, but gained ≥10% of the weight back were classified as regainers, while those who lost ≥5% BW and gained N=7 regainers (lost at least 5%, but gained ≤10% back were classified as non-regainers.	N=7 regainers N=11non-regainers, adults, specific age not provided All men from Spain	Leukocytes	MALDI-TOF mass spectrometry	The methylation level of *LEPR* CG 7 was lower in regainers, but did not correlate with the percentage weight regain. *POMC* CG's 10-11 showed higher methylation levels in regainers and was directly associated with weight regain. Regainers has lower methylation in CG4-5 and 8-9 of *NPY* than the non regainers and in the total region of *NPY* examined. Pretreatment methylation levels correlated inversely with weight loss regain.
2. (Duggan et al., 2014)	Examined global methylation in overweight and obese post-menopausal women after a one year RCT comparing independent and combined effects of reduced caloric intake and exercise program compared to controls.	N=300 (58±5.1 yrs.) women All from Seattle, WA	Leukocytes	*LINE-1* methylation via pyrosequencing	No significant differences in *LINE-1* methylation levels were detected in any intervention group v controls. The magnitude of weight loss was not associated with *LINE-1* methylation at one year.
3. (Martin-	Examine the effects of a	N= 155 control	Blood	Pyrosequencing	Both *LINE-1* and *SCD1* gene

Nunez et al., 2014)	one year weight loss intervention on both global methylation and the promoter region of *SCD1*. The intervention consisted of a control group only receiving general dietary recommendations and PA guidelines and an intervention group with intensive lifestyle intervention and followed the Mediterranean dietary pattern.	group (53.7±7.8 yrs.), 34.8% male N=155 intervention group (53.5±8.15 yrs.), 47.5% male All from Spain			promoter methylation, were similar at baseline. After one year methylation levels of both were higher in the control group. Weight loss was associated with higher levels of *SCD1* promoter methylation after the intervention. Weight change was associated with changes in *LINE-1* methylation regardless of the group, and those who did not lose weight showed higher levels of *LINE-1*
4. (Milagro et al., 2011)	Compare DNA methylation profiles of high and low responders to a hypocaloric 8 week diet intervention in overweight and obese men.	N=25 adults, age not provided, all men from Spain	PBMCs	HumanMethylation 27 BeadChip by Illumina MALDI-TOF for validation	DNA methylation levels in several CG sites in *ATP10A* and *CD44* had baseline differences depending on weight loss outcome. After the intervention, DNA methylation of several CGs in the *WT1* promoter had higher methylation levels in high responders. CG sites in *WT1* and *ATP10A* were modified as a result of the intervention.
5. (Nicoletti et al., 2015)	Determine if two weight loss by an energy restricted Mediterranean diet would affect global DNA methylation, hydroxymethylation or	N=9 control normal weight (35-65 yrs.) N=33 energy restricted obese (35-65 yrs.) All women from	Buffy coat DNA	Global hydroxyl-methylation by ELISA *LINE-1* for global methylation, and MS-HRM for gene	Baseline *LINE-1* methylation was associated with serum glucose levels whereas baseline hydroxymethylation was associated with BMI, WC, total cholesterol and TG.

	methylation patterns of inflammatory genes (*IL-6, SERPINE-1*).	Brazil and Spain		specific analysis	*LINE-1* and *SERPINE-1* methylation levels did not change after weight loss. *IL6* methylation was increased after energy restricted diet An association between *SERPINE-1* methylation and weight loss response was found.
6. (Huang et al., 2015b)	Assess methylation patterns in individuals who are obese, normal weight, or who were obese and lost sufficient weight to be of a normal weight (SWLM).	N=16 SWLM (44.4±8.5 yrs.), N=16 obese (47.9±7.7 yrs.), N=16 normal weight (49.8±10.3 yrs.) Mixed ethnicities, all 87.5% female	Buffy coat DNA	HumanMethylation 450 BeadChip by Illumina Used a correction for leukocyte types	No differences after FDR correction. Potentially differentiated methylated sites across the three groups were observed in *RYR1, MPZL3* and *TUBA3C*. In 32 obesity related genes, differential methylation profiles were found between groups in *BDNF*. In *RYR1, TUBA3C* and *BDNF*, SWLM differed from obese but not normal weight.
7. (Perez-Cornago et al., 2014)	Examined 20 CG sites associated with *HTR2A* after a RTC weight loss intervention. Study groups consisted of control diet and modified energy restricted diet (both 30% E restricted) for six months. Samples were pooled into one group for methylation	N=41 (49±1 yrs.), mixed males and females all with metabolic syndrome and all Caucasian	WBC	HumanMethylation 450 BeadChip by Illumina	A positive association of *HTR2A* methylation with waist circumference and insulin levels were observed at baseline. After the intervention, lower mean *HTR2A* gene methylation as baseline was associated with major reductions in BW, BMI and fat mass. Mean *HTR2A*

	analysis.				methylation at baseline significantly predicted the decrease in depressive symptoms after the weight loss treatment.
8. (Samblas et al., 2016)	Examine the DNA methylation profile of *BMAL1, CLOCK,* and *NR1D1* after an energy restricted weight loss intervention in overweight and obese women. Intervention included both dietary and exercise components for four months, followed by a five month maintenance period.	N=61 (42.2±11.4 yrs.) all women, from Spain	Blood	MassArray Epityper	The methylation status of different CGs in *BMAL1* and *NR1D1* was modified as a result of the intervention. Baseline methylation in *BMAL1* positively correlated with energy and carbohydrate intake and negatively correlated with the effect of the weight loss intervention on total cholesterol and LDL cholesterol.
9. (Campion et al., 2009a; Campion et al., 2009b)	Examine the association of the DNA methylation profile of *TNFα*'s promoter in obese men and women with an eight week energy restricted intervention.	N=24 (34±4 yrs.) 50% male All from Spain	PBMC	Bisulfite conversion and pyrosequencing	Obese men with successful weight loss (≥5% initial body weight) showed lower levels of total *TNFα* promoter methylation at baseline.
10. (Milagro et al., 2012)	Examined the association of DNA methylation of *CLOCK, BMAL1,* and *PER2* at baseline in normal weight, overweight and obese women with the success of a	N=60 (23-53 yrs.) all women, from Spain	WBCs	Sequome MassArray	Methylation pattern at different sites in these genes showed correlations with BMI, adiposity and Metabolic syndrome score. Baseline methylation levels of *CLOCK* (1 site) and *PER2* (4 sites) were correlated with the

	16 week weight loss program with energy restriction following the Mediterranean diet and PA recommendations.				magnitude of weight loss.
Adolescents (<18 yrs.)					
11. (Moleres et al., 2013)	Look at baseline changes in DNA methylation that could be associated with better weight loss following a 10 week multidisciplinary intervention program in overweight or obese adolescent boys.	N=24 (12-15 yrs. old) 42% male All from Spain	Blood	HumanMethylation 27 BeadChip by Illumina Validation with MALDI-TOF for all participants	After validation, five regions located in/near *AQp9, DUSP22, HIPK3, TNNT1* and *TNNI3* had different methylation levels between high and low responders. A calculated methylation score was significantly associated with changes in weight, BMI and body fat mass loss after intervention.

Studies assessing DNA methylation in blood cells with weight loss were included if the full text of the manuscript was available. Only the information relevant to methylation and weight loss in blood cells is included. Studies are separated by those performed in adults or adolescents.

CHAPTER 2

DNA CYTOSINE HYDROXYMETHYLATION LEVELS ARE DISTINCT AMOUNG NON-OVERLAPPING CLASSES OF PERIPHERAL LEUKOCYTES[1]

[1] Hohos NM, Lee K, Ji L, Yu M, Kandasamy MM, Philips BG, Baile CB, He C, Schmitz RJ, Meagher RB. 2016. *Journal of Immunological Methods.* Doi: 10.1016/j.jim.2016.05.003. Reprinted here with permission of the publisher.

Abstract

Background: Peripheral blood leukocytes are the most commonly used surrogates to study epigenome-induced risk and epigenomic response to disease-related stress. We considered the hypothesis that the various classes of peripheral leukocytes differentially regulate the synthesis of 5-methylcytosine (5mCG) and its removal via Ten-Eleven Translocation (TET) dioxygenase catalyzed hydroxymethylation to 5-hydroxymethylcytosine (5hmCG), reflecting their responsiveness to environment. Although it is known that reductions in TET1 and/or TET2 activity lead to the over-proliferation of various leukocyte precursors in bone marrow and in development of chronic myelomonocytic leukemia and myeloproliferative neoplasms, the role of 5mCG hydroxymethylation in peripheral blood is less well studied.

Results: We developed simplified protocols to rapidly and reiteratively isolate non-overlapping leukocyte populations from a single small sample of fresh or frozen whole blood. Among peripheral leukocyte types we found extreme variation in the levels of transcripts encoding proteins involved in cytosine methylation (DNMT1, 3A, 3B), the turnover of 5mC by demethylation (TET1, 2, 3), and DNA repair (GADD45A, B, G) and in the global and gene-region-specific levels of DNA 5hmCG (CD4+ T cells >> CD14+ monocytes > CD16+ neutrophils > CD19+ B cells > CD56+ NK cells > Siglec8+ eosinophils > CD8+ T cells).

Conclusions: Our data taken together suggest a potential hierarchy of responsiveness among classes of leukocytes with CD4+, CD8+ T cells and CD14+ monocytes being the most distinctly poised for a rapid methylome response to physiological stress and disease.

Key words: 5-hydroxymethylcytsine, surrogate cells, disease, epigenetic control, epigenome-induced risk, cellular memory

Highlights

- Reiterative isolation of several non-overlapping leukocyte types from fresh or frozen blood
- Leukocytes suitable for cell type specific epigenetic analysis
- Classes of peripheral leukocytes distinctly regulate factors for the synthesis and removal of 5mCG
- Classes of peripheral leukocytes are distinct in their levels and distribution of 5hmCG
- CD4+, CD8+ and CD14+ cells appear to be poised to respond via changes in 5mCG

1. Background[2]

Peripheral blood leukocytes are the most commonly used cell types to assess human disease states (Javierre et al., 2010; Huang et al., 2014; Di Francesco et al., 2015; Ellinger et al., 2015). Because of their accessibility, leukocytes are used in preference to other tissues such as the brain, muscle, adipose tissue, bone or various non-blood-borne cancer cells, even when these latter cell types are the focus of disease. As a result, the methylome and transcriptome of peripheral blood leukocytes often act as proxies for disease states centered in other tissues and cell types. One implicit assumption is that genetic and epigenetic reprograming caused by disease states in other tissues are systemically reflected in blood-borne leukocytes. There are numerous published studies examining the response of genome-methylation in whole blood

[2] ***Abbreviations:*** ACTB (cytoplasmic beta actin), DNMT1, 3A, 3B (DNA methyltransferase 1, 3A, 3B), CG (cytosine-guanine dinucleotide), CNS (conserved noncoding sequence), CT (cycle threshold), GADD45A, B, G **(**Growth arrest DNA damage inducible proteins 45a, 45b, 45g), 5hmC (5´-hydroxymethylcytosine), GO (gene ontology), 5hmCG (5-hydroxymethylcytosine-guanine dinucleotide), IFM (Immuno-Fluorescence Microscopy), 5mC (5´-methylcytosine), 5mCG (5´-methyl CG dinucleotide), MBD2, MBD4, MeCP2 (Methyl-CpG Binding Domain Proteins), NLP (natural log p-value), nt (nucleotide), PE (phycerythrin), RQ (Relative Quantity), SLE (systemic lupus erythematous), TAB-Seq (TET assisted bisulfite sequencing), TSS (transcription start site), TTS (transition termination site), TET1, 2, 3 (Ten-eleven Translocation Enzymes 1, 2, 3, methylcytosine dioxygenases 1, 2, 3).

leukocytes to diseases and disease progression (Javierre et al., 2010; Wang et al., 2010; Weiss et al., 2011; Saied et al., 2012; Schroeder et al., 2012; Smith et al., 2012; Sun et al., 2013; Xu et al., 2013; Almen et al., 2014; Smith et al., 2014). However, the genome-wide methylation data from these studies comprises the epigenomic information of several major leukocyte cell types combined as a weighted average of their fractional representation in blood.

Epigenetics, from its modern inception, predicts that different cell types within a tissue or organ are epigenetically distinct (Nanney, 1958). Reinius et al (2012) made pairwise comparisons of the methylomes of seven major leukocyte types to reveal that even the two most closely related peripheral blood mononuclear cells (PBMCs), CD4+/CD8± and CD8+/CD4± T cells, differ significantly in DNA methylation levels. Over 45,000 of the 485,000 cysteine-guanine dinucleotide (CG) sites measured (~9%), which are concentrated in gene regions, are distinct. Equally remarkable, the PBMC CD8+ T cells and granulocyte Siglec8+ eosinophils differ in approximately 193,000 of the CG sites measured (~40%) (Reinius et al., 2012). Different leukocyte types all have quantitatively different global methylation profiles, with the relatively hypomethylated granulocytes (Siglec8+ eosinophils, CD16+ neutrophils) showing 5 to 10 times less methylation across different gene regions than the hypermethylated PBMC lymphoid cell types (CD4+ and CD8+ T cells, CD19+ B cells, CD56+ NK cells, CD14+ monocytes) (Reinius et al., 2012). When these cell types are analyzed together in DNA methylation studies using total peripheral blood DNA, the methylation profiles from the seven leukocyte types with high and low levels of methylation, as well as their sequence specific differences in methylation, are obscured as a weighted averaged depending upon cell type frequency. Many statistically significant differences in the methylation profiles of individual cell

types are lost, making the results from whole blood less intelligible and less meaningful than they would be from individual cell types.

In the Reinius et al (2012) study they started with large whole blood samples, separated PBMCs and granulocytes by density centrifugation on Ficoll-Paque gradients and lysed the remaining red blood cells with NH_4Cl. Seven leukocyte subtypes were then isolated from separate aliquots of the PBMC and granulocyte fractions by immuno-paramagnetic bead capture. Fluorescence activated multichannel cell sorting (FACS) can also be used to isolate various classes of leukocytes after Ficoll gradient separation (Roederer et al., 1997; Melzer et al., 2015). Herein, as a potentially less expensive and rapid alternative to these two methods we develop the approach of first lysing red blood cells by NH_4Cl treatment or by freezing and thawing and then reiteratively isolating six or seven cell types on immuno-paramagnetic beads starting from a single small starting sample. A reiterative approach (Lyons et al., 2007) has the potential to eliminate or include specific cell types expressing overlapping markers in each isolated class, depending upon the order of isolation, and providing relatively pure distinct leukocyte populations for subsequent epigenetic analysis. The utility of examining non-overlapping populations of leukocytes is demonstrated herein by our own analysis of 5hmCG levels and supports the previous benefits of reiterative methods presented in Lyons et al (2007).

Recent evidence suggests the gene sequence-specific differences in 5mCG levels at 20% of CG sites are tissue specific and may be important to changes in gene regulation, whereas 80% of global 5mCGs appear to have little impact on gene expression (Lister et al., 2013; Meagher, 2014; Wu and Zhang, 2014; Gu et al., 2016). By contrast, 5hmCG levels are much lower than 5mCG levels, but highly correlated with differential gene regulation (Mellen et al., 2012; Lister et al., 2013; Tsagaratou et al., 2014). 5hmCG is concentrated in euchromatin (Ficz, 2015), which

has an open-chromatin conformation facilitating transcriptional regulation. Genes and gene regions enriched for 5hmCG are said to be "poised" to be differentially regulated (Pastor et al., 2011). For example, during chondrogenesis there is an increase in 5hmCG associated with important chondrogenic genes, while minimal changes in 5hmCG were observed in housekeeping genes (Taylor et al., 2016). Finally, 5hmCG enriched gene sets appear to be relatively distinct for each tissue (Nestor et al., 2012). Therefore, it is reasonable to propose that differences in gene region specific 5hmCG levels among peripheral blood might reflect their potential to respond to their environment with changes in gene expression.

We recently presented strong evidence that the majority of functional 5mCG sites, turn over rapidly (**Figure 2.1**), with half-lives of less than an hour (Meagher, 2014). Many of the proteins essential to the establishment and maintenance of 5mCG (DNA methyltransferases, DNMT1, DNMT3A, DNMT3B), removal of 5mCG by oxidation to 5hmCG (Ten eleven translocation dioxygenase, TET1, 2, 3), and repair back to cytosine (Growth arrest and DNA damage induced, GADD45A, B, G) are expressed in leukocytes (Calabrese et al., 2014). TET catalyzed oxidation may be a rate-limiting step in 5mCG removal (Sabag et al., 2014; Wu and Zhang, 2014), and hence, the model presented in **Figure 2.1** emphasizes the central importance of TET activity to the turnover of modified cytosine and its potential impact on gene regulation.

There are limited data showing the importance of the DNA methylation cycle (**Figure 2.1**) to peripheral blood leukocytes. TET enzyme oxidation of 5mCG to 5hmCG is critical in T cell development as well as in the expression of T cell lineage specific genes (Tsagaratou et al., 2014; Ichiyama et al., 2015). RNAi mediated silencing of TET2 in cord blood progenitor CD34+ cells lowers 5hmCG levels and skews differentiation toward granulocytes and away from lymphoid and erythroid lineages (Pronier et al., 2011). When both TET2 and TET3 are mutated in

zebrafish, there are reductions in the number of hematopoietic stem cells emerging during embryonic development (Li et al., 2015). Furthermore, TET2 mutations are implicated in the development of T-cell lymphomas, myeloproliferation, and myeloid malignancies, where bone marrow precursor cells are affected (Moran-Crusio et al., 2011; Pronier et al., 2011; Muto et al., 2014). Although the relationship of the cytosine modification cycle to health is limited, it is reasonable to consider that each of the divergent leukocyte lineages may regulate the cycle differently.

The roles for TETs and 5hmCG in leukocyte development led us to hypothesize that *various classes of peripheral leukocytes differentially regulate the establishment of 5mCG and its removal via oxidation to 5hmCG* (**Figure 2.1**). These and other data also point to potential cause-and-effect relationships, that these differences in sequence specific 5mCG and 5hmCG impart to each cell type more or less potential to respond to physiological stresses and disease and in a cell type specific manner. We looked for initial evidence that non-overlapping leukocyte classes, isolated by our reiterative isolation protocol, might vary in the expression of machinery controlling the rates of 5mCG turnover, through changes in their DNA cytosine hydroxymethylome. Our results identify CD4+ T cells and CD14+ monocytes as having the highest levels of 5hmCG, but identified CD8+ T cells as having the highest levels of TET gene expression that might reflect turnover rates.

2. Results

2.1 Isolation of cell populations

After a number of preliminary studies, we developed three different isolation methods to successively and rapidly isolate a few to seven leukocyte types (helper T cells, inflammatory T cells, monocytes, neutrophils, B cells, natural killer cells, and eosinophils) from single 5 ml

samples of fresh or frozen whole blood as summarized in **Figure 2.2.** The three methods included: (1) the isolation of CD4+ T cells, CD8+ T cells, and CD14+ monocytes directly from whole fresh blood, (2) the isolation of six or seven leukocyte types from whole blood using prior red blood cell lysis, and (3) the isolation of six or seven leukocyte types from frozen whole blood.

In determining the order of isolation that would yield the purest samples of the seven leukocyte types, we had to consider that each of the seven leukocyte populations are complex and often express more than one of the common plasma membrane antigens (PMAs) used to isolate each population (**Supplemental Table 2.S1)**. Our results represent an attempt to optimize isolation of defined leukocyte populations free of unwanted cell types without seriously compromising the recovery of cell types. Three different orders of isolation were identified, where isolation order A was used for isolation method 1 and isolation order B was found to yield the purest cell populations for the isolated cell types (methods 2 and 3) with the exception of one cell type, NK cells. Isolation order C resulted in relatively pure populations of some of the leukocyte types (e.g., CD16+ neutrophils), but not others, and is shown to highlight the importance of the order of isolation in recovering pure cell populations.

The efficiencies of recovery of leukocyte types from each isolation method are estimated in **Table 2.1.** Method 1 produced the highest recovery of CD4+ T cells and CD14+ monocytes while Method 2 generated the highest recovery of CD8+ T cells, CD16+ neutrophils, CD19+ B cells, CD56+ NK cells, and Siglec8+ eosinophils. In general there was a 30 to 80% reduction in recovery depending upon leukocyte types for Method 3, resulting from cell lysis during the freeze-thaw process.

2.1.2 Confirmation of purity of isolated cell types

Initial analysis of the purity of the seven isolated cell types was performed using the four distinct nuclear morphologies (round for CD4+, CD8+, CD19+, CD56+ cells; kidney shaped for CD14+ and CD56+ cells; multilobular for CD16+ cells; bilobed for Siglec8+ cells) of peripheral blood leukocytes (Alberts, 1994). Purity was assessed based on the absence of three uncharacteristic nuclear phenotypes for six cell types with relatively unique morphologies. For CD56+ NK cells the estimate is based on the absence of two nuclear morphologies, multilobed and biolobed. The fluorescent nuclear morphology we observed for the seven isolated leukocyte types after staining with DAPI and propidium iodide (PI) are shown in **Figure 2.3**. After examining several fields of cells by fluorescent microscopy, each cell type isolated by the methods and isolation orders described in **Figure 2.2** and **Table 2.1**, were found to be at least 95% pure, thus meeting our criteria to be initially considered as a highly enriched cell population pending qRT-PCR transcript analysis. Some of the isolated cell types were bound to numerous dynabeads during the isolation process, making the nuclear morphology of these cells difficult to assess. Cells with obscured nuclear morphologies are not included in our estimates of purity. Purity was further confirmed through qRT-PCR analysis of leukocyte-specific transcripts.

Most studies of isolated leukocytes use cytometry to demonstrate that the cell types express common PMAs consistent with expectations (i.e., CD4+ T cells are CD4+/CD3+/CD14-) (Reinius et al., 2012). However, this approach does not quantitatively address the level of contamination from other unwanted cell types, without the use of many other marker antibodies during cytometry. The binding of multiple large Dynabeads to cells complicates cytometry and bead removal may damage cells. Therefore, we developed qRT-PCR assays using a panel of eight leukocyte-specific marker transcripts to assess the purity of the seven isolated cell types. The relative quantity (RQ) of expression of each transcript in each cell type is shown in

Supplemental Figure 2.S1A (isolation order B, yielding the most pure leukocyte populations) and isolation order C (shown to demonstrate the importance of isolation order) in **Supplemental Figure 2.S1B**. Unexpectedly high levels of CD4 mRNA were observed in CD14+ monocytes and CD16+ neutrophils (**Supplemental Figure 2.S1A**). Therefore, IFM was used to assess CD4 protein localization in these cell types and in CD4+ T cells (**Supplemental Figure 2.S2**)**.** We found CD4 protein in the membrane of isolated CD4 cells, but only in the cytoplasm of CD14+ monocytes and CD16+ neutrophils. The results of this analysis provided evidence that the CD4 protein is only trafficked to the membrane in the CD4+ T cells. Considering the IFM analysis of nuclear morphology showed no round nuclei in either the isolated monocytes or neutrophils, we can conclude that there is no contamination of CD4+ T cells in these isolated cell populations.

As shown in **Supplemental Figure 2.S1**, NK cells captured using the CD56+ antigen express transcripts for many of the common PMAs used for cell isolation (**Supplemental Table 2.S1**), and thus represent a heterogeneous population of cells (Kelly-Rogers et al., 2006; Poli et al., 2009). If the order of the isolation is manipulated to isolate CD56+ NK cells last, very few cells remain, as the majority of CD56+ cells have been isolated in the other cell populations as sub-types of these cell populations. This is due to the co-expression of CD16 (Kelly-Rogers et al., 2006; Poli et al., 2009; Accomando et al., 2014), CD4 (Kelly-Rogers et al., 2006; Zloza and Al-Harthi, 2006; Gruenbacher et al., 2009), CD8 (Kelly-Rogers et al., 2006; Zloza and Al-Harthi, 2006; Gruenbacher et al., 2009), and CD14 (Gruenbacher et al., 2009) surface markers in some CD56+ NK cells. For the cells assayed in **Supplemental Figure 2.S1A**, CD56+ cells were isolated first, hence, CD56+ cells expressing all these other markers were included.

2.2 Utilization of isolated leukocytes for epigenetic analysis

Based on the purity of the isolated cell populations as defined by nuclear morphology and the expression of cell type specific transcripts, isolation order B was selected and used for the remaining experimental analysis in this manuscript, with the exception of only the first six cell types were isolated following this order, while CD56+ NK cells were isolated in parallel from an additional blood sample.

2.2.1 5hmCG levels among leukocytes

To explore the differences in 5hmCG levels and gene-region specific distribution of 5hmCG among leukocytes, we performed a TET assisted bisulfite sequencing (TAB-Seq) genome wide analysis on DNA isolated from each of the seven isolated cell types. The majority of the 5hmCG modification is found in the CG dinucleotide context, resulting from the specificity of TET enzymes and associated DNA binding machinery to CG dinucleotides (Lister et al., 2013; Wu and Zhang, 2014). And thus, we first quantified the overall level of 5hmCG in each of the cell types (**Table 2.2**) and found that CD4+ T cells had by far the highest level of 5hmCG at CG dinucleotides: 3.67% of CG sites assayed contained a 5hmCG. Monocytes, neutrophils, and B cells had intermediate levels of 5hmCG: 2.69%, 2.62% and 2.38%, respectively. NK cells, eosinophils and CD8+ T cells had the lowest levels with 2.12%, 1.99%, and 1.91%, respectively. These values were based on the analysis of ~$10x10^6$ different CG dinucleotides from each cell type.

As an independent confirmation of this range of 5hmCG levels among classes of leukocytes we performed immuno-fluorescent microscopy (IFM) (**Figure 2.4**) of 5hmC in preparations of total leukocytes co-stained with DAPI for DNA. Preparations of leukocytes where prepared from both fresh (method 2, **Figure 2.4A**) and frozen (method 3, **Figure 2.4B**) blood to determine the impact of using freezing on recovery. Observation of the lowest levels of

5hmC in some cells required longer photographic exposures and a further assessment of antibody reagent controls (**Supplemental Figure 2.S3**), however all leukocyte nuclei, as identified by DAPI staining (**Supplemental Figure 2.S3A**), expressed 5hmC (**Supplemental Figure 2.S3B-C**) in varying degrees after this assessment, agreeing with our TAB-Seq data. After quantification of the 5hmC signal of at least 100 cells of each of the four distinct nuclear morphologies (round: T cells, B cells and NK cells; kidney: monocytes and NK cells; multilobed: neutrophils; bilobed: eosinophils) the 5hmC signal for each morphology was classified as minimal, low, medium or high (**Figure 2.4C**). A two-way MANOVA revealed that freezing had no significant effect on the distribution of 5hmC signal among nuclear types. However, as expected, the various nuclear morphologies were a significant determinant of 5hmC levels ($p=0.001$). In particular, among nuclei with the high 5hmC signal, 92.7% of the variance in 5hmC levels was explained by the different nuclear morphologies ($p=2.07x10^{-7}$) with the kidney and round morphologies much more commonly represented than the others. Furthermore, nuclear morphology significantly impacted 5hmC signal in the medium, low and minimal groups ($p<0.05$, explaining 61.6%, 76.3%, and 45.5% of the variance in 5hmC signal respectively). However, all the different nuclear morphologies were represented in these groups. The differences identified were similar to the TAB-seq analysis, where the CD4+ T cells with round nuclei and monocytes with kidney shaped nuclei have the highest level of 5hmC, and lower levels of 5hmC are found among all nuclear morphologies (i.e. all cell types).

2.2.2 Gene-region specific 5hmCG distribution

We next assessed the gene-region specific 5hmCG profile of the seven different leukocyte types (**Figure 2.5**). Three regions were defined for all genes, 100 kb upstream of the transcription start site (TSS), the gene body (i.e., start to stop of transcript) and 100 kb

downstream of the transcription stop site (TTS). The gene regions examined are the same as those used to examine gene region distribution of 5hmCG in brain (Lister et al., 2013). 18,000 transcripts detected in leukocytes (Palmer et al., 2006) were broken into five expression quintiles (5 of 5 representing transcripts with the highest steady state level and 1 of 5 representing transcripts with the lowest levels). All of the leukocyte types had roughly the same pattern of gene-region 5hmCG distribution per transcript expression quintile with the most highly expressed transcripts having the highest levels of 5hmCG, with decreasing 5hmCG correlating with decreasing quintile of expression (**Figure 2.5B)**. Although the highest expression quintile for each cell type had the highest level of 5hmCG, the absolute level was different for each cell type. CD4+ T cells had by far the highest levels of 5hmCG with a peak at 4.53% and CD8+ T cells had the lowest levels of 5hmCG with a peak at 2.57% (**Figure 2.5B**). The relative differences among cell types held true across gene location within each quintile of expression, where for the highest expression transcript quintile, the level of 5hmCG was highest in the CD4+ T cells and lowest in the CD8+ T cells following the same pattern as the global levels presented in **Table 2.2.** The highest quintile data are shown in **Figure 2.5C** and lower quintile data in **Supplemental Figure 2.S4**. The highest 5hmCG peak for each cell type and transcript expression quintiles occurred immediately after the TSS, at the beginning of the gene body, following a valley of 5hmCG just prior to the TSS site. A dip in the level of 5hmCG across all cell types and expression quintiles can be seen at the end of the gene body with a second smaller peak of 5hmCG at or near the TTS (**Figure 2.5B**).

We then examined the differences in the levels of 5hmCG for several different leukocyte-relevant gene-ontology (GO) term categories (**Figure 2.6**). For GO category analysis each gene was divided into seven smaller gene sequence locations (**Figure 2.6**) than used in **Figure 2.5**.

This analysis allowed us to examine both the total variance in 5hmCG levels in all cell types assayed for each GO term, and also assess how different each cell type is from all the others in each gene sequence location. The total leukocyte data for all but two of the GO term based gene lists (Adaptive Immune Response and Leukocyte Migration) showed a dip in 5hmCG levels around the TTS, in agreement with this analysis of larger gene regions in **Figure 2.5**. By pooling the exons into one single bin in the box plot we no longer resolve the spike in 5hmCG at the start of the gene body (**Figure 2.5**). With a few exceptions, the two regions immediately flanking the TTS had some of the highest levels of 5hmCG for all leukocyte types, which was obscured when we looked at the larger downstream region in **Figure 2.5**.

When the differences in the weighted average of 5hmCG for all genes in the GO term list for each cell type are examined in relation to the 5hmCG levels observed in all leukocytes, striking differences from the total leukocyte population emerge. As an example, for the Leukocyte Differentiation genes, CD16+ neutrophils had much higher weighted average of 5hmCG levels in the two regions around the TSS, far exceeding 1.5 times the interquartile range (IQR) for this region of the 5hmCG levels observed in the total leukocytes (**Figure 2.6**). As an additional example, CD4+ and CD8+ T cells also distinguish themselves from the other leukocyte types with much higher and much lower levels of 5hmCG, respectively, within exons of Inflammatory Response genes (**Figure 2.6**).

Not only are there differences among cell types for the different GO terms, but there are also differences in the level of 5hmCG for total leukocytes across GO terms. Most of the GO terms related to leukocyte function had variable levels of 5hmCG across the different gene regions assayed, with the exception of the Adaptive Immune Response gene list, where the level of 5hmCG is relatively constant across gene regions with the exception of the 100 nt downstream

of the TTS, where levels of 5hmCG are three times higher (**Figure 2.6**). The peak level of 5hmCG across these smaller gene regions among the gene lists are variable with some reaching more than 7.5% (Cytokine Production and Adaptive Immune Response), while others only reach ~4% (Immune System Process, Leukocyte Differentiation, and Leukocyte Activation). Additional GO terms related to general cell function were also examined and are presented in **Supplemental Figure 2.S5**.

It seemed possible that any one of three factors (1) the genomic bin (the gene-region specific location), (2) the cell type, and/or (3) the GO term-based gene set might account disproportionately for the variance in our data, weighting its biological relevance. Therefore, we examined the variance in 5hmCG levels separately for these three factors (**Supplemental Figure 2.S6**). For all leukocyte types, the gene-region specific location and the cell type explained approximately 31% of the variance in 5hmCG levels, ~16% and ~15%, respectively. The different gene ontology gene lists explained less (~7%) of the variance in 5hmCG levels. Hence, there are many factors controlling 5hmCG levels and its gene-region distribution in leukocytes.

2.2.3 Expression of factors involved in regulation of DNA 5hmCG levels in peripheral leukocyte types

We used qRT-PCR to determine the relative quantity (RQ) of each transcript for nine factors involved in the DNA cytosine methylation cycle (DNMT1, 3A and 3B, TET1, 2, and 3, GADD45A, B, and G) as shown in **Figure 2.7**. *DNMT1* was 8- to 16-fold more highly expressed in the CD4+ and CD8+ T cells ($p<0.0005$), while *DNMT3A* and *3B* were 8- and 4-fold more highly expressed in the CD4+ T cells ($p<0.0005$) and CD8+ T cells ($p<0.05$), respectively, than in the 5 other cells types (**Figure 2.7A**). *TET1* was 50-fold more highly expressed in the two T cell types than other leukocytes ($p<0.0005$). *TET2* was 10- to 50-fold higher in the CD8+ T cells

and eosinophils than in 4 other cell types ($p<0.005$). *TET3* was most highly expressed in eosinophils, monocytes, and CD4+ T cells than the other leukocyte types ($p<0.05$) (**Figure 2.7B**). *GADD45A* was 50-fold higher in CD8+ T cells than in all other cell types ($p<0.0005$). *GADD45B* was 8- to 16-fold higher in both T cell populations relative to all other cell types ($p<0.0005$), except for eosinophils, where it was modestly expressed. *GADD45G* was 15-fold higher in the CD4+ T cells than in the other cell types ($p<0.0005$), again with exception of modest expression in eosinophils (**Figure 2.7C**). In summary, there are dramatically different expression profiles for many of the enzymes central to the turnover of modified cytosine residues (**Figure 2.1**) among classes of leukocytes.

3. Discussion

3.1 Isolating sub-populations of leukocytes

Before testing our hypothesis that the *various classes of peripheral leukocytes differentially regulate the establishment of 5mCG and its removal via oxidation to 5hmCG*, we first optimized methods to quickly and reiteratively isolate a few to seven of the peripheral blood leukocyte types from small samples of fresh or frozen stored blood. Our approach represents an extension of the partially reiterative methods of Lyons et al. (2007), who started with two aliquots of PBMCs and then successively isolated CD14+ monocytes and then CD4+ T cells from one aliquot and the CD19+ B cells followed by CD8+ T cells from the other. Most other reports of blood cell methylome analysis start with large amounts of blood (e.g., 100 to 450 ml) and isolate single cell types from separate aliquots (Reinius et al., 2012; Zilbauer et al., 2013). Such large blood samples are not easily adapted to the analysis of large patient populations particularly if multiple cell types are to be isolated. Furthermore, typical protocols for leukocyte isolation rely on Ficoll density centrifugation to first purify the leukocyte population from the red blood cells

prior to individual cell type isolation, which takes time and effort and results in a significant loss of cells. This is of concern with epigenetic analysis, as the half-life of promoter region specific DNA methylation for many genes is measured in fractions of an hour (Meagher, 2014). Hence, any additional time of manipulation may result in the loss of the original methylation profile. Our protocol(s) allows for rapid isolation of several relatively pure leukocyte populations with minimal time and labor compared to the current methods.

If multiple cell types are to be isolated from one blood sample, six to ten color flow cytometry and sorting has the potential to identify and eliminate contamination of subpopulations expressing multiple markers (Roederer et al., 1997; Granja et al., 2015; Melzer et al., 2015) and provide more information about the cell populations than magnetic bead isolation. However, multichannel sorting is more technically challenging and more costly. In addition, during cytometry "contamination" of one cell type with another depends not only upon co-expressed markers, but also on the choice of fluorophores and their overlapping emission spectra. While the reiterative isolation of cell types employed herein is less complex, less expensive, and more rapid, it may not achieve the level of purity possible using multichannel FACS.

The current common criterion for assessing purity of isolated leukocyte types relies on fluorescently labeling isolated cells with a panel of antibodies and subsequent analysis by flow cytometry (Reinius et al., 2012; Accomando et al., 2014). However, we were confronted with the problem that the large 2.8 or 4.5 micron diameter Dynabeads used to capture cells would have to be dissociated from cells before cytometric analyses. Therefore, to confirm purity by a technique independent of cytometry we employed qRT-PCR analysis of transcripts encoding eight defining PMA marker proteins.

3.2 Evidence for extreme variability in the regulation of 5mCG and 5hmCG levels among classes of leukocytes

We examined the levels of 5hmCG and related proteins regulating synthesis and decay of 5mCG levels among leukocytes. We were exploring the idea that the leukocyte types with higher levels of 5hmCG and enzymes leading to 5hmCG synthesis and removal would be more readily poised to respond to different environmental stimuli via changes in DNA demethylation and rapid changes in transcription. We hoped these data would begin to define those classes of blood-borne leukocytes with the greatest potential to respond rapidly to changes in the cell, tissue, and blood environment. However, we have not yet directly tested the response of the methylome of different cell types to environmental stress.

Our goal was a broad survey of 5hmCG across all of the peripheral leukocyte types. When we examined 5hmC by IFM in a total leukocyte preparation, we found that all cells examined expressed 5hmC at some level (**Supplemental Figure 2.S3**), and that the level of 5hmC varied dramatically among leukocyte types (**Figure 2.4**). However, there was no difference in the 5hmC levels as determined by IFM for total leukocytes isolated with either method 2 (fresh blood) or 3 (frozen blood) (**Figure 2.4**) and thus we only examined one isolation method in our further analysis. The analysis of 5hmC levels was expanded and quantified using TAB-Seq and showed a wide variation among leukocytes (**Table 2.2**). The modestly high levels of 5hmCG in all peripheral leukocyte types suggests that all peripheral leukocytes are able to alter their 5mCG levels via hydroxymethylation, presumably in response to different physiological signals.

However, it should be mentioned that a direct relationship between the loss of 5mCG and the gain of 5hmCG should not be expected, as there are many regulated steps in the cycle, and the half-lives of all the intermediates may vary in a site specific manner. In other words, these

two modifications may change independently in different gene sequences and in different cell types in a tissue (Ruzov et al., 2011; Salvaing et al., 2012; Hahn et al., 2013). With a few exceptions, we did not observe a clear relationship between the relative levels of 5hmCG among the 7 leukocyte types and the levels of 5mCG reported previously (Reinius et al., 2012). This comparison supports the view that the cytosine modification cycle is quite distinctly regulated in each cell type.

Further, we did not find a simple correlation between the expression of transcripts encoding enzymes involved in the turnover of modified cytosine residues (e.g., TETs, GADD45s, **Figure 2.1**) and the levels of 5hmCG among the seven leukocyte types, although some speculation about these relationships seems warranted. CD4+ T cells have by far the highest levels of 5hmCG (**Table 2.2, Figure 2.5**) and they have the highest levels of 5mCG of any leukocyte type assayed (Reinius et al., 2012). It is tempting to speculate then, that they are the most highly potentiated to respond to stress by 5hmCG mediated changes in 5mCG levels followed by changes in gene expression. Yet, CD4+ T cells did not distinguish themselves with high levels of expression of those factors controlling the synthesis of 5hmCG. They did highly express *TET1, DNMT1, DNMT3A,* and *GADD45G*. These data suggest there is another level of regulation controlling the balance among the factors in the turnover cycle of cytosine modification (**Figure 2.1**).

Overall, CD8+ T cells expressed relatively high steady state levels of transcripts encoding most of the factors involved in this cycle (DNMT1, DNMT3B, TET1, TET2, GADD45A, and GADD45B). Despite this, CD8+ T cells had the lowest levels of 5hmCG for all quintiles of transcript expression. CD8+ T cells have moderately high levels of 5mCG relative to other PBMCs (Reinius et al., 2012). Hence, the balance among these modifying activities (examine **Figures 2.1 and 2.7**) for CD8+ T cells must be weighted toward accumulation of 5mCG and

rapid removal of 5hmCG. The high levels of TET and GADD45 suggests these cells may be highly responsive in changing 5mCG levels, despite having low levels of 5hmCG. It has been shown there are dynamic changes in DNA cytosine methylation in CD8+ T cells in response to acute infection (Scharer et al., 2013), supporting the view that the cycle turns over rapidly in these cells. This emphasizes the importance of both DNA demethylation and methylation in the ability of CD8+ T cells to respond to changes in their environment (Scharer et al., 2013).

CD14+ monocytes have relatively low levels of all the factors assayed with the exception of TET3, and yet they have the second highest levels of 5hmCG. By contrast they have 2 to 3 times lower levels of 5mCG relative to other PBMCs (Reinius et al., 2012). Hence, these modifying activities must be weighted toward removal of 5mCG and accumulation of 5hmCG. Granulocytes, CD16+ neutrophils and Siglec8+ eosinophils have the lowest levels of 5mCG (Reinius et al., 2012), but have moderate and low levels of 5hmCG, respectively **(Table 2.2, Figure 2.5)**.

Interestingly, the two cell types with the highest levels of 5hmCG, CD4+ T cells and CD14+ monocytes, both expressed high levels of transcripts involved in the removal of 5hmCG (TETs and GADDs), which may seem counterintuitive. Perhaps the TET activity in these two cell types efficiently carry out the first oxidation step from 5mC to 5hmC, but are less efficient at further oxidation. Those sites that are oxidized further to 5fC and 5caC may be repaired by the base excision repair (BER) pathway back to C and then further modified to 5mC and then back to 5hmC. This proposed mechanism emphasizes the importance of the modification cycle for DNA cytosine (**Figure 2.1**). Both of these cell types also express high to modest levels of the DNMTs, further strengthening this explanation.

When we examined the 5hmCG distribution across gene regions among the quintiles of gene expression (**Figure 2.5**) for the seven leukocyte types, we observed a strong depletion of 5hmCG at the TSS and an enrichment over the gene body. These data agree with previous work assessing 5hmCG distribution in T cells (Tsagaratou et al., 2014; Ichiyama et al., 2015), as well as other non-leukocyte cell types and tissues (Song et al., 2011; Mellen et al., 2012; Yu et al., 2012b; Chapman et al., 2015; Taylor et al., 2016). In genes with the highest levels of expression (**Figure 2.5**), we observed an enrichment of 5hmCG in the gene body, suggesting that higher gene body 5hmCG correlates with the activation of these genes. Gene body enrichment of 5hmCG and its association with highly expressed cell type specific genes have been observed in studies of the brain, neurons, colonocytes, and chondrocytes (Mellen et al., 2012; Hahn et al., 2013; Chapman et al., 2015; Taylor et al., 2016). Higher and lower levels of 5hmCG in both the total leukocytes and the individual cell types were observed in our analysis of smaller gene regions (**Figure 2.6**) than what we had observed in larger gene regions (**Figure 2.5**), because the extreme levels are averaged out by adjacent regions when larger gene regions are examined. The highest 5hmCG level in the different gene regions and GO terms were not always in the CD4+ T cells as we have observed for large gene regions **Figure 2.5**. For example, in the Cytokine Production GO gene list in the region upstream of the TTS, CD14+ monocytes have high 5hmCG levels (~8%) and in the region downstream of the TTS, Siglec8+ eosinophils and CD19+ B cells have levels reaching ~8.5% (**Figure 2.6**). The Siglec8+ eosinophils have the second lowest global 5hmCG level as well as the second lowest peak 5hmCG level when examined by transcript expression quintile and larger gene regions. This may suggest that although globally there are generally low levels of 5hmCG in the eosinophils, there are gene-region and gene-type differences in 5hmCG in this cell type (and others) that may be important

to gene regulation of specific genes and would be missed by both global analysis and transcript expression quintile analysis of larger gene regions.

Cell type specific changes in 5hmCG in relation to different GO classes of genes and gene region may be relevant to the role of 5hmCG in potentiating gene expression. For example, during chondrogenisis those genes for which increases in 5hmCG correlate with increases in gene expression there was a enrichment of 5hmCG both prior to the TSS and in the gene body and a sharp drop in between (Taylor et al., 2016). This distribution of 5hmCG was not observed in the genes with either no change in expression or a decrease in expression associated with large changes in 5hmCG (Taylor et al., 2016). Our data (**Figure 2.6**) also shows this relationship. For example, when neutrophils were examined for the leukocyte activation GO gene list we saw enrichment of 5hmCG in the region prior to the TSS and in the gene body with a valley in between. By contrast, when the adaptive immune response GO gene list is applied to neutrophils, an innate immune cell type, this enrichment pattern of 5hmCG is not observed. These data support the idea that gene region specific enrichment of 5hmCG is cell type specific in leukocytes, potentiating the expression of cell type specific genes for regulated gene expression.

3.4 The role of hydroxymethylcytosine in potentiating gene regulation

A current view is that high levels of gene region specific 5hmCG potentiate genes for "*on demand gene regulation*"(Irier et al., 2014). Further, there is direct evidence that this cytosine modification is essential for normal regulation of gene expression. For example, during erythropoiesis in zebra fish, there is both increased expression and demethylation of *scl*, *gata-1*, and *cmyb*. However, if TET2 is knocked down there is increased methylation and decreased expression of these genes that results in anemia (Ge et al., 2014) suggesting a direct role for 5mCG oxidation in essential gene regulation. In embryonic stem cells, when TET1 and TET2 are

knocked down, there is an increase in methylation of the pluripotency related genes with a corresponding decrease in expression, altering their differentiation potential (Ficz et al., 2011).

5hmCG rich regions are associated with a potentiated, open chromatin state allowing access to various transcription and chromatin remodeling factors. For example, in embryonic stem cells enhancer regions enriched for 5hmCG are also enriched for nucleosomal histone modifications H3K4Me1 and H3K27Ac, which are associated with active transcription (Szulwach et al., 2011). In addition, in CD4+/CD8+ double positive thymocytes, 5hmCG is enriched in active thymus-specific enhancers, which have high levels of H3K4Me1 and H3K27Ac (Tsagaratou et al., 2014). There is also a negative correlation between 5hmC and the repressive histone modification H3K27me3 (Tsagaratou et al., 2014). It has been suggested that the combination of these chromatin structures indicates genes that are poised for transcriptional activation or silencing in response to environmental cues (Lister et al., 2013; Tsagaratou and Rao, 2013; Tsagaratou et al., 2014).

Considering that oxidation of 5mC to 5hmC must impact 5mC levels it is worth considering the contribution of 5mC binding protein to the concept of 5hmC function. 5mCG dinucleotides attract a wide variety of chromatin remodeling machinery such as methyl-CG binding proteins MBD4 and MeCP2 (Du et al., 2015). MBD4 recognizes 5hmCG and has a glycosylase domain (Otani et al., 2013) (see Figure 2.1, TDG) and therefore, may contribute to the cyclic modification and removal of cytosine (Figure 2.1) in various classes of leukocytes. The binding and activity of MeCP2 appears enhanced at promoter 5hmCG sites in the brain (Zhubi et al., 2014) and its activity is considered particularly important, because in the brain MeCP2 activity responds to external stimuli (e.g., cocaine, ethanol) (Pol Bodetto et al., 2013; Liyanage et al., 2015). MeCP2 binding in the brain to modified C residues can work to enhance

transcription if bound to 5hmCG, or repress transcription if bound to 5mCG (Mellen et al., 2012). Appropriate levels of MeCP2 activity are essential for the differentiation of naive CD4+ T cells into a variety of T cell types (Yang et al., 2012; Jiang et al., 2014). Thus it appears that the balance between 5mCG and 5hmCG is allowing the DNA sequence to be poised for activation and expression or repression via a combination of interactions with methyl-binding proteins.

In summary, Pastor et al., 2011 (Pastor et al., 2011) presented an initial simple view of the role of 5hmCG that may still be valid, stating that "*5hmC contributes to the 'poised' chromatin signature found at developmentally-regulated genes*". On the other hand, Nestor et al. (Nestor et al., 2012) presented solid evidence that "*tissue type*" was "*a major modifier of the 5-hydroxymethylcytosine content*" of genes. It is reasonable for us to interpret tissue type as leukocyte type and expect gene specific differences among cell types when a genome-wide analysis is performed. Our data herein and that from mouse brain (Lister et al., 2013) agree with both views, suggesting that gene expression levels and cell type are both significant determinants of overall 5hmCG levels. In short, gene region-specific 5hmCG level differences between active and inactive chromatin regions are preserved within a background of higher or lower total 5hmCG, which is determined by the cell type. Perhaps it is important to recall that the seven major classes of leukocytes examined are indeed quite divergent.

3.5 Conclusions

In summary, we optimized protocols for the reiterative isolation of the major leukocyte types from single small whole blood samples that are rapid and efficient. The isolated populations of the various leukocyte types appear sufficiently pure for epigenome and

transcriptome studies. This was confirmed with the analysis of both nuclear morphology and of leukocyte cell-type specific transcripts.

We showed that these seven isolated leukocyte types, (1) differentially express factors involved in the cycle of DNA cytosine methylation and demethylation, (2) have rank order differences in 5hmCG levels and gene region dispersal, and (3) collectively our data suggest that the CD4+ and CD8+ T cells and CD14+ monocytes may be potentiated to turnover their 5mCG more rapidly via oxidation to 5hmCG than other leukocytes. However, there is not a simple relationship between TET expression levels and 5hmCG levels as suggested by our hypothesis, implying that a much better understanding of every step in the cytosine modification cycle is needed. Recall that TETs catalyze the further oxidation of 5hmCG to 5fCG and 5caCG (Figure 2.1). Taken together our data suggest that each leukocyte type uniquely regulates their cycle of DNA cytosine modification, which imparts to each cell type a distinct ability to regulate gene expression in response to different physiological cues. Definitive identification of the optimal surrogate leukocyte cell types in peripheral blood to report the methylome's response to health status awaits further experimental analysis. It still needs to be established that 5hmCG levels and components of the turnover cycle for cytosine modification enable some cell types to respond more rapidly or more definitively than others to distinct physiological stresses and diseases.

4. Materials and Methods

4.1 Methods of reiteratively isolating leukocytes

Building on previous efforts to reiteratively isolate some classes of peripheral blood leukocytes (Lyons et al., 2007) we developed simplified protocols that could be executed in 5 hours on six or seven leukocyte classes (**Figures 2.2-2.3, Supplemental Figure 2.S1-2.S2, Table 2.1)**. Venous blood samples were collected in EDTA tubes in the morning between 8 and

9AM from a nonfasted healthy 65-year-old male volunteer free of cardiovascular and other diseases with a BMI of 29. These studies were approved by the Institutional Review Board at the University of Georgia. Analyzing the methylome of seven cell types from one individual in this initial study eliminated genetic variation so as to strengthen the interpretation of complex data, as was done with the early studies of bisulfite conversion for analyzing 5mCG residues, TET-assisted bisulfite sequencing for analyzing 5hmCG residues, and six color sorting for the analysis of multiple leukocyte types (Frommer et al., 1992; Roederer et al., 1997; Yu et al., 2012a). However, we have not yet addressed the natural variability in the leukocyte populations or in chromatin structures that may exist among healthy individuals differing in age and/or sex.

Leukocytes were isolated following the methods described in detail below and are depicted in **Figure 2.2**. For each method the cells were isolated in the following order: (A) CD4+ T cells, CD8+ T cells, and CD14+ monocytes, respectively, (B) CD16+ neutrophils, Siglec8+ eosinophils, CD4+ T cells, CD8+ T cells, CD19+ B cells, CD14+ monocytes, and CD56+ NK cells, respectively, and (C) CD14+ monocytes, CD4+ T cells, CD16+ neutrophils, CD56+ NK cells, CD8+ T cells, CD19+ B cell, and Siglec8+ eosinophils, respectively. The majority of leukocyte data presented in the text utilize Method 2 in which six of the cell types were reiteratively isolated from one 5 ml fresh blood sample in isolation order B, while the seventh type, CD56+ NK cells were isolated in parallel from a second 5 ml blood sample, because NK cells are a highly mixed cell type in terms of the affinity markers being utilized, their recovery is poor when isolated at the end of isolation order B.

4.1.2 Dynabead Preparation

Anti-CD4, anti-CD8, and anti-CD14 antibodies bound to 4.5 µm paramagenetic Dynabeads were obtained from Life Technologies (Grand Island, NY, USA Cat# 11145D, 11147D,

11149D). 25 µl of suspended anti-CD4+ and anti-CD8+ Dynabeads and 20 µl of suspended anti-CD14+ Dynabeads were used per 5 ml of blood. The Dynabeads we prepared per manufacturer's instructions and stored in 50 µl of PBSBE (phosphate buffered saline, 1% BSA, 2mM EDTA, pH7.4) on ice until needed. Protein G Dynabeads (2.8 µm, Life Technologies, Grand Island, NY, USA Cat# 10009D) were coupled to anti-CD16 (Santa Cruz Biotech Dallas, TX, USA Cat# sc-19620), anti-CD19 (Bio legend San Diego, CA, USA Cat# 302202), anti-CD56 (Bio Legend, San Diego, CA, USA Cat# 31824), or anti-Siglec8 (Bio Legend, San Diego, CA, USA Cat# 347102) antibodies for the isolation of 4 other leukocyte classes. Protein G Dynabeads were first washed with 1 ml of PBSBE, resuspended in 200 µl of PBSBE where the antibody was then added (40 µl Dynabeads and 4 µl anti-CD16 or 10 µl Dynabeads and 0.5 µl of anti-CD19, anti-CD56, and anti-Siglec8). Beads and antibodies were incubated at room temperature with rotation for 15 min, washed twice with 1 ml PBSBE, resuspended in 50 µl of PBSBE and stored on ice for no more than 4 h until use. Washing was implemented with the use of strong neodymium magnets that pulled the Dynabead-bound cells to the side of a 1.5 ml microfuge tube (MagnaRack, Invitrogen, Grand Island, NY, USA Cat # CS15000) in a minute.

4.1.3 Method 1: Isolation of leukocytes from fresh whole blood without red blood cell lysis **(Figure 2.2)**

Five ml of venous blood samples were collected in EDTA tubes in the morning between 8 and 9AM in a nonfasted male subject (kept on ice immediately after collection until leukocyte isolation) and were diluted 1:2 with PBSBE and centrifuged at 300 x g for 30 min at 4ºC. Supernatant was discarded, and the cell pellet was resuspended in 1 ml of PBSBE. Cells were centrifuged at 400 x g for 2 min, supernatant was discarded, and cells resuspended in 1 ml of PBSTBE (phosphate buffered saline, 2% Tween 20, 1% BSA, 2mM EDTA, pH7.4). Three

leukocyte types (CD4+, CD8+, and CD14+) were then successively isolated following the general isolation protocol (see below).

4.1.4 Method 2: Isolation from fresh whole blood with controlled red blood cell lysis **(Figure 2.2)**

Five ml of venous blood samples were collected in EDTA tubes in the morning between 8 and 9AM in a nonfasted male subject (kept on ice immediately after collection until leukocyte isolation) and were diluted 1:10 with freshly prepared 1x cold red blood cell lysis solution form a 10x stock (10x red blood cell lysis solution: 1.5 M NH_4Cl 100mM $NaHCO_3$, 10 mM EDTA, pH 7.4) (Bossuyt et al., 1997) and placed on ice for 20 min. Samples were then centrifuged at 300 x g for 20 min at 4ºC. Supernatant was discarded, and cells were resuspended in 10 ml of PBSBE and centrifuged at 300 x g for 10 min at 4ºC. Supernatant was again discarded, and cells were resuspended in 1 ml of PBSTBE. Six to seven leukocyte types were then successively isolated following the general isolation protocol.

4.1.5 Method 3: Isolation from frozen whole blood **(Figure 2.2)**

Venus blood samples were collected in EDTA tubes in the morning between 8 and 9AM in nonfasted a male subject and frozen at -80 ºC. Five ml of frozen blood (at -80ºC) was thawed on ice. Approximately 90% of red blood cells lyse during the freeze-thaw process, but most white cells do not (Fiebig et al., 1997). Thawed blood was diluted 1:2 with PBSBE and centrifuged at 300 x g for 30 min at 4ºC. The supernatant was discarded, and cells were resuspended in 1 ml of PBSBE. Cells were centrifuged at 400 x g for 2 min, supernatant was discarded, and cells were resuspended in 1 ml of PBSTBE. Again, six to seven leukocyte types were then successively isolated following the general isolation protocol.

4.1.6 General Isolation Protocol

Dynabeads prepared for the first cell type to be isolated were added to the cells. Cells were incubated with Dynabeads with rotation at 4ºC for 30 min for CD4+ T cells, CD8+ T cells, CD14+ monocytes, CD19+ B cells, CD56+ natural killer (NK) cells, and Siglec8+ eosinophils or 1 hour for CD16+ neutrophils. Samples were then placed on a magnetic rack (Invitrogen, Grand Island, NY, USA Cat # CS15000) for 2 min, and the supernatant was carefully removed, placed in a fresh microcentrifuge tube for the next leukocyte type to be isolated, and stored on ice until processing. The cells bound to paramagnetic beads were resuspended in 1 ml of PBSBE and washed three times with PBSBE using the magnetic rack. Cells were finally resuspended in 200 µl of PBS (phosphate buffered saline, pH 7.4) and stored according to future use (e.g., the cell pellet was frozen with liquid nitrogen and stored at -80ºC for RNA, frozen at -80 ºC for DNA extraction or fixed with 3.7% formaldehyde for Immuno-Fluorescent Microscopy (IFM)). The Dynabeads prepared for the next cell type to be isolated were added to the microcentrifuge tube containing the uncaptured cells, and the next cell type was isolated following the same protocol. This process was repeated until all desired leukocyte types had been isolated. When we tried to use this capture method with the white blood cells suspended in 10 ml the recovery was not quantitative, even with longer incubation times, using twice as much antibody and beads, and larger neodymium magnets.

4.1.7 Determination of cell recovery for each isolation method

Ten µl of the formalin fixed cells of each cell type were incubated for 20 min in the dark at 4ºC with 0.25 µl of Propidium Iodine (PI, 1mg/ml). Stained cells were examined on a hemocytometer under combined fluorescence and DIC, and cells were counted following standard protocols. Florescence microscopy was performed on a Leica TR600 epifluorescence microscope using Hamamatsu SimplePCI Image Analysis software or on a confocal microscope

(Zeiss SM710) using ZEN 2011 software. A one-way ANOVA followed by tukeys HSD post hoc was performed using SPSS software (IBM) to determine differences in cell recovery between the three methods of isolation for the three cell types where cells were recovered for all three methods. Significance was set at $p < 0.05$. A two tailed t test was performed on the cell recoveries between methods 2 and 3 to determine any significant differences in cell recovery between the two methods for the four cell types in which cells were only recovered in these methods. Significance was set at $p<0.05$. Data are presented in **Table 2.1.**

4.1.8 Assessment of nuclear morphology by fluorescent microscopy

Initial cell purity was assessed by microscope analysis of the four distinct nuclear morphologies characteristic of the various leukocyte types (Alberts, 1994). Samples for analysis were prepared the same as used to determine cell recovery. Analysis of at least 100 cells showed that all cell preparations assayed were greater than 95% pure, based on the presence of the correct nuclear morphology and the absence of the alternate morphologies. Red blood cells were seldom observed. Data are presented in **Figure 2.3.**

To confirm the purity of CD16+ neutrophils and CD14+ monocytes, we fluorescently labeled the total leukocyte fraction with DAPI and anti-CD4-Texas RPE (Invitrogen, Grand Island, NY, USA Cat# MHCD0417). First the cells were blocked for 45 min with PBSBE, washed 3x, and stained with DAPI and the fluorescent antibody for 1 hour with rotation in the dark. Cells were then washed 3x with PBSBE and a 20 µl aliquot was set aside for immediate microscope analysis while the remaining cells were split into three equal aliquots. CD4+, CD14+, and CD16+ were then isolated from one of the three samples of labeled leukocytes and then immediately analyzed by fluorescent microscopy. Cells positive for the CD4 antibody were classified by their nuclear morphology and all cells were counted into their respective

classifications. The percentage of each cell type was then calculated. A one-way ANOVA followed by tukey HSD post hoc test was used to determine significant differences between each of cell populations for each cell type using SPSS software (IBM).

4.1.9 qRT-PCR analysis of transcript levels

To further confirm the purity of the cell types, we performed qRT-PCR assays for eight transcripts specific to the seven isolated cell types (CD3, CD4, CD8, CD14, CD16, CD19, CD56 and Siglec8. Leukocytes bound to Dynabeads (Method 3, Order B) were washed in PBS and frozen in liquid nitrogen and stored at -80ºC. RNA was extracted using RNeasy Mini Kit (Qiagen, Frederick, MD, USA Cat# 74104) following manufacture instructions. RNA concentrations were quantified using Qubit RNA assay kit (Life technologies, Grand Island, NY, USA Cat# Q32855) and 400 ng of RNA was used for cDNA synthesis using qScript cDNA synthesis supermix (Quanta Biosciences, Gaitthersburg, MD, USA Cat# 95148-100). Relative quantities (RQ) of cell type specific transcripts were normalized to an endogenous control, Beta Actin (ACTB) (Vandesompele et al., 2002) using the dCT method (Livak and Schmittgen, 2001). *ACTB* was used when we were examining the expression profile of the transcripts of cell type markers within a single cell type, and not making comparisons among cell types. ACTB mRNA abundance, and hence, its CT values were closer to those of the target transcripts being examined, however expression was variable across cell types (**Supplemental Figure 2.S7**). Oligonucleotide primer sequences (**Supplemental Table 2.S2**) were synthesized by Integrated DNA Technologies (Coralville, IA, USA). Two to six primer sets were tested for each of the target genes, ensuring the specific gene target was being amplified, and those having a single sharp dissociation peak and the lowest CT values were selected for subsequent use. A 25µl reaction using SYBR green master mix (Life Technologies, Grand Island, NY, USA Cat#

43677659) and 4 ng of cDNA was used for analysis of the eight gene panel in all seven isolated leukocyte types. To determine statistical relevance of differences in transcript levels, a one-way ANOVA was used to examine the effects of cell type on expression with Tukey's HSD test as a post hoc using Statistica software 7.1 (StatSoft; Tulsa, OK, USA).

4.2 TAB-Seq library preparation and sequencing

DNA (0.5 to 1 ug) was prepared from all seven isolated cell types (1- to 2- x 10^5 cells) while still attached to Dynabeads using DNeasy kit (Qiagen, Frederick, MD, USA #69506) according to the manufacturer's recommendations. DNA was quantified using a Qubit 2.0 Instrument and dsDNA HS Reagent (Life Technologies, Grand Island, NY, USA #Q32866 and #Q32851 respectively). TET-enzyme assisted bisulfite sequencing (TAB-Seq) was performed as described previously (Yu et al., 2012a). Briefly, 0.5 ng of M. *SssI* methylated Lambda DNA and 0.25 ng of hydroxymethylated pUC19 DNA was added per 1 ug of DNA prior to treatment as C/5mCG and 5hmCG control respectively and then sequencing libraries were prepared following the MethylC-seq protocol (Urich et al., 2015) (**Supplemental Table 2.S3**). Deep sequencing was performed using an Illumina NextSeq500 Instrument at the University of Georgia Genomics Facility. The limitation of our TAB-Seq analysis is that there is only moderate to low coverage of the genome and thus it is only statistically valid to look at groupings of genes and not individual genes. Our coverage for the different cell types ranged from 34% to 55% of the human genome (**Supplemental Table 2.S3**), which is considerably above accepted levels for a meta-analysis (Popp et al., 2010) and hence the analysis of gene groups with more than 100 genes gave statistically sound results.

4.2.1 TAB-Seq data analysis

The raw sequence reads were trimmed for adapters, preprocessed to remove low quality reads and aligned as previously described in (Yu et al., 2012b) to the *H. sapiens* GRCh38 reference genome. Fully unmethylated lambda DNA was treated by M. SssI to methylate all cytosines in the CG context to 5mCG. These 5mCG sites in CG contexts were used to calculate the 5mCG non-conversion rate upon TET and bisulfite treatment. Non-CG sites were used to compute the non-conversion rate of unmodified cytosines upon bisulfite treatment. For the 5hmCG control, a ~1.64 kb region of the pUC19 vector was constructed by PCR amplification with all cytosines being synthesized as 5hmCGs. These 5hmCG sites were used to evaluate the protection rate of 5hmCGs. For this analysis, only cytosines in the CG context were considered (**Supplemental Table 2.S3**). The TAB-Seq data set supporting the results of this article is available in NCBI GEO repository, accession number **GSE70519.**

For the data plotted in **Figure 2.5** and **Supplemental Figure 2.S4**, we grouped genes into five quintile groups based on the ranking of expression levels for ~18,000 transcripts in peripheral blood mononuclear cells in a highly cited previous study (Palmer et al., 2006). For each quintile (~3,600 transcripts), the level of 5hmCG was determined using weighted methylation level calculations (Schultz et al., 2012) for each of 20 bins upstream, 20 bins within genes (between annotated TSS and TTS), and 20 bins downstream of the gene region. Each of the upstream and downstream bins spanned 5 kb of the total of 100kb. The within-gene regions, which varied in length, were evenly divided between the 20 bins. Figures were prepared using ggplot2 (Wickham, 2009).

A related analysis was used to investigate the level of the 5hmCG marks in 100 bp windows in the context of transcriptionally- and translationally-important locations: around the TSS, exon boundaries, and around the TTS and in a larger window containing the collated exons

(i.e., seven genomic bins in all). For this analysis, numerous immune cell-relevant GO terms were used. The genes in each GO term set were extracted, and all CG sites within the bins were examined to determine the level of 5hmCG. The total levels of 5hmCG in all leukocytes were presented as box plots in order to show the variance in the level of 5hmCG in each region. The weighted average of 5hmCG for each cell type was also plotted as a colored dot within the diagram. A table describing the GO terms used and the lists of genes is provided in **Supplemental File 1** (available at http://www.sciencedirect.com/science/article/pii/S0022175916300941)**.** In order to determine the amount of variance in 5hmCG levels explained by genomic bin, cell type, and GO term set, an ANOVA was performed between the three factors using R and the amount of variance explained by each factor was determined by the proportion of their total sum of square (**Supplemental Figure 2.S6**).

4.3 Fluorescent labeling of 5hmCG

Total white blood cells were isolated from both fresh (method 2) and frozen blood (method 3) (**Figure 2.4**), fixed with 3.7% formaldehyde for 15 min, and then centrifuged at 400 x g for 2 min, after which the supernatant was discarded. Cells were resuspended in 500 µl of 50% methanol in PBS (phosphate buffered saline, pH7.4), incubated for 1 min at room temperature, and centrifuged at 400 x g for 3 min, after which the supernatant was discarded. Cells were heated to 95^{o}C for 5 min, snap cooled on ice, washed two times with PBSBE (phosphate buffered saline, 1% BSA, 2mM EDTA, pH7.4), and finally resuspended in 200 µl PBSBE and incubated for 45 min to block non-specific binding of antibodies. Rabbit anti-5hmC polyclonal antibody (Active Motif, Carlsbad, CA, USA Cat# 39769) was added to cells at a 1:200 dilution in PBSBE and incubated at room temperature with rotation for 1 hour. Cells were washed three

times with PBSBE and resuspended in 1:500 dilution of goat anti-rabbit conjugated with phycerythrin (PE) (Abcam, Cambridge, MA, USA Cat# ab97070) in PBSBE and incubated at room temperature with rotation in the dark for 1 hour. Cells were washed three times with PBSBE and finally resuspended in 30µl of PBSBE. 10 µl of labeled cells were stained with 1 µl of DAPI (1mg/ml) for ten minutes and examined with fluorescence microscopy, all under the same exposure conditions. Cells were classified by nuclear morphology into four groups, round nuclei, kidney shaped nuclei, multilobed nuclei, or bilobed nuclei, and Hamamatsu SimplePCI Image Analysis software was used to assess the 5hmCG signal for at least 100 cells for each morphology in each isolation method. Background fluorescent was subtracted from each raw 5hmCG signal. The 5hmGC signal for each of the morphologies and each isolation method was divided into 4 quintiles of data, labeled as minimal, low, medium or high 5hmCG signal. The cells for each morphology were randomly separated into three groups and the percent of cells falling in each 5hmCG signal range were identified for each morphology in both isolation methods. To determine if there was an interaction between the isolation method and the nuclear morphology on the number of cells in each 5hmCG signal group a two-way MANOVA was performed using pillia trace test using SPSS software (IBM). A significant effect of only nuclear morphology was identified and Bonferroni post-hoc analysis was used to assess differences between nuclear morphology and 5hmCG signal for this analysis with significance set at $p<0.05$.

4.4 qRT-PCR analysis of transcript levels

To assess the potential for turnover of 5mCG, three transcripts related to DNA methylation (*DNMT1, 3A,* and *3B*) and six related to DNA demethylation (*TET1, 2, 3* and *GADD45A, B, G*) were also assayed. Isolated leukocytes bound to Dynabeads (Method 3, Order B) were washed in PBS and frozen in liquid nitrogen and stored at -80ºC. RNA was extracted using RNeasy Mini

Kit (Qiagen, Frederick, MD, USA Cat# 74104) following the manufacturer's instructions. RNA concentrations were quantified using the Qubit RNA assay kit (Life technologies, Grand Island, NY, USA Cat# Q32855) and 400 ng of RNA was used for cDNA synthesis using qScript cDNA synthesis supermix (Quanta Biosciences, Gaithersburg, MD, USA Cat# 95148-100). Relative quantities (RQ) of cell type specific transcripts were normalized to endogenous control ribosomal *18S rRNA* (Vandesompele et al., 2002) using the dCT method (Livak and Schmittgen, 2001). We found *beta-actin* was far more variably expressed across the seven cell types than 18S rRNA, relative to a constant cDNA input (**Supplemental Figure 2.S7**). Therefore, *18S rRNA* was used as an endogenous control when comparing transcripts related to establishment and removal of 5mCG across cell types.18S rRNA has the disadvantage as an endogenous control that it is 1,000-fold more abundant than any mRNAs being assayed. Therefore, the low number of PCR cycles needed to reach cycle threshold levels are far removed from those of the target, which could be another source of error. Oligonucleotide primer sequences (**Supplemental Table 2.S2**) were synthesized by Integrated DNA Technologies (Coralville, IA, USA). Two to six primer sets were tested for each of the target genes and those having a single sharp dissociation peak, ensuring the specific gene target was being amplified, and the lowest CT values were selected for subsequent use. A 25 µl reaction using SYBR green master mix (Life Technologies, Grand Island, NY, USA Cat# 43677659) and 4 ng of cDNA was used for analysis of the gene panel. To determine statistical relevance of differences in transcript levels, a one-way ANOVA was used to examine the effects of cell type on expression with Tukey's HSD test as a post hoc using Statistica software 7.1 (StatSoft; Tulsa, OK, USA).

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Table 2.1. Efficiency of recovery of each isolation method.

Method	CD4 + T cells	CD8+ T cells	CD14+ monocytes	CD16+ neutrophils	CD19+ B cells	CD56+ NK cells	Siglec8+ eosinophils
1	72,300 ± 6,500[a]	34,500 ± 7,400[a,b]	11,100 ± 1,600[a]	NR	NR	NR	NR
2	44,900 ± 4,700[b]	39,300 ± 10,600[a]	3,500 ± 1,600[b]	211,000 ± 53,500[a]	3,900 ± 800[a]	54,300* ± 11,100[a]	6,400 ± 2,900[a]
3	13,800 ± 2,100[c]	7,100 ± 2,300[b]	4,800 ± 1,500[a,b]	43,200 ± 6,700[b]	2,800 ± 900[a]	14,100* ±4,500[b]	3,600 ± 200[a]

Numbers of cells recovered from each isolation method starting with 5 ml of blood reported as Mean ±SEM (N=3). Fresh blood with RBC lysis and frozen blood protocols were isolated following isolation order A, except *CD56+ cells which were isolated directly from white blood cells isolated with the respective method, as they are unable to be recovered in isolation order A. NR: Not Recovered. A one-way ANOVA followed by Tukey HSD post hoc was performed between methods 1, 2 and 3 for the three cell types where cells were recovered in all three methods. A two tailed t test was performed between methods 2 and 3 for the four cell types where cells were only recovered in these methods. Significant differences between the different methods for each of the seven cell types are designated by having different letters.

Table 2.2. 5hmC levels are distinct among the seven classes of peripheral leukocytes

Leukocyte Type	Total 5hmCG sites	Total CGs sites	% 5hmCG	Scaled % 5hmCG
CD4+ T cell	268,707	12,379,005	2.17%	3.67%
CD14+ monocyte	155,335	10,332,947	1.50%	2.69%
CD16+ neutrophil	176,386	12,621,471	1.40%	2.62%
CD19+ B cell	144,885	10,154,660	1.43%	2.38%
CD56+ NK cell	174,264	12,397,889	1.41%	2.12%
Siglec8+ eosinophil	117,257	8,357,101	1.40%	1.99%
CD8+ T cell	158,512	13,045,103	1.22%	1.91%

Quantification of 5hmC levels from TAB-Seq data demonstrated a modestly wide range of 5hmC levels among the peripheral leukocytes. See Materials and Methods.

Supplemental Table 2.S1. Expression of common antigen markers used for cell isolation of peripheral leukocytes

Cell Type	Co-Expression of Other Markers
CD4+ T cells	CD8 (Sullivan et al., 2001; Zloza and Al-Harthi, 2006); CD16 (Zloza and Al-Harthi, 2006); CD56 (Zloza and Al-Harthi, 2006)
CD8+ T cells	CD4 (Sullivan et al., 2001; Zloza and Al-Harthi, 2006); CD56 (Ohkawa et al., 2001)
CD14+ monocytes	CD4 (Biswas et al., 2003; Lyons et al., 2007); CD8 (Gibbings et al., 2007); CD16 (Ziegler-Heitbrock, 2007); CD56 (Grip et al., 2007)
CD16+ neutrophils	CD4 (Biswas et al., 2003); CD14 (Kuuliala et al., 2007)
CD19+ B Cells	CD14(Ziegler-Heitbrock et al., 1994)
CD56+ NK cells	CD4 (Subset) (Biswas et al., 2003); CD8 (Subset) (Kelly-Rogers et al., 2006); CD16 (Schleypen et al., 2006); CD14 (Subset) (Gruenbacher et al., 2009)
Siglec8+ eosinophils	CD4 (Biswas et al., 2003); CD16 (Davoine et al., 2002)

Many of the peripheral leukocyte types express many of the common antigen markers that were used for cell isolation and are described in this table.

Supplemental Table 2.S2. Oligonucleotide sequences for qRT-PCR

Oligonucleotide Name	Oligonucleotide sequence
BACTN	GGATCAGCAAGCAGGAGTATG AGAAAGGGTGTAACGCAACTAA
CD4	ACCTTTGCCTCCTTGTTCTC CTCCAGAAAAATTTGACCTGTGAG
CD8	CAGTTTGAAGTAATGTAGTGGCT GAACCGAAGACGTGTTTGC
CD3	TGCTGGTACCCAGTCCTAAA CCAAAGGGAAGGGAGTGAATAG
CD14	GCCTCAAGGTACTGAGCATT AGACAGGTCTAGGCTGGTAAG
CD16	GTTCAAGGAGGAAGACCCTATTC ACTTCCTGTCTTTGCCATTCT
CD19	GCTCAGGAAGTCCATTGTCC CAGCTCTCCCAGGATGG
CD56	GAGGCTTCACAGGTAAGAGTG GACCATCCACCTCAAAGTCTT
Silgec8	CAGGGCACAGAGCAGGT ATTACAAAACTAAGCAGCTGTCTG
18s	CACGGACAGGATTGACAGATT GCCAGAGTCTCGTTCGTTATC
DNMT1	CCAGGATGAGAAGAGACGTAGA AGTGCGCGTTCCTGATTT
DNMT3A	GCCCAAGGTCAAGGAGATTATT GAGATGCAGATGTCCTCAATGT
DNMT3B	AGACAGTGGAGATGGAGACA CAGGAGAAGCCCTTGATCTTT
TET1	ATTGATCCAAGCTCTCCCTTAC GCTACTGGAGCATACTGCTTAT
TET2	GCACTCTGAATGGTGGAGTT GCCTCAGGTTTACCCTCTATTT
TET3	CGATTGCGTCGAACAAATAGTG TCCATGAGTTCCCGGATAGA
GADD45A	CGGTGATGGCATCTGAATGA GCATCAGTGTAGGGAGTAACTG
GADD45B	GTCGGCCAAGTTGATGAATG GATGAGCGTGAAGTGGATTTG
GADD45G	ACTTGGTACAGTTGCAGGAG CTTCAACAGCAGCATCCTTTAG

Description of data: Oligonucleotide pairs for each assayed transcript are listed in order of sense (S) followed by antisense (A) oligonucleotides.

Supplemental Table 2.S3. TAB-Seq Analysis metrics

Sample	Mapped reads		Lambda						pUC19 (5hmC)			Genome coverage
			CG sites (5mC)			non-CG sites (unmodified C)						
	number	%	methylated Cs	mapped Cs	%	methylated Cs	mapped Cs	%	methylated Cs	mapped Cs	%	
CD4 + T cell	11,375,778	50.49%	2,759	129,604	2.13%	3,822	492,185	0.78%	1,501	2,540	59.1%	0.53
CD8+ T cell	11,932,983	45.80%	3,213	319,140	1.01%	7,596	995,267	0.76%	1,732	2,722	63.6%	0.55
CD14+ monocyte	9,226,507	46.84%	2,961	308,976	0.96%	6,922	959,506	0.72%	1,051	1,881	55.9%	0.43
CD16+ neutrophil	10,215,886	49.17%	3,615	366,496	0.99%	7,470	1,103,681	0.68%	874	1,640	53.3%	0.47
CD19+ B cell	8,853,607	47.84%	2,724	289,549	0.94%	6,242	891,022	0.70%	1,196	1,995	59.9%	0.41
CD56+ NK cell	11,713,710	51.16%	2,989	289,667	1.03%	6,691	915,692	0.73%	1,376	2,076	66.3%	0.54
Siglec8+ eosinophil	7,462,495	47.07%	2,409	243,724	0.99%	5,641	757,979	0.74%	623	883	70.6%	0.35

Description of data: Provided in the table are the metrics related to the TAB-Seq analysis. The genome coverage achieved by our Tab-Seq meta-analysis is listed in the last column as a fraction of our coverage to the human genome (GRCh38).

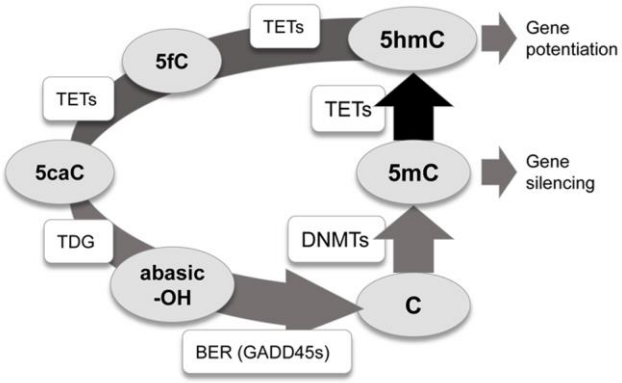


Figure 2.1. The dynamic modification cycle of DNA cytosine and its impact on gene activity. This model of the turnover of modified cytosine (C) residues emphasizes the central role of DNMTs in the methylation of C to 5-methylcytosine (5mC) and TETs in the rate limiting removal of 5mC by oxidation to 5-hydroxymethylcytosine (5hmC). The dynamic turnover of 5mC appears critical to regulating rapid changes in linked gene expression (Meagher, 2014; Wu and Zhang, 2014). TETs may further oxidize 5hmC to 5-formalcytosine (5fC) and 5-carboxycytosine (5caC). Thymine DNA glycosidase TDG removes the modified 5fC or 5caC bases leaving an abasic nucleotide (-OH). Base excision repair (BER) repairs the single nucleotide gap in double stranded DNA back to a C residue. Enzymes are in square boxes and nucleotide bases are in ovals. The diagram was modified from (Kohli and Zhang, 2013), based on the data in (Chen et al., 2012; Ramon et al., 2012; Dubois-Chevalier et al., 2014; Haseeb et al., 2014; Oger et al., 2014; Dubois-Chevalier et al., 2015).

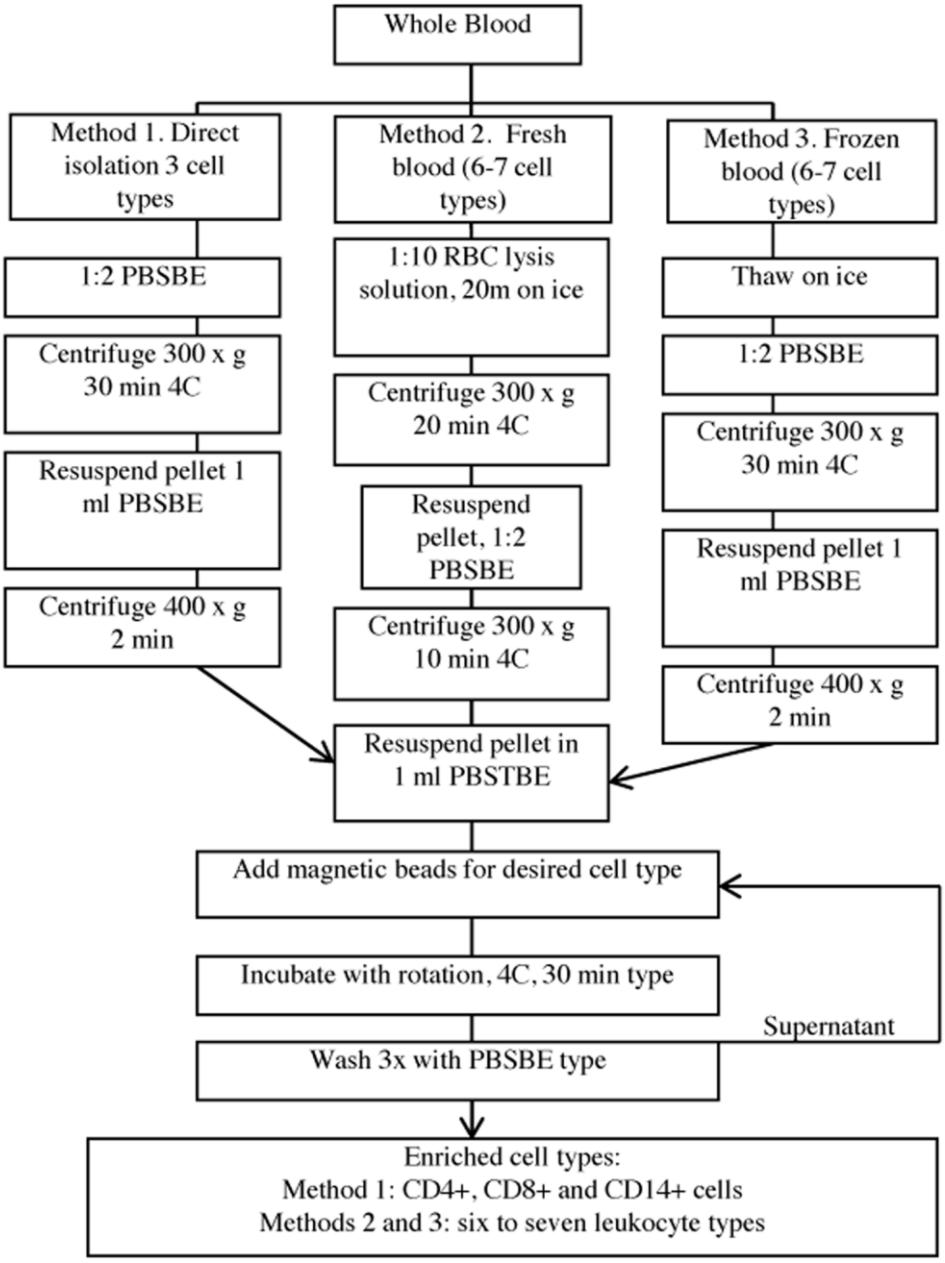


Figure 2.2. Description of isolation protocols. Graphical outline of the three isolation methods (1, 2, 3) each starting with 5 ml of peripheral blood.

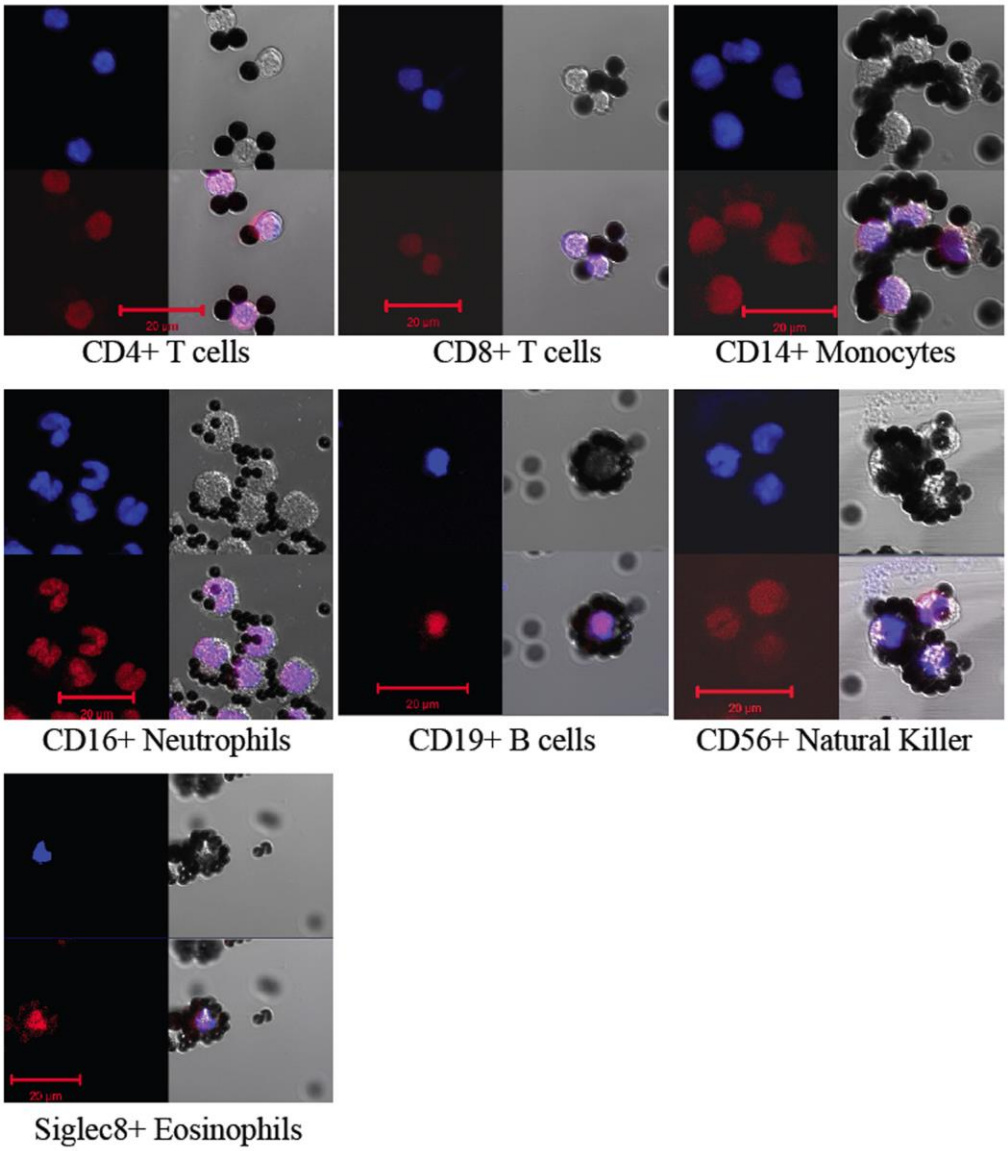


Figure 2.3. Nuclear morphologies of isolated cell types. Isolated leukocytes, bound to Dynabeads were stained with: DAPI (upper left blue) and PI (lower left red) and photographed by fluorescence (left) and DIC microscopy (upper right) of each of the seven panels. The three images were merged to yield the image in the lower right. Scale bar = 20 microns.

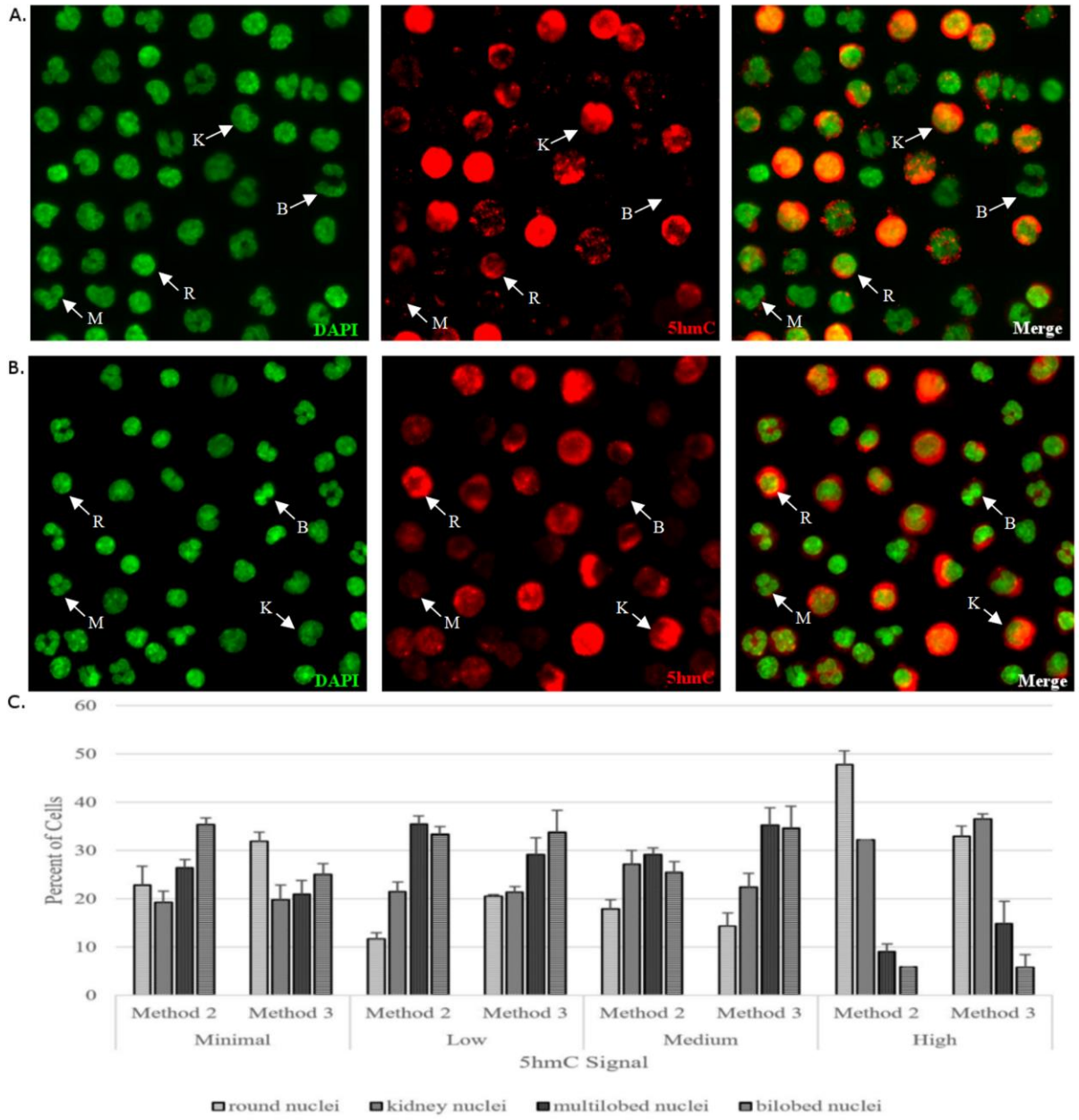


Figure 2.4. Immuno-fluorescent analysis showed a wide distribution of 5hmC levels among various classes of leukocytes. **A.** Total human leukocyte fraction from fresh peripheral blood (method 2) were labeled with DAPI for DNA (fluorescent green), primary antibody to 5hmC and secondary R-PE (red fluorescence), and then the merged image of DAPI and 5hmC is also presented. The same field of cells is shown in all images. **B**. Total human leukocyte fraction

from frozen peripheral blood (method 3) were labeled with DAPI for DNA (fluorescent green), primary antibody to 5hmC and secondary R-PE (red fluorescence), and then the merged image of DAPI and 5hmC is also presented. The same field of cells is shown in all images. Example cells are labeled based on nuclear morphologies. K: kidney shaped (monocytes or natural killer cells), R: Round (T cells and B cells), M: Multilobed (neutrophils), B: Bilobed (eosinophils). **C**. 5hmC signal was quantified, and categorized as minimal, low, medium or high for each of the nuclear morphologies in each isolation method (fresh blood: method 2, frozen blood: method 3) and the percent of cells for each nuclear morphology was plotted for each 5hmC signal. Error bars represent standard error of the mean.

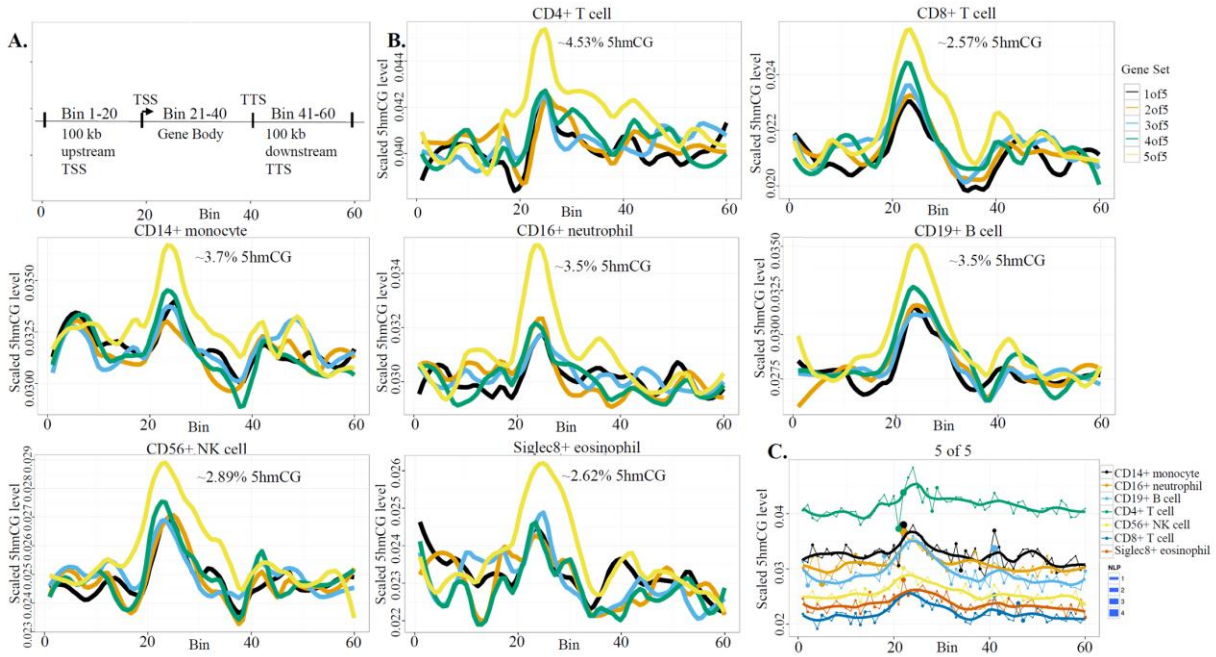


Figure 2.5. Gene-region-specific 5hmCG levels are distinct among the peripheral leukocyte types and vary by transcript level. **A.** Map defining the three gene regions assayed (Lister et al., 2013). **B**. 5hmCG levels were plotted for each of the seven leukocyte types by quintile of transcript expression level. The peak percentage of 5hmCG relative to total CG content for each cell type is estimated at the top of each graph. **C.** The 5hmCG levels for the quintile of the highest quintile of transcript for each cell type was plotted together. The dots plotted with each line on the graph represent the degree of change from the previous regions level of 5hmCG to the current level. The figure legend to the right of panel C shows the varying levels of significance of this change as determined by NLP. The relative position of each cell type is the same when these data are plotted for the other 4 quintiles of transcript expression (**Supplemental Figure 2.S4**).

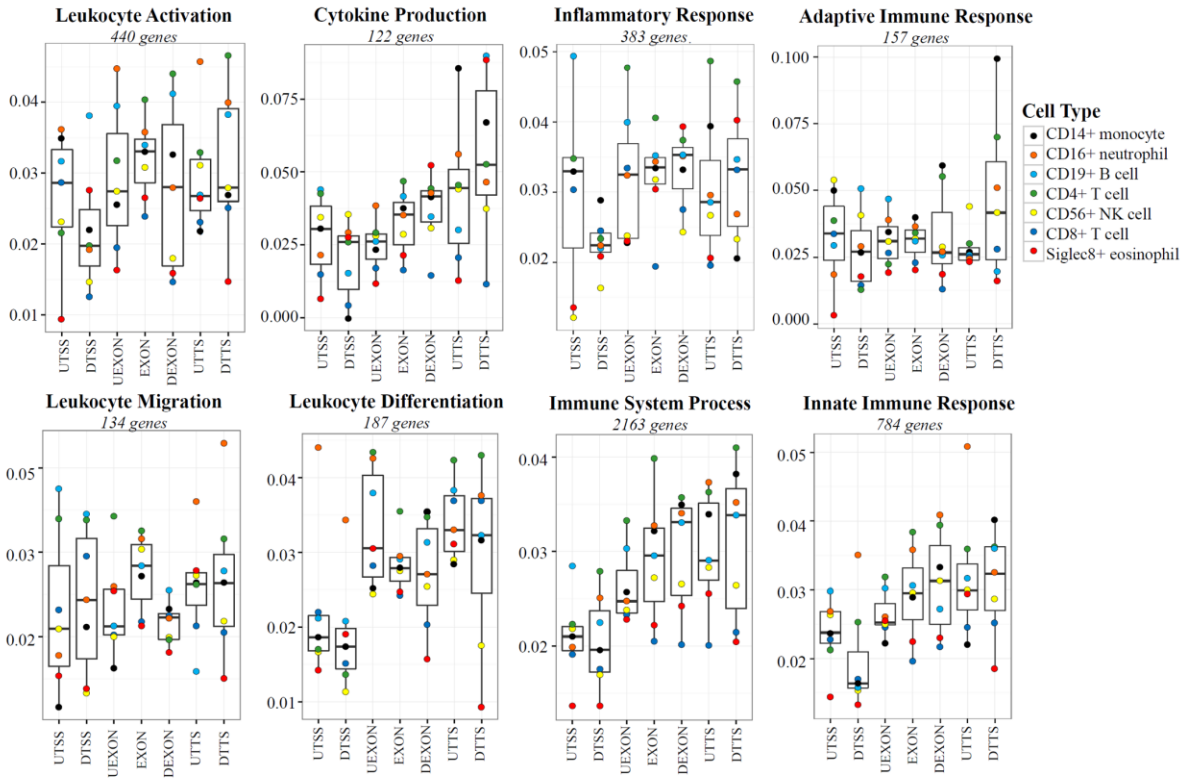


Figure 2.6. Gene-sequence-specific distribution of 5hmCG levels in the peripheral leukocytes for relevant GO term gene lists. The fraction of 5hmCG relative to all CG dinucleotides for seven gene sequence locations in all leukocytes (100 bp upstream of the TSS (UTSS), 100 bp downstream TSS (DTSS), 100 bp upstream of all exons (UEXON), within exons (EXON), 100 bp downstream of exons (DEXON), 100 bp upstream TTS (UTTS), and 100 bp downstream TTS (DTTS)) were plotted for different GO terms related to leukocyte function as box plots with the bar representing the median, and the box extending from the 25th to 75th percentiles. The whiskers represent +/- 1.5 times the interquartile range. Within each box plot the weighted average of 5hmCG for each leukocyte type was plotted as a colored dot, while the box is the weighted average for all 7 cell types. Similarly plotted data for several other genes grouped by GO terms are presented in **Supplemental Figure 2.S5**.

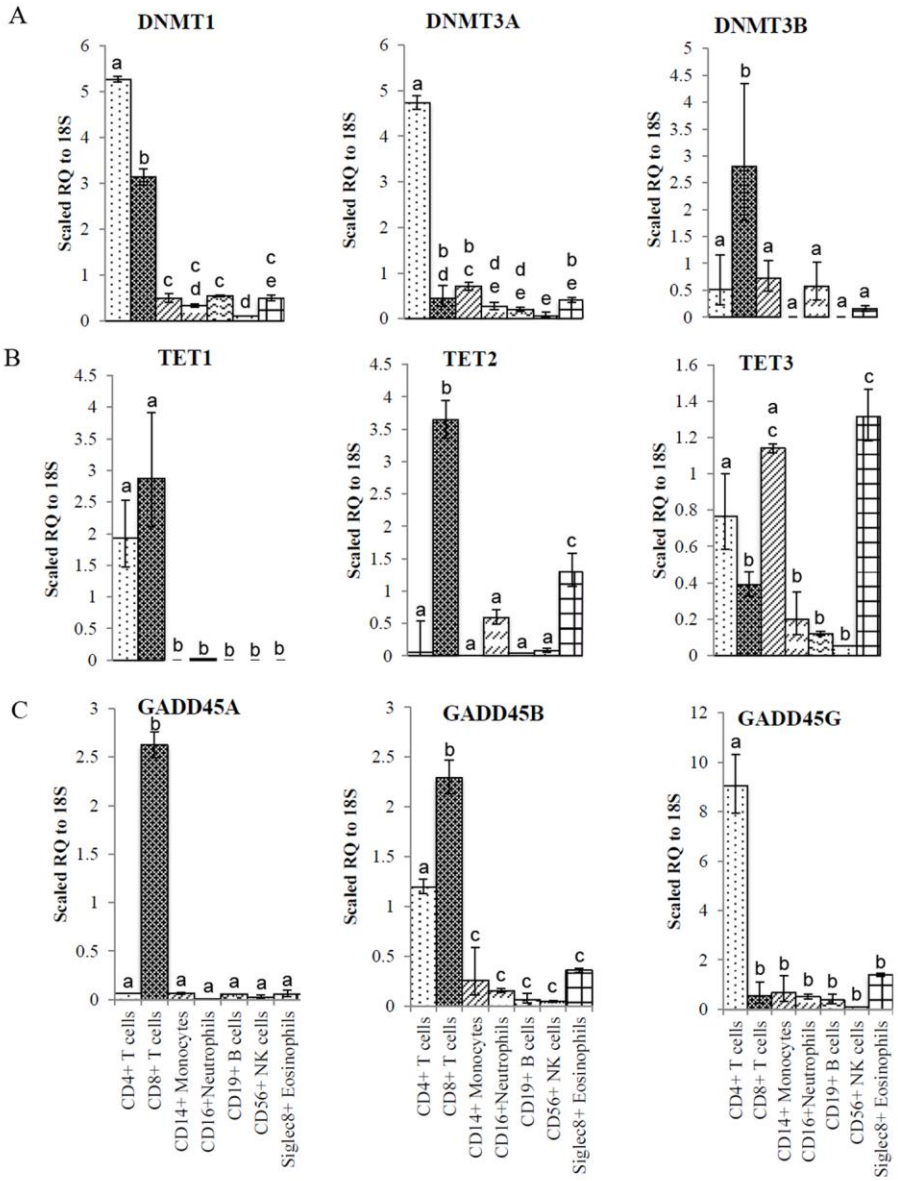
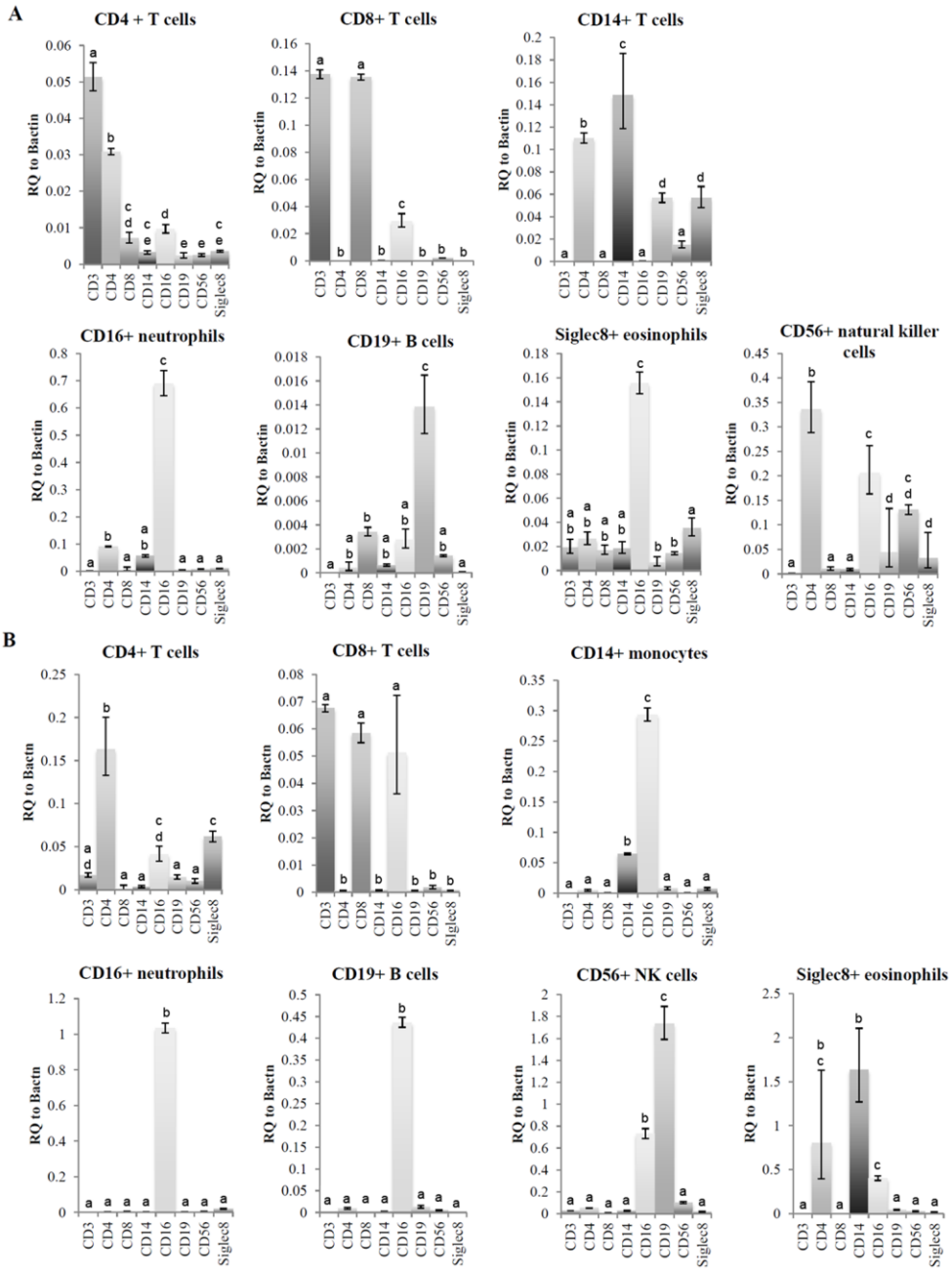


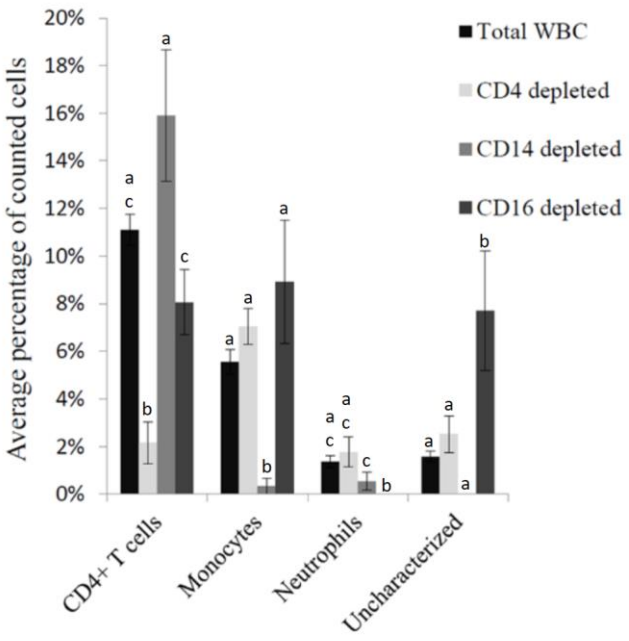
Figure 2.7. Expression of transcripts encoding enzymes involved in the establishment and removal of modified DNA cytosine. **A-C**. qRT-PCR analysis of the relative transcript expression was performed on cDNA prepared from seven leukocyte types. Values are expressed as a scaled Relative Quantity (RQ) of transcript in each cell type using the dCT method. Letters designate significant differences of at least $p<0.05$. **A**. Analysis of transcripts for DNMTs (**Figure 2.1**). The RQ value for each cell type is presented as a scaled value of 10^4, 10^5, and 10^6 times their RQ

value for *DNMT1*, *DNMT3A*, and *DNMT3B*, respectively. **B**. Analysis of transcripts for TETs (**Figure 2.1**). The RQ value for each cell type is presented as a scaled value of 10^6, 10^5, and 10^5 times their RQ value for *TET1*, *TET2*, and *TET3*, respectively. **C**. Analysis of transcripts for GADD45s (**Figure 2.1**). The RQ value for each cell type is presented as a scaled value of 10^5, 10^5, and 10^6 times their RQ value for *GADD45A*, *GADD45B*, and *GADD45G*, respectively.



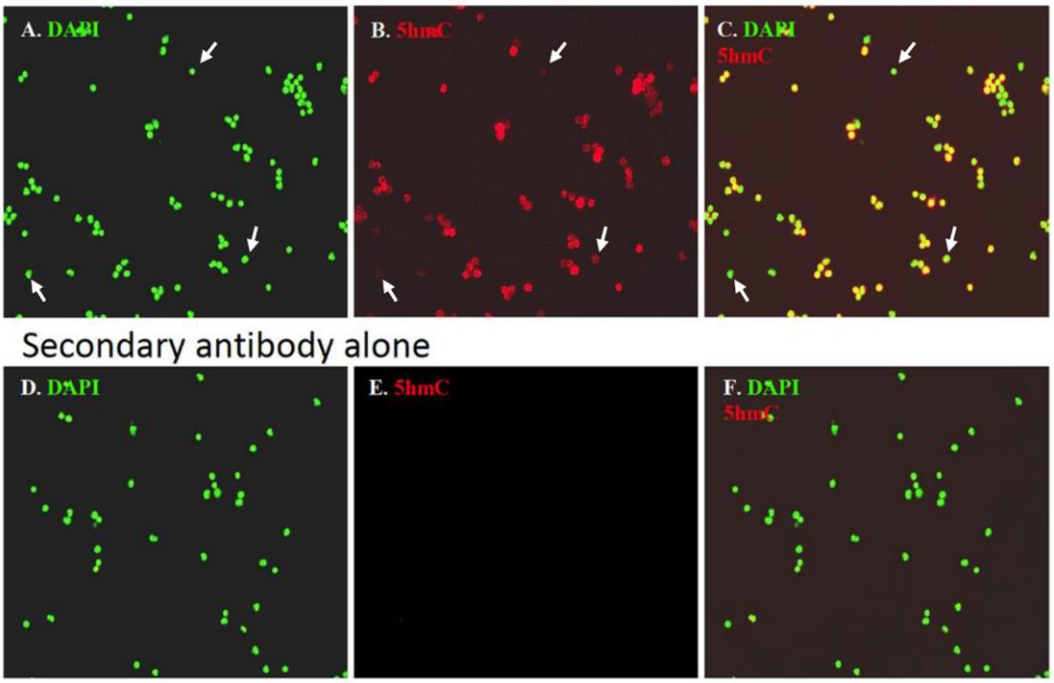
Supplemental Figure 2.S1. Cell-type specific transcript analysis among peripheral leukocyte types. **A-B.** qRT-PCR analysis of mRNA levels for eight leukocyte specific transcripts were used to further assess leukocyte purity. Values are expressed as Relative Quantity (RQ) of transcript using the dCT method, with actin transcript levels set to 1. Letters designate significant

differences of at least $p<0.05$. **A**. Assessment of purity of cell types isolated by Method 2, cell type order B (Table 2.1). Cytoplasmic *beta-actin* was used as the endogenous control. Cell marker transcript analysis of CD56+ NK cells isolated first from fresh blood using Method 2. It should be noted that beta actin mRNA levels vary widely among leukocyte cell type, and hence, the RQ values cannot be compared across cell types. **B.** Assessment of purity of isolated cell types isolated by Method 2, cell type order C. A one-way ANOVA followed by tukeys HSD post hoc was performed, and significant differences ($p<0.05$) are represented as having different letters.



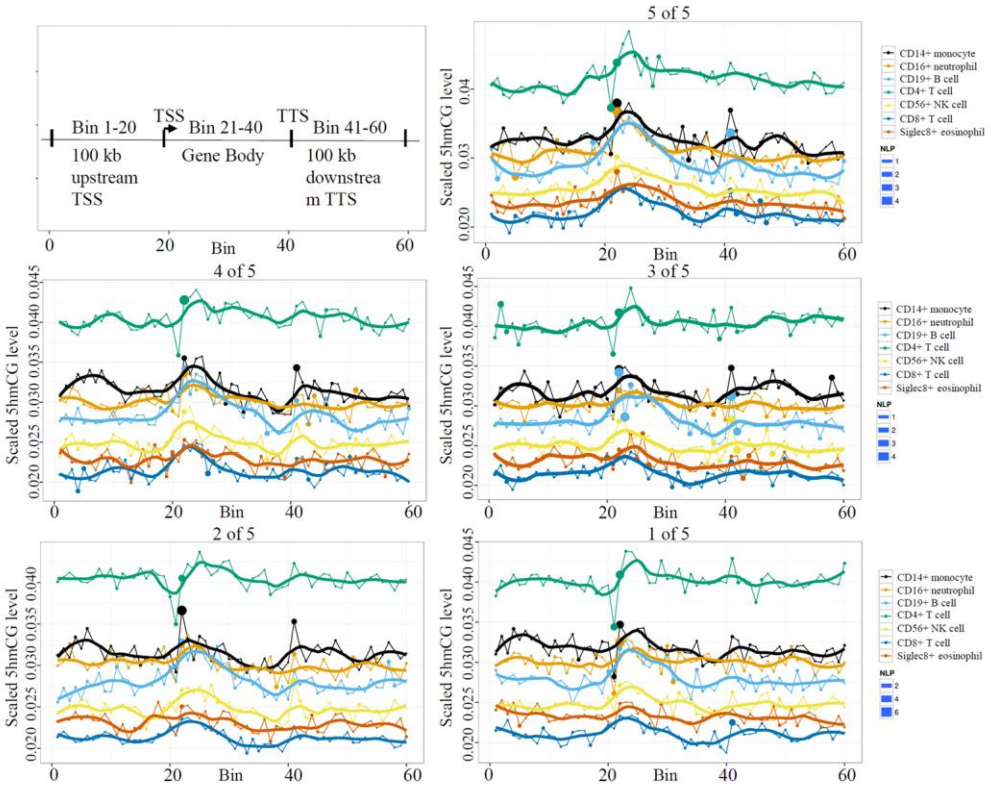
Supplemental Figure 2.S2. Assessment of monocyte and neutrophil purity in the light of detecting transcripts encoding CD4. **A.** Total leukocyte fraction from peripheral blood was isolated with Method 3 and labeled with DAPI for DNA and with anti-CD4-Texas RPE. The total leukocyte fraction shown was split into three equal aliquots and CD4+, CD14+, and CD16+ cells were each depleted from one aliquot. Cells positive for CD4 in each aliquot were classified

based on their CD4 immunostained nuclear morphologies assayed and quantified. Mean percentage of counted cells and the standard error of the mean are plotted. A one-way ANOVA followed by tukeys HSD post hoc was used to determine differences between samples, significant ($p<0.05$) differences are depicted as having differing letter.



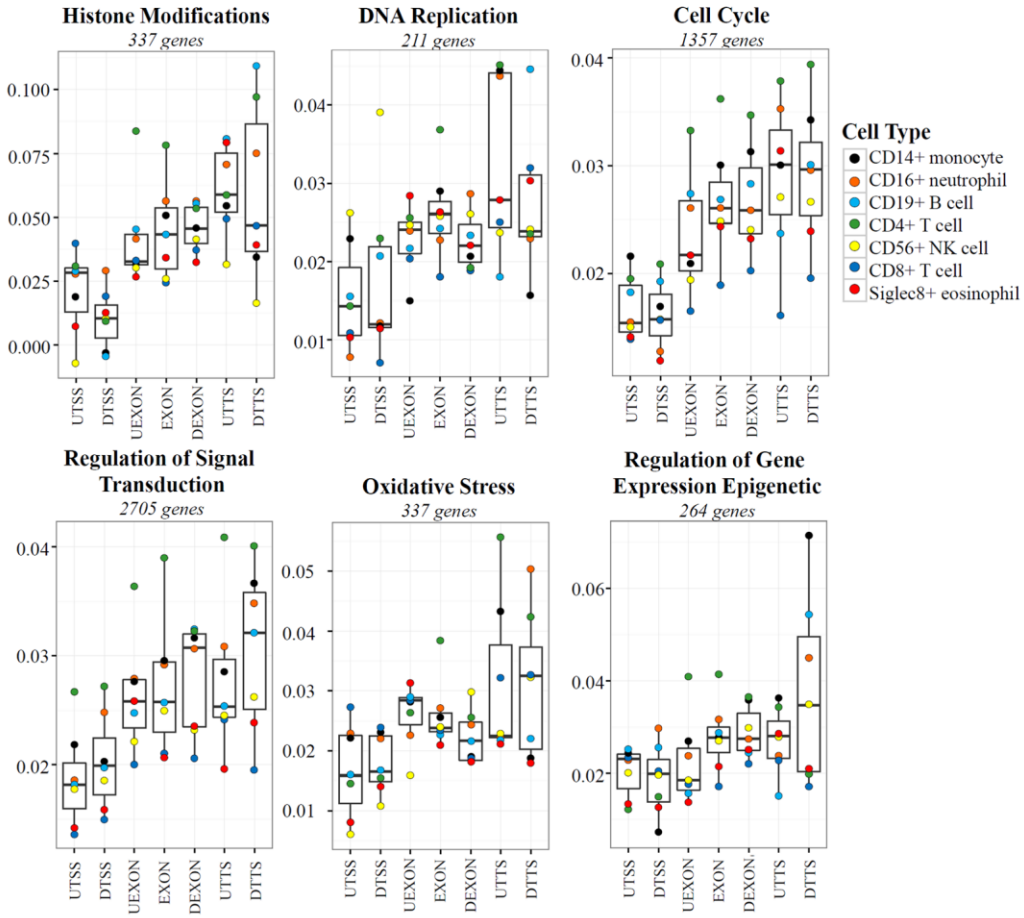
Supplemental Figure 2.S3. 5hmC is detected in the nuclei of essentially all peripheral leukocytes. **A-C.** Total leukocyte fraction from blood was formalin fixed and labeled with rabbit anti-5hmC primary antibody and goat anti-rabbit secondary antibody conjugated to PE. **D-F**. Total leukocyte fraction from blood was formalin fixed and only labeled with secondary goat anti-rabbit antibody conjugated to PE omitting the primary antibody to 5hmC (i.e., primary antibody negative control). **A-F**. All images were photographed with the exact same exposure time for DAPI and PE (5hmC) and images enhanced to with the exact same parameters. **A and D**. Cells were labeled with DAPI for DNA (green fluorescence). **B and E** differ only in that anti-5hmC was omitted from E. **C and F** represent the merged images of A and B and D and E

respectively. White arrows point to three cells in panel C that have weak 5hmC staining, and in panels A and B white arrows are in the same location pointing to the same cells labeled for DAPI alone in panel A and 5hmC alone in panel B clarifying that these cells and nearly all leukocytes examined express 5hmC, even though the weak 5hmC labeling is hard to see in the merged image in panel C. Results for A-C. Nearly all leukocytes showed some expression of 5hmC, which was not observed in the negative control (D-F). This is an extension of **Figure 2.4.**



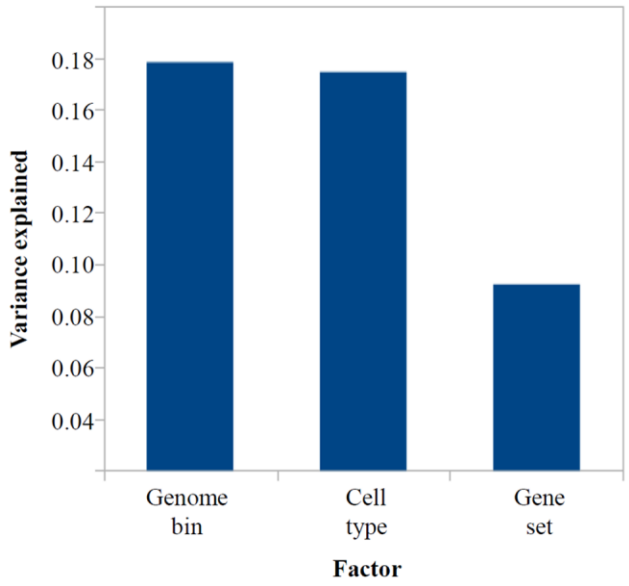
Supplemental Figure 2.S4. 5hmCG by quintile of transcript expression level among the seven peripheral leukocytes. This is an extension of figure 2.4C. The dots plotted with each line on the graph represent the degree of change from the previous regions level of 5hmCG to the current

level. The figure legend to the right of panel C shows the varying levels of significance of this change as determined by NLP.

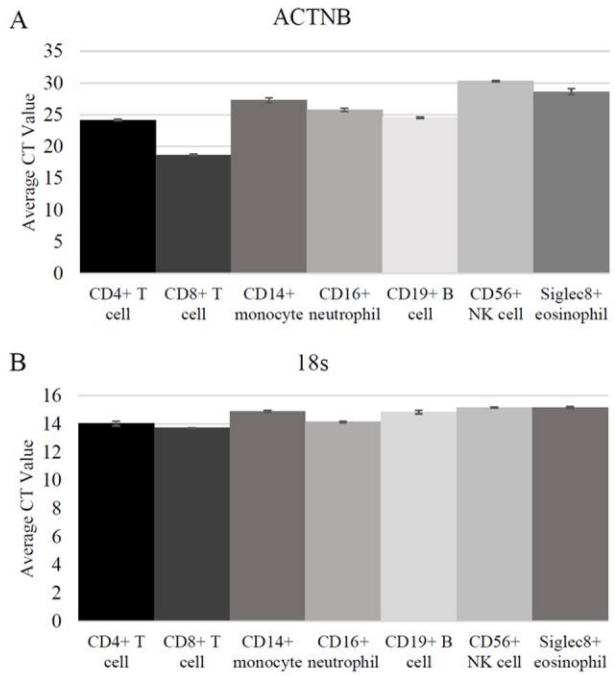


Supplemental Figure 2.S5. Additional gene ontology analysis by gene-sequence-specific distribution of 5hmCG levels among the peripheral leukocytes. Percent 5hmC by gene sequence location (upstream TSS 100 nt, downstream TSS 100 nt, upstream of exon 100 nt, within exon, downstream of exon 100 nt, upstream TTS 100 nt, and downstream TTS 100 nt) was plotted for

total leukocytes for different GO terms relevant to peripheral leukocyte function as box plots with the median 5hmCG level plotted as the bar, and the box extending from the 25th to 75th percentile of 5hmCG levels. The whiskers represent 1.5 times the IQR. Within each boxplot, the weighted average of 5hmCG for that gene region in that GO term was plotted as a colored dot for each leukocyte type. This is an extension of Figure 2.6.



Supplemental Figure 2.S6. Variance explained by genome bin, cell type and GO term gene lists. The variance explained by each of the three factors used in Figure 2.6 and Additional Figure 2.S6 were presented as a bar graph.



Supplemental Figure 2.S7. Endogenous controls. **A.** The average cycle threshold (CT) values of *ACTNB* for each of the seven leukocyte types. The error bars represent the standard error of the mean. **B.** The average CT values of *18S* for each of the seven leukocyte types. The error bars represent the standard error of the mean.

CHAPTER 3

LEUKOCYTE-SPECIFIC DNA CYTOSINE METHYLATION DIFFERENCES ASSOCIATED WITH OBESITY[3]

[3] Hohos NM, Smith AK, Kilaru V, Park HJ, Hausman DB, Bailey LB, Lewis RD, Phillips BG, Meagher RB. To be submitted to a peer reviewed journal

Abstract

Epigenome-regulated gene expression may be altered by diet, nutrition, and other lifestyle factors associated with obesity and increased adiposity. Most dietary and nutritional studies analyze DNA 5´-methylcytosine (5mC) differences in whole blood, and are a weighted average of the profiles of several distantly related classes of leukocytes. To examine leukocyte-specific differences in obesity, we examined 5mC profiles from three distinct cell types isolated from peripheral blood in normal weight (BMI 18.5-24.9 kg/m^2) and obese (BMI >30 kg/m^2) women (18-35 y). CD4+ T cells, CD8+ T cells and CD16+ neutrophils were reiteratively isolated from blood samples and 5mC levels were measured across > 485,000 CG sites in each cell type using the HumanMethylation450 BeadChip. After controlling for the false discovery rate (q-value < 0.05), 19 CG sites were differentially methylated between the obese and normal weight women in CD4+ T cells, 16 CG sites in CD8+ T cells and 0 CG sites in CD16+ neutrophils. None of the differentially methylated sites were in common between the CD4+ and CD8+ cells. The amount of visceral adipose tissue (VAT) was strongly associated with the level of methylation in 79 CG sites (q-value < 0.05) in CD4+ T cells, while no associations with VAT were identified in either of the other cell types. Gene-specific associations of 5mC site changes with obesity and VAT were highly significant, ranging from $10^{-5} < p < 10^{-9}$. Methylation increased in 4 CG sites in *CLSTN1*'s promoter with increasing VAT in CD4+ T cells. We demonstrated that inhibiting the maintenance of methylation in CD4+ T cells for 24 hr with 5-azacytidine increased *CLSTN1* transcript levels, suggesting the observed changes in the study population were important to *CLSTN1* expression. It appears that the methylome of these three cell types each respond quite differently to obesity and the levels of VAT, perhaps illustrating the importance of examining epigenetic marks in single cell types. In summary, by examining DNA

methylation differences in three cell types, we identified highly significant sites that were differentially methylated between the normal and obese weight women with biological relevance to obesity.

Introduction[4]

Obesity is a well know public health problem and is associated with many negative health consequences (Field et al., 2001; Sturm, 2002; Poirier et al., 2006; Apostolopoulos et al., 2016). Obesity results from many factors including both internal (genetic) and external (i.e. lifestyle) influences (Franks and Ling, 2010; McCarthy, 2010; de Mello et al., 2014; Apostolopoulos et al., 2016). External factors involved in the pathogenesis of obesity include environmental aspects such as dietary intake and physical activity. Diet and physical activity appear to act via epigenetic mechanisms (i.e., changes to chromatin structures) (Franks and Ling, 2010; de Mello et al., 2014; Martin-Nunez et al., 2014; Martinez et al., 2014; Kim et al., 2015). One such change in chromatin structure is the 5´ methylation of DNA cytosine, which often results in altered gene expression and corresponding altered physiology (Bird and Wolffe, 1999; Klose and Bird, 2006; Suzuki and Bird, 2008; Paluch et al., 2016). Generally, hypermethylation of the promoter region is associated with repressed transcription, while hypomethylation of this region is associated with active transcription (Klose and Bird, 2006; Suzuki and Bird, 2008; Siegfried and Simon, 2010; Pinnick and Karpe, 2011). However, gene body methylation is proposed to also have an

[4] **Abbreviations:** 5mC (5-methylcytosine), BMI (body mass index), CG (cytosine guanine dinucleotide), CGI (GC island), *CLSTN1* (calsyntenin 1), DMS (differentially methylated site), DNMT (DNA methyl transferase), *HOXB5* (homeobox 5), IFN-γ (interferon gamma), IRF1 (interferon regulatory factor 1), IRF2 (interferon regulatory factor 2), PBMCs (peripheral blood mononuclear cells), T2D (type 2 diabetes), *TMEM18* (transmembrane protein 18), TNFα (tumor necrosis factor alpha), TSS (transcription start site), VAT (visceral adipose tissue), WBC (white blood cells)

effect on epigenetically-regulated gene expression, yet the relationship here is still unclear (Bird and Wolffe, 1999; Klose and Bird, 2006; Suzuki and Bird, 2008). DNA methylation appears to be involved in obesity as it has been associated with altered methylation of specific genes in human tissues (Hermsdorff et al., 2013; de Mello et al., 2014; Dick et al., 2014; Remely et al., 2014; Houde et al., 2015), with some alterations in methylation associated with corresponding changes in gene expression (Barres et al., 2013).

Once obese or overweight, the main treatment goal is weight loss primarily through dietary modifications and increased physical activity (Jensen et al., 2014; Apovian et al., 2015). Even though there is strong evidence that caloric restriction with or without a comprehensive lifestyle program will lead to weight loss (Jensen et al., 2014), they are not successful with long term weight maintenance (Stevens et al., 2001; Kraschnewski et al., 2010). It is thought that the difficulty in maintaining weight loss stems from the reprograming of the body in the obese state through biological adaptations (i.e. suppressed energy expenditure, altered endocrine signaling, increased appetite) that favor weight re-gain (Maclean et al., 2011; Leung et al., 2016). This suggests that the reprograming that occurs in obese individuals might be carried out through semi-stable modifications in their epigenome. The dynamic nature of DNA methylation, its response to environmental influences (Jaenisch and Bird, 2003; Jacobsen et al., 2012; Kim et al., 2015) and the existence of mechanisms for its maintenance once established (Meagher, 2014) all suggests a potential connection to obesity (Campion et al., 2010; Franks and Ling, 2010; Lavebratt et al., 2012; van Dijk et al., 2015).

Most work examining the association of obesity and DNA cytosine methylation have utilized mixed cell populations, taking samples from adipose tissue (Dick et al., 2014; Guenard et al., 2014; Keller et al., 2014; Houde et al., 2015), skeletal muscle (Barres et al., 2013), and

peripheral blood leukocytes (Hermsdorff et al., 2013; Almen et al., 2014; Dick et al., 2014; Keller et al., 2014; Remely et al., 2014), all of which are comprised of many cell types. Each cell type is distinct in its DNA methylome reflecting not only tissue specificity but also cell type specific epigenetically controlled gene expression (Lister et al., 2013; Wu and Zhang, 2014; Gu et al., 2016). For example, when the DNA methylome of the seven major leukocyte types in whole blood was examined, distinct methylation profiles were revealed in each cell type (Reinius et al., 2012). The granulocytes were found to be hypomethylated while the peripheral blood mononuclear cells (PBMCs) were found to be hypermethylated (Reinius et al., 2012). CD4+ and CD8+ T cells, two closely related PBMCs differed in more than 45,000 of the 485,000 sites assayed (9%), while the more distantly related CD8+ T cells and the Siglec8+ eosinophils differed in >40% of the sites assayed (193,000 sites) (Reinius et al., 2012). Thus, analyzing leukocytes together in whole blood results in a methylation profile that is a weighted average of all included cell types. When the global methylation of the peripheral leukocytes was examined in relation to obesity, it revealed that there are only changes in global methylation in the B cells in obese individuals (Simar et al., 2014). There was no association with obesity and methylation in the PBMCs (peripheral blood mononuclear cells), which contains the weighted average of the T cells, B cells, monocytes and natural killer cells global methylation levels, providing an example of the loss of data when examining mixed cell types in relation to obesity (Simar et al., 2014). The problem is only made worse when comparing normal to obese adipose tissue, as the numbers of leukocytes is dramatically increased in obese tissue (Apostolopoulos et al., 2016). Thus methylation levels when comparing lean and obese adipose tissue may only reflect the weighted average of changes different cell populations, and not informative changes in methylation of adipocytes. The data obtained through analyzing the individual leukocyte types,

or other individual cell types from tissues (i.e. differentiated adipocytes only from adipose tissue) will yield more meaningful and insightful data to further understand the role of DNA methylation in obesity related health risks.

Obesity is characterized by a chronic low-grade systemic inflammatory state, which is thought to contribute to the development of related co-morbidities (Lee and Pratley, 2005; Anderson et al., 2010; Huh et al., 2014; Pecht et al., 2014; Apostolopoulos et al., 2016). Central adiposity further exacerbates this relationship and is considered a more unhealthy fat depot (Pinnick and Karpe, 2011; Gerriets and MacIver, 2014). Although the adipose tissue is the main organ responsible for the development of this inflammatory state (Cildir et al., 2013; Apostolopoulos et al., 2016), the resulting systemic inflammation affects the circulating leukocytes. For example, circulating levels of neutrophils are increased in obesity as are the levels of myeloperoxidase and calprotectin, which are released from activated neutrophils (Nijhuis et al., 2009; Pecht et al., 2014; Apostolopoulos et al., 2016). The neutrophil activation marker CD66b is also increased in obesity (Nijhuis et al., 2009). Additionally, in obese adipose tissue CD8+ T cells increase while CD4+ T cells decrease, and in circulation there is an increase in CD4+ T cells while the effect on CD8+ T cell levels has been mixed (Womack et al., 2007; Nishimura et al., 2009; Ilavska et al., 2012; Pecht et al., 2014). There is also an increase in the ratio of circulating pro-inflammatory Th1 to anti-inflammatory Th2 CD4+ T cells with obesity, which may further potentiate the inflammatory cascade (Viardot et al., 2012; Apostolopoulos et al., 2016). Human adipose tissue samples are difficult to collect, however different classes of leukocytes from peripheral blood are easy to obtain, making them ideal surrogate cell types to assay DNA methylation (Terry et al., 2011; Adalsteinsson et al., 2012; Crujeiras et al., 2013). .

Thus, we choose to examine CD4+ and CD8+ T cells, and CD16+ neutrophils to examine the DNA methylome among obese and normal weight women.

The physiological stress of obesity and the distinct roles of these three classes of leukocytes led us to hypothesize that (1) there will be differences in DNA methylation that are associated with both obesity and levels of adiposity and (2) the differences in methylation will be distinct to each of the three classes of leukocyte. We assayed DNA methylation of >450,000 sites in each leukocyte type in a group of obese and normal BMI women. Our results identified cell type specific differences in DNA cytosine methylation between the obese and normal weight women in both the T cell types, but not in the neutrophils. We also identified an association of DNA methylation with the amount of VAT in the CD4+ T cells, while no associations were found between VAT and the other two cell types.

Results

The obese and normal weight groups in this study differed in weight, BMI, percent body fat, amount of VAT, and VAT normalized to body weight ($p<0.05$) (**Table 3.1**). Although the groups were not significantly different in age, age was added as a covariate in all subsequent analysis of DNA methylation, as age is independently associated with altered DNA methylation (Boks et al., 2009; Hannum et al., 2013; Jenkins et al., 2013; Jones et al., 2015).

Assessment of DNA methylation differences between obese and normal weight women in three leukocyte types.

DNA methylation differences between women classified as obese (BMI ³ 30 kg/m^2) and normal weight (BMI 18.5 £ 24.9 kg/m^2) for all sites on the array were analyzed for each of the three leukocyte types assayed (CD4+ T cells, CD8+ T cells, and CD16+ neutrophils). There were 19 significantly differentially methylated sites (DMS) identified in CD4+ T cells ($q < 0.05$), 16

in CD8+ T cells, and no sites were significantly differentially methylated in the CD16+ neutrophils (**Table 3.2**).

Within the DMS in the CD4+ T cells, eight had decreased methylation and 11 had increased methylation in the obese women. In addition, eight of the sites were associated with promoter regions of the associated gene. The most significantly DMS ($q<0.005$) was cg06384413 which is associated with both the *HOXB5* and *LOC404266* genes. Within the significantly DMS in the CD8+ T cells, 10 had decreased methylation and six had increased methylation in the obese women. Five of the sites were associated with promoter regions. The most significantly DMS ($q<0.002$) in the CD8+ T cells was cg26655295, which is associated with *TMEM18.* There were no significantly differentially methylated sites common to both cell types.

To further characterize the differences in methylation between the obese and normal BMI women, the absolute difference in methylation between the two groups was calculated for the 19 DMS in the CD4+ T cells and the 16 DMS in the CD8+ T cells (**Figure 3.1A**). The CD8+ T cells had much higher differences in the magnitude of methylation change between the two groups with over 40% of the DMS having at least a 10% difference in methylation. The differences in methylation between the two BMI groups in CD4+ T cells were smaller, with over 70% of the DMS having a difference in methylation between 2.5 to 5%.

The 19 and 16 sites with differential methylation in the obese women for the CD4+ and CD8+ T cells respectively were then characterized with respect to the percent of sites associated with each aspect of CG island (CGI) (**Figure 3.1B**). Over 60% of the 19 DMS in CD4+ T cells were located in a CGI, while the majority of the 16 DMS in the CD8+ T cells were located in the flanking regions of CGI and very few within the island themselves. Only a small percentage of

the DMS were located outside of a CGI in the open sea, 15.8% in CD4+ T cells and 25% in CD8+ T cells.

Functional enrichment analysis identified 57.9% and 42.1% of the genes associated with the 19 sites with differential methylation between the two BMI groups in the CD4+ T cells as having transcription factor binding sites for Interferon Regulatory Factor 2 (IRF2) and Interferon Regulatory Factor 1 (IRF1) respectively (**Table 3.3**). IRF2 and IRF1 are members of the interferon regulatory transcription factor family which have various roles in regulating the immune response and in hematopoietic differentiation (Taniguchi et al., 2001; Battistini, 2009; Huang et al., 2010).

DNA methylation levels correlated with the amount of VAT

Because higher levels of VAT are associated with a more negative health outcome (Freedland, 2004; Revelo et al., 2014; Lee et al., 2015), we looked for an association between methylation levels and the amount of VAT in each of the three peripheral leukocyte types. Only for the CD4+ T cells were such differences identified. The methylation of 79 CG sites were significantly associated with the amount of VAT (q-value<0.05) in this cell type (**Table 3.4**).

Of the 79 DMS that were identified, 61 displayed decreasing methylation with increasing amount of VAT. 26 of these sites were associated with enhancer regions and 5 with promoter regions. None of these 79 sites were found to be differentially methylated between the obese and normal BMI women in either CD4+ or CD8+ T cells (**Table 3.2**). The 79 sites with methylation levels associated with the amount of VAT were plotted to show the distribution of the percent of sites falling into each aspect of CGI (**Figure 3.1C**). The majority of the sites were either in a CGI, or in the flanking regions, although more sites were identified in the open sea than the sites with differential methylation between the obese and normal BMI groups in either of the two T

cell types. One gene, *CLSTN1*, had four CG sites that increased with the amount of VAT, all of which were associated with the transcription start site region (**Figure 3.2)**.

Functional enrichment analysis of these genes containing these 79 DMS (**Table 3.5**) identified enriched biological processes including those related to phosphate and phosphorus metabolic processes, phosphorylation, negative regulation of signal transduction and cell communication, and intracellular transport.

Validating a potential role for DNA methylation changes in regulating gene expression

We identified one gene, *CLSTN1* with four sites where methylation levels increased with the amount of VAT. All four sites were located prior to the TSS, a region where methylation levels have been shown to affect gene expression (Suzuki and Bird, 2008). As this gene has been previously shown to have differential methylation associated with obesity (Huang et al., 2015b), as well as altered expression in adipose tissue of morbidly obese women (Dahlman et al., 2012), we choose to further look into the role of methylation in *CLSTN1*'s gene regulation in CD4+ T cells.

There is accumulating evidence to suggest that DNA methylation of CG sites may turn over relatively rapidly with half-lives measured in hours, going through the methylation cycle of oxidation, base removal and remethylation, even though steady state methylation levels may be relatively constant (Meagher, 2014). Maintenance of methylation status is enabled by the retention of hemi-methylated base in complementary CG site recognized by the maintenance methylase DNMT1. Inhibiting cultured cells for 2 hours with a DNMT inhibitor 5-aza-deoxycytosine decreased genome-wide methylation levels by 10% whereas longer treatment produced insignificantly greater decrease (Yamagata et al., 2012). These data suggest a subset of sites turnover rapidly and that that their re-methylation is inhibited by 5-azacytidine. It seemed

possible that we could make use of the methylation cycle to demonstrate its relevance to gene regulation, even in non-activated peripheral CD4 T cells. WBCs were isolated from freshly drawn peripheral blood and cultured for 24 hours with or without the DNMT inhibitor 5azacytidine (5azaC, 2µM) followed by isolation of the CD4+ T cells. We were hypothesizing that if DNA methylation is repressing transcription of *CLSTN1*, 5azaC treatment should result in increased mRNA. The expression of *CLSTN1* transcript was significantly increased in the CD4+ T cells as compared to those cultured without the drug treatment (**Table 3.6**, Supplemental **Figure 3.5**). The other four genes assayed, all of which had only one CG methylation difference correlating with VAT levels were not significantly altered in expression in response to 5azaC treatment (**Table 3.6**).

Discussion

We examined DNA cytosine methylation differences between obese and normal weight women and as a function of VAT mass among CD4+ T cells, CD8+ T cells, and CD16+ neutrophils. We identified CG sites with changes in CG methylation levels associated with both BMI classification in CD4+ and CD8+ T cells and with VAT mass levels in CD4+ T cells. To our knowledge, only two prior studies have been performed examining DNA methylation in obesity in single leukocyte types. When global 5mC was examined, there were only obesity related differences in the B cells (Simar et al., 2014). We also did not observe global methylation changes in the three cell types assayed in this study, suggesting that the methylation changes associated with obesity in the T cell types is site specific. Additionally, CD4+ T cells 5mC profile has been examined in a mixed population of adults and eight DMSs were identified to be correlated with obesity and five with waist circumference (a measure of central adiposity) (Aslibekyan et al., 2015). However, none of the DMSs identified in their study were also

identified in our analysis of CD4+ T cells. This may be due to the fact that only women were examined in this study, while both sexes were included in Aslibekyan et al. (2015), and they looked for associations with BMI as a continuous variable, where we looked between obese and normal BMI groups. None the less, this previous data and ours supports the idea that 5mC levels in CD4+ T cells respond to obesity.

It is important to consider that the obese women included in this study had no metabolic comorbidities of obesity, and were overall healthy obese women. Thus, the DNA methylation differences we observed have occurred before the development of insulin resistance and other obvious associated comorbidities and are only associated with increased adiposity. This is important when comparing DNA methylation studies in obesity in which the participants have developed metabolic disturbances (i.e. insulin resistance) and comorbidities (i.e. T2D) (Keller et al., 2014; Su et al., 2014; Kurylowicz et al., 2015; Al Muftah et al., 2016).

T Cells as surrogates to study obesity.

It is not surprising that we observed DMSs in the CD4+ and CD8+ T cells in the obese women. The two T cell populations evaluated in this study have been shown to have high methylation levels in healthy adults, 56.1% methylated in CD4+ T cells and 33.4% methylated in CD8+ T cells (Reinius et al., 2012). Thus, there is room for both increases and decreases in methylation of site specific residues in these cell types. The high methylation levels may also suggest that DNA methylation is important in these cell types. Additionally, CD4+ T cells 5mC profile has been associated with BMI and waist circumference (Aslibekyan et al., 2015). Furthermore, DNA methylation has been implicated in the differentiation of both CD4+ and CD8+ T cells from their precursor cells (Tsagaratou et al., 2014) and in obesity there is altered differentiation of these cells into their various subtypes. For example, there is an alteration in the

lineage of CD4+ T cell subsets to higher levels of Th17 cells, which release pro-inflammatory cytokines in obesity (Viardot et al., 2012; Apostolopoulos et al., 2016).

Although differences in DNA methylation associated with obesity were identified in both the T cell types assayed in this study, there was no overlap in the differentially methylated sites identified between them. While CD4+ and CD8+ cells both are involved in the adaptive immune system, they carry out very different roles. CD4+ T cells bind antigens that are in MHC class II complexes (Apostolopoulos et al., 2016), while CD8+ T cells bind to MHC class II complexes and are involved in the cytolysis of target cells (Huh et al., 2014). In response to *mycobacterium tuberculosis* CD4+ T cells play a protective role through their secretion of TNFα and IFN-γ to recruit and activate other innate immune cells, while CD8+ T cells have a more 'multifunctional' role including their ability to target cells for apoptosis (Prezzemolo et al., 2014). Additionally, in response to *Listeria* infection, CD4+ T cells have limited proliferative capacity while the CD8+ cells have extensive proliferative capacity, thought to facilitate their roles as regulator and effector T cells (Foulds et al., 2002). Finally, CD8+ T cells have longer sustained memory cell responses than CD4+ T cells in response to viral infection (Seder and Ahmed, 2003). Considering that each of these cell types has its own 5mC (Reinius et al., 2012) and 5-hydroxymethylcytosine (Hohos et al., 2016) profile, our data showing no overlapping sites of DNA methylation differences may not be so surprising. These distinct cell type specific differences provide further evidence of the importance of examining epigenetic marks in relation to obesity in single cell types. Considering that T cells types make up only 7 to 24% of the peripheral blood leukocyte population, the significance of these differences would undoubtedly have been missed in an analysis of whole blood methylation.

The limitations of neutrophils as cell type to study DMSs.

No associations between DNA methylation and obesity were identified in neutrophils. There are several aspects of this observation worthy of discussion. First, neutrophils have very low levels of methylation to begin with in healthy adults (5.7% methylated) (Reinius et al., 2012), so only an increase in methylation in obesity is reasonably possible. Second, we used a stringent cut off to determine significance after controlling for multiple testing ($q < 0.05$), there were sites with p-values as low as $1x10^{-5}$ but were no longer associated with the phenotype. Third, we recently published data showing that neutrophils have low levels of the machinery needed for methylation and de-methylation, suggesting they are not involved in rapid turnover of their methylomes (Hohos et al., 2016). Forth, neutrophils have a short half-life in the blood (Pillay et al., 2010) and thus they may have no need or time to alter their methylome in response to physiological changes. Instead, methylation may be more important to their pre cursor cells in the bone marrow. Finally, perhaps neutrophils integrate physiological changes into to genome thorough other epigenetic mechanism, such as histone modifications not assessed herein.

Associations with VAT

To further characterize DNA methylation differences in obesity, we assessed the relationship between 5mC level and the amount of VAT. VAT is known to be a fat depot with a significant relationship with the development of obesity and the chronic low grade inflammatory state (Shu et al., 2012; Thomas et al., 2012). BMI is a height to weight ration and does not provide information of either percent body fat or adipose distribution (Gallagher et al., 1996; Shah and Braverman, 2012) thus, our analysis of 5mC between the obese and normal BMI groups may have missed relationships with VAT and DNA methylation. VAT experiences changes to its cellular makeup with increasing adiposity as well as releases a milieu of cytokines that affect the overall inflammatory state involving both CD4+ and CD8+ T cells

(Apostolopoulos et al., 2016), and likely affect the peripheral leukocytes. However, we only identified DNA methylation correlating with the amount of VAT in one of the peripheral leukocyte types, the CD4+ T cells. In comparison to the differences we observed between the obese and normal weight women, the 79 sites with methylation levels correlating to the amount of VAT were unique to this analysis. Interestingly, when we normalized the amount of VAT to body fat or mass we did not observe any associations with methylation (data not shown). This suggests that the amount of VAT, regardless of total body mass or adiposity, has an impact at the molecular level.

However, unlike in the analysis between the obese and normal weight women we only identified correlations between methylation and visceral adiposity in one cell type, the CD4+ T cells. Again, the same explanations for why we did not observe differences in the neutrophils apply here (low levels of methylation, low levels of machinery for methylation and demethylation, and short half-lives), however we might expect to see the correlation with DNA methylation and VAT in the CD8+ T cells. This is especially true because CD8+ T cells have been shown to be involved in the early stages of increased adiposity, where they infiltrate VAT before macrophages and are involved in macrophage recruitment into adipose tissue (Nishimura et al., 2009).

Relevance of DMS's to gene expression

We identified four sites within the gene *CLSTN1,* calsyntenin 1, to have methylation levels correlating with the amount of VAT in CD4+ T cells, all located prior to the TSS. We show that in CD4+ T cells when DNMT-dependent remethlation of hemi-methylated DNAs was inhibited and thus unable to methylate DNA, there was an increase in *CLSTN1* gene expression, suggesting the DNA methylation is involved in its gene regulation. We examined 4 other genes

with only one DMS, but found no other changes in gene expression. *CLSTN1* interacts with kinesin-1 motor for the transport of certain vesicles and has a cytoplasmic calcium binding domain (Vogt et al., 2001; Vagnoni et al., 2012). The majority of the work on *CLSTN1* has been performed in the brain where it has been shown to be involved in the trafficking of the amyloid precursor protein and the pathogenesis of Alzheimer's disease (Vagnoni et al., 2012). However, there is also evidence of this protein having an effect in CD4+ T cells, as in some patients with acute myeloid leukemia (AML), calsyntenin 1 peptides are able to produce the CD4+ T cell response (Stickel et al., 2013).

Other studies have suggested that *CLSTN1* may be relevant to diet and obesity. In rats fed a low protein diet with vitamin D, there is also increased expression of *CLSTN1* in the kidneys (Chen et al., 2010). Additionally, when human islet cells are exposed to the saturated fatty acid palmitate, there is decreased expression of *CLSTN1* (Cnop et al., 2014). In the subcutaneous adipose tissue of morbidly obese women, there is increased expression of *CLSTN1* (Dahlman et al., 2012). Finally, in PBMCs differential methylation of one site of *CLSTN1* was identified between obese and normal weight participants, obese and successful weight loss maintainers, and between normal weight and successful weight loss maintainers (Huang et al., 2015b). This evidence in addition to our data suggest that *CLSTN1* methylation levels are increased with increasing amounts of VAT, potentially related to its gene expression, and may be a novel gene related to adiposity and obesity .

Conclusions

In our exploratory study we identified differences in DNA methylation in both CD4+ and CD8+ T cells in obese women and in CD4+ T cells with increasing amounts of VAT. The differences we observed were unique to each cell type and revealed no overlaps in methylation

changes between the different analyses. The data herein provide further evidence of the importance of examining DNA methylation in single cell types in relation to obesity. As neutrophils are the majority cell type in WBCs and we observed no methylation differences in this cell type, if we had performed these experiments in WBCs we may have not observed the cell type specific differences in the two T cell types as they would have been obscured by the heavily weighted methylation profile of neutrophils.

Materials and Methods

Study participants:

Fourteen normal weight (BMI 18.5 to 24.9 kg/m^2) and eight obese (BMI >30.0 females kg/m^2) (age 18-35 years old) women were recruited from the Athens, GA area. Exclusion criteria included pregnancy in the last year, chronic disease, prescription drug use other than oral contraceptives, smoking, heavy alcohol consumption, and weight change greater than 10% in the past six months. To limit genetic variability, only those women who self-identified as Caucasian were selected for this study. As this study was a subset of a larger folate supplementation trial, all included women had serum folate levels <60 nmol/L and the CC or CT *MTHFR* 677 (Rs1801133) genotype (sequenced using real-time PCR by the Georgia Genomics Facility in Athens, GA). All data used in the presented study are from baseline appointments, before the folate supplementation or any other intervention occurred. The University of Georgia Institutional Review Board approved this protocol and all subjects provided written informed consent, after being made aware as to the design of the study.

The participant's height and weight were obtained by standard protocols and used for the calculation of their BMI (kg/m^2). Body composition was also determined for the participants through duel-energy X-ray absorptiometry (DXA) (Hologic Discovery A, Hologic Inc.,

Waltham, MA). DXA data was only available for N = 13 of the normal weight women and N=7 of the obese women.

To determine differences in regards to the biometrical parameters between the obese and normal weight women, a two tailed t-test was performed with significance set at $p<0.05$.

Cell isolation

10 mL of venous blood samples were collected from all participants after an overnight fast. The samples were stored on ice after collection and processed within four hours of collection. CD4+ T cells, CD8+ T cells, and CD16+ neutrophils were reiteratively isolated from the whole blood following the protocol published in Hohos et al. (2016). Isolated cells were stored at -80ºC in 200 µl of PBS (Phosphate Buffered Saline) until genomic DNA extraction with the DNeasy Kit (Cat # 69506, QIAGEN). The extracted DNA was then quantified using Quant-iT PicoGreen dsDNA assay kit (Cat #P7589, Life Technologies) following manufacture protocol and by nanodrop. Limitations in cell isolation and DNA yield resulted in a reduced sample size for the obese CD8+ T cell samples (N=7), normal weight neutrophils (N=12) and obese neutrophils (N=6).

DNA methylation analysis

In total 61 samples of genomic DNA (~1.6 µg) were loaded onto the Illumina-provided, midi deep well, barcoded plate and sent to Illumina for processing of the HumanMethylation450 BeadChip to interrogate >485,000 independent CG sites throughout the genome with 99% coverage of RefSeq genes following Illumina's instructions (Illumina, San Diego, CA). CpGassoc was used for implementation of quality control parameters (Barfield et al., 2012). Samples with probe detection call rates < 90% were excluded, as were those with an average intensity value of either < 50% of the experiment-wide sample mean or < 2,000 arbitrary units.

Data points with detection p-values > 0.001 were set as missing, and probes that cross hybridize between autosomes and sex chromosomes were excluded (Chen et al., 2013). No individual subject samples were excluded. BMIQ was then used to normalize the probe distributions and background signals (Teschendorff et al., 2013). Estimated DNA methylation proportions (the ration of methylated signal to total signal) or β-values were then computed for each CpG site.

MethLAB (Kilaru et al., 2012) was used to test for association with BMI class (normal or obese) in each of the three leukocyte types via linear regressions that modeled the M-values (log(beta-value/(1-beta-value))) as the outcome and the BMI class as a categorical independent variable, or VAT g as a continuous independent variable for each CG site on the array. Age was added as a covariate in all regression analysis. To control for false positives due to multiple testing, associated sites were only considered significant after controlling the false discovery rate with a q-value < 0.05. Methylation levels associated with the phenotype as determined with the use of M-values are discussed as ß-values in the manuscript, where a ß-value of 0 is equal to 0% methylation and a ß-value of 1 is equal to 100% methylation (Du et al., 2010). Functional enrichment analysis was performed using DAVID 6.7 (Huang da et al., 2009a; Huang da et al., 2009b). Terms were considered enriched in the data set if the EASE score (a modified fishers exact p-value) was <0.05 and the fold enrichment was >1.5 (Huang da et al., 2009b).

Methylation and gene expression assay

Venous blood samples were collected in EDTA tubes in the morning from a nonfasted healthy 65-year-old male volunteer free of cardiovascular and other diseases with a BMI of 29. These studies were approved by the Institutional Review Board at the University of Georgia. White blood cells were immediately isolated. Following isolation cells were washed 1X with 10 ml of DMEM (at 37°C) at 100 x g for 10 min. Cells were resuspended in DMEM+ (DMEM, 1%

penicillin-streptomycin, 10% FBS) and plated in a volume of 2 ml (~500,000 WBCs) in a 6 well culture dish. 2 µM 5azaC in DMEM was added to drug treated samples. N=6 control (no drug) and N=6 treatment (5azaC) were incubated in a 37°C incubator for 24 hours. Cells were then washed with 1X PBSBE (phosphate buffered saline, 1% BSA, 2mM EDTA, pH7.4) and CD4+ T cells were isolated as described in (Hohos et al., 2016). RNA was extracted from the isolated CD4+ T cells with RNeasy mini kit (Cat#74101, QIAGEN) following manufactures protocol. RNA was quantified with Quibit RNA assay kit (Cat # Q32855, Life Technologies) and 400 ng of RNA was used for cDNA synthesis with qScript cDNA synthesis supermix (Quanta Biosciences, Gaitthersburg, MD, USA Cat# 95148-100). Oligonucleotide primer sequences (**Supplemental Table 3.S1**) were synthesized by Integrated DNA Technologies (Coralville, IA, USA). Two primer sets were tested for each of the target genes and those having a single sharp dissociation peak, ensuring the specific gene target was being amplified, and the lowest CT values were selected for subsequent use. A 25 µl reaction using SYBR green master mix (Life Technologies, Grand Island, NY, USA Cat# 43677659) and 4 ng of cDNA was used for analysis of the gene panel. All reactions were repeated in triplicate. All data was normalized to the endogenous control 18s mRNA and then to the relative expression of the control samples by the ddCT method. A one-tailed t-test was performed to determine if there was an increase in expression of the 5azaC treatment, with significance set to $p<0.05$.

Table 3.1. Descriptive characteristics of study participants

	Age	Weight (kg)	BMI	% Body Fat*	VAT (g)*	VAT (g) per Body fat (kg)*	Vat (g) per Body weight (kg)*
CD4+ T cells							
Normal BMI	26.2 ± 1.4	59.7 ± 1.7	21.6 ± 0.3	29.2 ± 1.4	154.8 ± 20.6	8.7 ± 0.9	2.6 ± 0.3
Obese BMI	30.9 ± 1.7	102.8 ± 3.7	36.9 ± 1.1	44.9 ± 0.4	502.5 ± 74.1	10.8 ± 1.6	4.9 ± 0.7
p-value	0.06	4.3E-10	7.4E-13	3.9E-7	0.001	0.3	0.006
CD8+ T cells							
Normal BMI	26.2 ± 1.4	59.7 ± 1.7	21.6 ± 0.3	29.2 ± 1.4	154.8 ± 20.6	8.7 ± 0.9	2.6 ± 0.3
Obese BMI	30.6 ± 1.9	105.2 ± 3	37.2 ± 1.2	44.9 ± 0.5	537.7 ± 71.8	11.4 ± 1.7	5.2 ± 0.8
p-value	0.1	1.8E-10	2.2E-12	1.8E-6	1.6-5	0.2	0.004
CD16+ neutrophils							
Normal BMI	27.1 ± 1.5	59.4 ± 1.8	21.6 ± 0.4	29.3 ± 1.5	158.7 ± 22.0	8.9 ± 1	2.7 ± 0.3
Obese BMI	31.3 ± 1.3	101.3 ± 3.6	37.6 ± 0.9	44.9 ± 0.5	502.25 ± 86.4	10.9 ± 1.8	5.0 ± .9
p-value	0.1	7.2E-9	4.9E-12	4.3E-6	0.0002	0.3	0.01

Values are reported as mean ± sem. Reported p-values were calculated from a two tailed t-test and were considered significant at $p < 0.05$. VAT: visceral adipose tissue. Limited amounts of DNA isolated from the CD8+ T cells and CD16+ neutrophils resulted in slightly reduced sample size for the respective analysis (CD4+ T cells normal BMI N = 14 and obese BMI N = 8; CD8+ T cells normal BMI N = 14 and obese BMI N = 7; CD16= neutrophils normal BMI N = 12 and obsess BMI N = 6). *Data from DXA was only available for N=13 of the normal BMI participants in the CD4+ and CD8+ cells; N=7 for obese CD4+ T cells and N=6 for obese CD8+ T cells.

Table 3.2. DMS between obese and normal BMI women in CD4+ and CD8+ T cells

Cell Type	CG sites	Associated Genes
CD4+ T cells	cg06384413, cg07321536, cg06352483, cg03056766, cg25350057, cg08913530, cg17213381, cg09248007, cg12227505, cg06090383, cg03704653, cg10318313, cg15418826, cg02466749, cg27659478, cg25291941, cg22068822, cg19180156, cg07790826	LOC404266, HOXB5, LIAS, RPL9, FAM76A, SCAMP1, GPR177, C10orf129, AGPAT1, MKL2, SLC26A11, SGSH, SAP30, FAM9A, NAP1L4, KIF21A, FANCC, TRIM65, POP1, HRSP12, UBTD2, FADD
CD8+ T cells	cg26655295, cg21579726, cg19235307, cg17191443, cg08426200, cg01419670, cg089164477, cg01059398, cg18449739, cg01560407, cg16248435, cg25732252, cg11844737, cg06544310, cg06074534, cg11088051	TMEM18, ABT1, IFT122, MBD4, MATN4, AGPHD1, TNFSF10, DTX1, ITFG3, JARID2, ST6GALNAC4, BCOR, HNRNPUL1, ZDHHC7, SLC25A3

The sites with differential methylation ($q < 0.05$) between the obese and normal BMI classified women in both CD4+ T cells and CD8+ T cells and their associated genes are listed above. Not all sites were associated with a named gene. Some sites were associated with two genes. Sites are listed in descending order of significance.

Table 3.3. Functional enrichment analysis of DMS between obese and normal BMI women in CD4+ T cells

Enriched TFBS	Number of genes	Percent of gene list	p-value	Fold Enrichment
IRF2 sites	11	57.9%	0.0099	2.02
IRF1 sites	8	42.1%	0.022	2.41

Functional enrichment analysis was performed for UCSC transcription factor binding sites with the associated genes of the DMS in obesity in the CD4+ T cells. The p-value listed is an EASE score, a modified fisher exact p-value, and terms were considered enriched at $p<0.05$ (Huang da et al., 2009b). The magnitude of enrichment of the UCSC transcription factor term to the total genes in the human genome is listed as the fold enrichment value (Huang da et al., 2009b). Fold enrichment values of greater than 1.5 and lower EASE scores are considered enriched in the data set (Huang da et al., 2009b).

Table 3.4. CG sites associated with VAT (g) in CD4+ T cells

CG sites	Associated Genes
cg05942022, cg03340649, cg19143282, cg14287443, cg20329085, cg19670290, cg12005412, cg26317237, cg05114959, cg24551579, cg22053720, cg25133192, cg01543179, cg23936609, cg02835977, cg09082287, cg18803110, cg17177074, cg22221131, cg01447854, cg07442105, cg19858017, cg20388707, cg14373988, cg06745684, cg07521668, cg26345916, cg26639906, cg11643442, cg12990575, cg02494246, cg14559176, cg22614521, cg20029881, cg24339043, cg11954030, cg10070328, cg25649895, cg18446069, cg21497780, cg06330289, cg05312779, cg09213124, cg14552010, cg22512973, cg06815003, cg23712458, cg01281450, cg01800926, cg17028259, cg23279792, cg00583861, cg08151292, cg10928257, cg05897809, cg16091292, cg04682699, cg24033558, cg02936679, cg00123104, cg05455971, cg01967642, cg24138916, cg15007123, cg03470671, cg19423175, cg23400715, cg11679124, cg04486919, cg13576552, cg01161042, cg23673974, cg18431489, cg01312828, cg16630259, cg04527989, cg13932865, cg07873325, cg27166993	SLC2A1, ZNF660, CTDP1, ASXL3, HDDC3, UNC45A, CLSTN1, PTK7, DHX9, NKX3-1, BRD4, DNAJC6, PRKCZ7, CASZ1, RNASEH2B, OBSCN, NGEF, PEX10, CLDN14, MACROD1, CACNA1G, SNORA38, BAT2, KLC4, ALDH3B1, LRP1, SPRYD3, MYO10, TMEM92, WNT5B, ANPEP, IGFBP4, AFF3, STX1A, RPH3AL, IFNG, SCARF1, SPEF1, MIR449C, CDC20B, C11orf35, SLC38A3, SHF, DLGAP2, EPHA10, SMTNL2, FAM109A, PRDM11, MAP2K2, FAM19A5M FRMD4A, MAD1L1, ZFYVE28, TBKBP1, TNXB, WIPF2, PTCD2, MRPS27, KRCC1, LGR5

The CG sites and the associated genes with differential methylated ($q < 0.05$) with amount of VAT in CD4+ T cells are listed. Not all CG sites were associated with a named gene. Some CG sites were associated with two genes. CG sites are listed in descending order of significance.

Table 3.5. Functional enrichment analysis of CG sites with methylation levels correlating with the amount of VAT in CD4+ T cells

Enriched GO: Biological Process	Number of genes	Percent of gene list	p-value	Fold Enrichment
GO:0006468 protein amino acid phosphorylation	7	12.07	0.01	3.74
GO:0006796 phosphate metabolic process	8	13.79	0.015	2.93
GO:0006793 phosphorus metabolic process	8	13.79	0.015	2.93
GO:0016310 phosphorylation	7	12.07	0.02	3.12
GO:0009968 negative regulation of signal transduction	4	6.90	0.02	6.44
GO:0010648 negative regulation of cell communication	4	6.90	0.03	5.74
GO:0046907 intracellular transport	6	10.34	0.03	3.25

Functional enrichment analysis was performed for GO: biological processes with the associated genes of the sites with methylation levels correlating with VAT in CD4+ T cells. The p-value listed is an EASE score, a modified fisher exact p-value, terms were considered enriched at $p<0.05$ (Huang da et al., 2009b). The magnitude of enrichment of the biological process term to the total genes in the human genome is listed as the fold enrichment value (Huang da et al., 2009b). Fold enrichment values of greater than 1.5 and lower EASE scores are considered enriched in the data set (Huang da et al., 2009b).

Table 3.6. Effect of 5azaC on gene expression in CD4+ T cells

Gene Name	DMS associated with phenotype	q-value for association with phenotype	DMS gene-region location	Direction of methylation change	Effect of 5azaC on gene expression	p-value for 5azaC treatment
IFNG	1	0.045	3'UTR	Decreases with increasing VAT	No effect	0.35
CLSTN1	4	0.034 0.045 0.045 0.045	TSS200 TSS200 TSS200 TSS200	Increases with increasing VAT	Increased	**0.037**
NAP1L4	1	0.043	TSS1500	Decreased in Obese	No effect	0.33
POP1	1	0.048	TSS1500/ 1st exon	Increased in Obese	No effect	0.15
LIAS	1	0.011	TSS1500	Increased in Obese	No effect	0.41

The five genes chosen to determine if DNA methylation is involved in the regulation of their expression in CD4+ T cells are listed. A one-tailed t-test was performed between control and 5azaC samples at 24 hours. Significance was set to $p<0.05$ (bold), and the p-values for this analysis are listed.

Supplemental Table 3.S1. Oligonucleotide sequences

Oligonucleotide Name	Oligonucleotide sequence
18s	CACGGACAGGATTGACAGATT GCCAGAGTCTCGTTCGTTATC
IFNG	GGGTTCTCTTGGCTGTTACT GAGTTCCATTATCCGCTACATCT
CLSTN1	TCCCGAGTGTGACTCTCTATG CACCACGAGCTGAGTTTCTATC
LIAS	CAGTCCCTACGTGTACTGAAAC TGCTCATCATTCTCGCCTAAA
NAP1L4	CTGCGGGTCACCTCATATTT CAAGGTGGTTCAGAAACGTTAAG
POP1	CTCTCCAACCACAGGCATTAT CAGTTAGGATGGAGTGGGAAAG

Oligonucleotide pairs for each assayed transcript are listed in order of sense (S) followed by antisense (A) oligonucleotides.

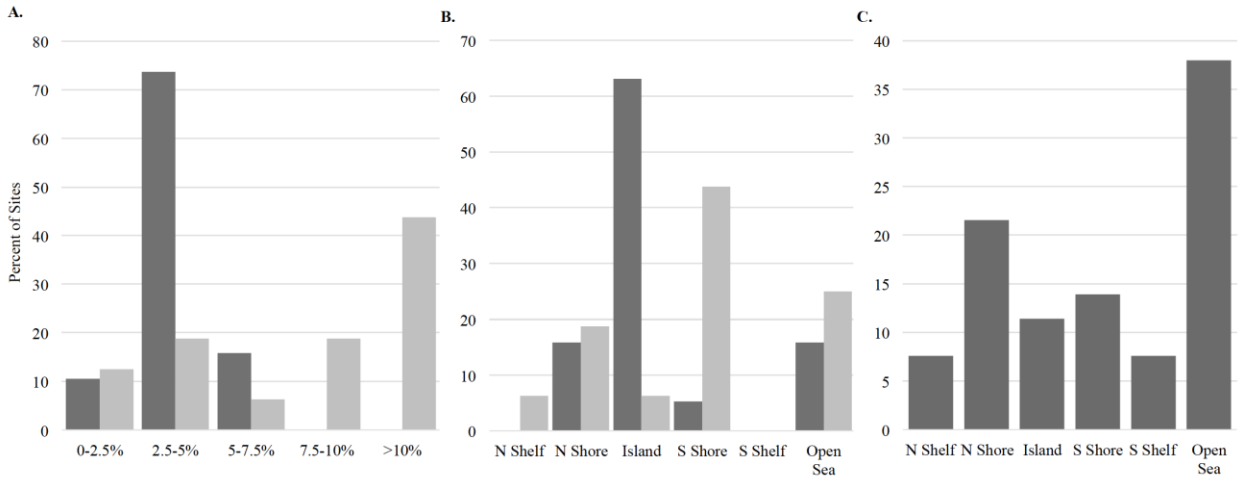


Figure 3.1. Distribution of methylation differences. **A-C** the percent of sites is plotted on the y-axis. Data from the CD4+ T cells is displayed in the dark grey bars and data from the CD8+ T cells is displayed in the light grey bars. **A.** The absolute difference in methylation between the obese and normal weight women was calculated for each of the differentially methylated sites in the CD4+ and CD8+ T cells. The differences in methylation between the two groups was categorized into five different ranges of methylation differences (0-2.5%, 2.5-5%, 5-7.5%, 7.5-10%, and >10%), and the percent of sites falling in each range for each cell type is presented. **B.** Each DMS between the obese and normal BMI women in the two T cell types were classified by its associated CGI region. The percent of sites in each cell type in each region are presented. **C.** Each DMS with methylation levels correlated to the amount of VAT (g) in the CD4+ T cells were classified by its associated CGI region. The percent of sites in each region are presented. The regions of the CGI (B-C) are defined as previously reported in (Bibikova et al., 2011; Ronn et al., 2013)

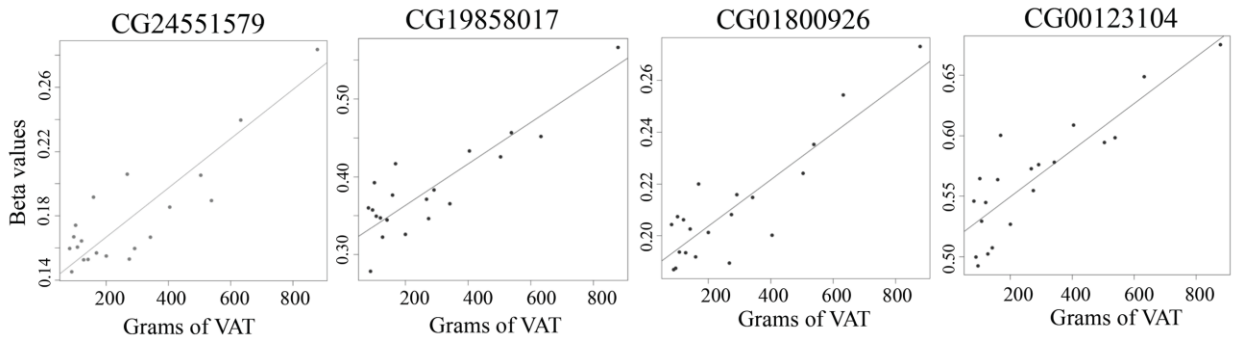
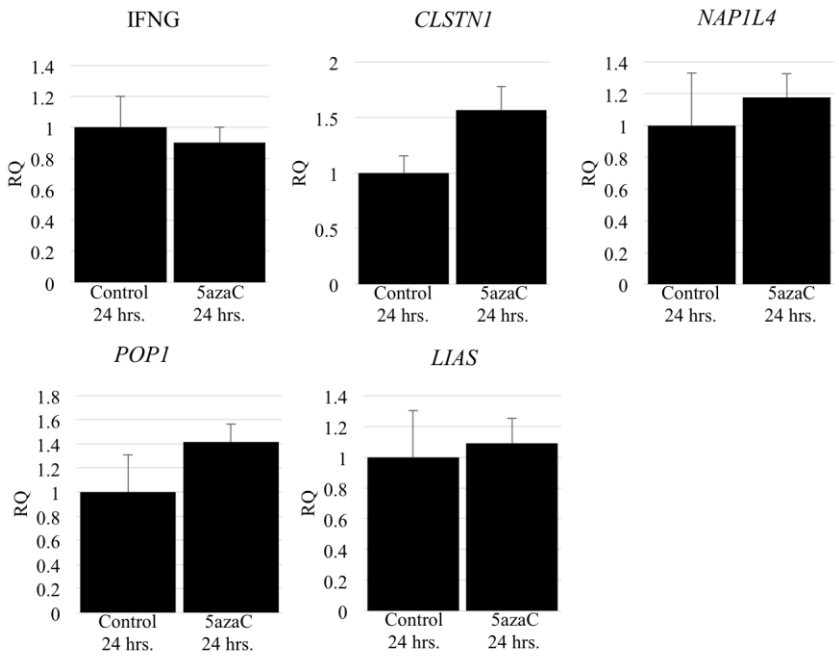


Figure 3.2. Methylation levels of the four CG sites associated with the amount of VAT in CD4+ T cells in the *CLSTN1* gene. The methylation level (beta values, 0: 0% methylated, 1:100% methylated) and the amount of VAT are plotted for the four DMS in *CLSTN1*. These DMS were positively correlated with the amount of VAT.



Supplemental Figure 3.S1. Changes in gene expression following 24 hour inhibition of DNMTs with 5azaC. The relative expression of transcripts as determined by qRT-PCR for the five genes with DMS associated with obesity or VAT in CD4+ T cells. The relative expression of each gene with 5azaC treatment (black) in CD4+ T cells in comparison to CD4+ T cells with no drug treatment (grey) is shown. All genes consist of the average of six independent drug treatments or control preparations. Error bars represent the standard error of the mean.

CHAPTER 4

WEGHT LOSS AND DNA METHYLATION CHANGES IN CD4+ T CELLS IN OVERWEIGHT AND OBESE WOMEN[5]

[5] Hohos NM, Johnson KB, Berg AC, Smith AK, Kilaru V, Johnson MA, Evans E, Phillips BG, Meagher RBM. To be submitted to a peer reviewed journal.

Abstract

We were interested in studying genome-wide DNA methylation changes in relation to diet and weight loss. Most studies examining the relationship between DNA methylation and weight loss have used peripheral whole blood DNA. Statistically meaningful data for any one cell type may be lost as these data are the weighted average of the gene specific methylation data of the various cell types in blood. We examined DNA methylation and weight loss in CD4+ T cells at baseline and after a six month weight loss intervention in 14 overweight and obese women (59.8±1 years) using the HumanMethylation450 BeadChip. Following the intervention, participants lost 11.99± 0.87 percent of starting body weight. After correcting for multiple testing (FDR, $q<0.05$) across our population, no differences in methylation were observed from pre- to post- intervention, except in relation to central adiposity (i.e., android fat). For example, 448 sites whose methylation levels after the intervention correlated with the amount of android fat lost. Further, participants with the lowest amount of android fat at baseline had significant changes in their DNA methylome following the intervention. One gene in particular, *CACNA1G* encoding the Calcium Channel, Voltage-Dependent, T Type, Alpha 1G Subunit, had two sites decrease in methylation following weight loss in participants with the lowest android fat prior to the intervention. Treating CD4+ T cells with the DNA methylation inhibitor 5-azacytidine increased *CACNAIG* expression supporting its regulation by DNA methylation. Our data show that DNA methylation in CD4+ T cells is associated with measures of central adiposity, but there is not a simple relationship between weight loss and changes of the methylome.

Introduction[6]

Obesity has become a global health problem that is associated with the development of a number of comorbidities that negatively affect health status (Field et al., 2001; Sturm, 2002; Poirier et al., 2006; Apostolopoulos et al., 2016). Weight loss can decrease the risk and development of these comorbidities, including insulin resistance and type 2 diabetes (Niskanen et al., 1996; Tuomilehto et al., 2001). Accordingly, weight loss is commonly prescribed to obese and overweight patients (Goldstein, 1992; Case et al., 2002; Grundy et al., 2005; Phelan et al., 2007; Jensen et al., 2014; Kushner, 2014; Apovian et al., 2015). However, weight loss interventions do not always have equal success for all subjects (King et al., 2008; Napolitano et

[6] **Abbreviations**

5azaC (5-azacytadine), *AGAP1* (ArfGAP with GTPase domain, ankyrin repeat and Ph domain 1), *ATP6V1B2* (ATPase, H+ transporting, lysosomal 56/58kDa, V1 subunit B2), *ATP10A* (ATPase, class V, type 10A), *AQP9* (Aquaporin 9), *BMAL1* (aka *ARNT1* arly hydrocarbon receptor nuclear translocator-like), *CACNA1G* (Calcium channel, voltage-dependent, T type, alpha 1G subunit), *CD44* (CD44 molecule), CG (cytosine guanine dinucleotide), CGI (CG island), DMR (differentially methylated region), DMS (differentially methylated site), *CLOCK1* (Clock circadian regulator 1), DNMTs (DNA methyltransferases), *DUSP22* (dual specificity phosphatase 22), GADD45s (growth arrest and DNA-damage-inducible protein 45), *GAS7* (growth arrest-specific 7), GO (gene ontology), *HIPK3* (homeodomain interacting protein kinase 3), *HTR2A* (5-Hydroxytyrpatine (Serotonin) receptor 2A, G protein coupled), *IL-6* (interleukin 6), *KIAA1731* (aka *CEP295*, Centrosomal protein 295 kDa), LDL (low density lipoprotein), *NR1D1* (nuclear receptor subfamily 1, group D, member 1), *NPY* (Neuropeptide Y), PBMCs (peripheral blood mononuclear cells), *PER2* (Period circadian clock 2), *PIP5K1C* (phosphatidylinositol-4-phosphate 5-kinase, type 1 gamma), *POMC* (Proopiomelanocortin), *PRDM16* (PR domain containing 16), *PPT2* (Palmitoyl-protein thioesterase 2), *SCD1* (Stearoyl-CoA desaturase 1 (delta-9-desaturase)), *SEPT9* (Septin 9), TDG (thymine DNA glycosylase), TETs (ten eleven translocase methyl-dioxygenases), *TNFα* (tumor necrosis factor α), *TNNT1* (Troponin T type 1), *TNNI3* (Troponin 1 type 3), VAT (visceral adipose tissue), WBCs (white blood cells)

al., 2012). There are large differences in response to weight loss interventions that are in part due to an individual's genetic differences (Tholin et al., 2005; Hainer et al., 2008). Further, most individuals who do achieve weight loss have difficulty keeping the weight off (Stevens et al., 2001; Curioni and Lourenco, 2005; Kraschnewski et al., 2010; Atallah et al., 2014; Fothergill et al., 2016). If weight loss is maintained, there are lasting metabolic adaptations that favor weight regain in individuals with sustained weight loss (Rosenbaum et al., 2008). Some metabolic adaptations have been proposed to be associated with the difficulty in maintaining weight loss, including a sustained decrease in resting metabolic rate (Rosenbaum et al., 2008), altered endocrine signaling, increased appetite, suppressed energy expenditure and other effects that are well described in the review by Maclean et al (2011).

As an individual's genetic profile has been shown to influence the success of weight loss, it seems reasonable to consider that the epigenome may also contribute to weight loss success (Hainer et al., 2008; Deram and Villares, 2009; Martinez et al., 2014; Nicoletti et al., 2015). For example, epigenetic modifications may reprogram the cells of an obese person, which may be sustained through weight loss, or impact the effectiveness of weight loss. This reprograming may reflect changes that have been imprinted into the genome via epigenetic modifications in the obese or overweight state that are sustained regardless of weight loss. Epigenetic modifications lead to changes in chromatin structure which ultimately affect gene expression and the resulting physiological function. And thus, the biological changes that prevent weight loss and make it difficult to maintain (Maclean et al., 2011), may manifest from epigenetic modifications.

DNA cytosine methylation in peripheral blood has been studied in relation to different aspects of weight loss. This includes studies looking at changes from baseline to after weight loss (Duggan et al., 2014; Martin-Nunez et al., 2014; Perez-Cornago et al., 2014; Nicoletti et al.,

2015; Samblas et al., 2016), differences between responders and non-responders to a specific weight loss intervention (Bouchard et al., 2010; Cordero et al., 2011; Milagro et al., 2011; Crujeiras et al., 2013), and as biomarkers that are predictive of weight loss (Campion et al., 2009c; Bouchard et al., 2010; Milagro et al., 2012; Moleres et al., 2013; Perez-Cornago et al., 2014; Nicoletti et al., 2015; Samblas et al., 2016). The data from these studies have yielded mixed results. Both decreases and no changes in global methylation have been observed (Duggan et al., 2014; Martin-Nunez et al., 2014; Nicoletti et al., 2015). While gene-specific changes in methylation have been consistently identified, the specific genes altered in relation to weight loss vary among studies (Campion et al., 2009c; Milagro et al., 2011; Milagro et al., 2012; Crujeiras et al., 2013; Moleres et al., 2013; Martin-Nunez et al., 2014; Perez-Cornago et al., 2014; Nicoletti et al., 2015; Samblas et al., 2016). These mixed results may be due to the fact that this work has been performed on samples composed of mixed blood cell types, including either total peripheral blood/leukocytes (Crujeiras et al., 2013; Moleres et al., 2013; Duggan et al., 2014; Martin-Nunez et al., 2014; Perez-Cornago et al., 2014; Nicoletti et al., 2015; Samblas et al., 2016) or PBMCs (peripheral blood mononuclear cells) (Campion et al., 2009c; Milagro et al., 2011). The PBMC fraction is comprised of CD4+ and CD8+ T cells, B cells, NK cells and monocytes. Epigenetics, including DNA methylation, is a cell type specific phenomena, reflecting not only the cells ontogeny, but also in the regulation of cell-type-specific genes (Reinius et al., 2012; Lister et al., 2013; Wu and Zhang, 2014; Gu et al., 2016). When the methylation profile of mixed cell types is examined, the data is a weighted average of all cell types. Biologically and statistically meaningful data from individual cell type are obscured by opposing changes in other cell types. Thus, weight loss studies examining DNA methylation in mixed cell types may be yielding mixed results as there is a heterogeneous population of cells

methylation profiles examined and compared, each unique to the study population. To circumvent this issue and to discover more information about the DNA methylation profile associated with or predictive of weight loss, we examined DNA methylation in CD4+ T cells before and after a six-month weight loss intervention.

CD4+ T cells are commonly used as a surrogate cell to study DNA methylation in relation to different phenotypes including obesity, psoriasis, juvenile idiopathic arthritis, systemic sclerosis, and systemic lupus erythematosus (Jeffries et al., 2011; Ellis et al., 2012; Park et al., 2014; Wang et al., 2014; Aslibekyan et al., 2015). Blood is an easy and minimally invasive tissue source to obtain from participants, of which CD4+ T cells can be easily and directly isolated from either whole blood (Hohos et al., 2016) or from the PBMC fraction. CD4+ T cell levels are altered in obesity in both circulation and adipose tissue (Feuerer et al., 2009; Ilavska et al., 2012; Cildir et al., 2013; Wagner et al., 2013), and have been shown in a recent study to have alterations in DNA methylation with increasing BMI and waist circumference (Aslibekyan et al., 2015). Additionally, CD4+ T cells have also been suggested to be potentiated to respond to changes in physiological cues via their methylome (Hohos et al., 2016), which likely occur in the altered inflammatory state in obesity.

We hypothesized that we would identify DNA methylation differences in CD4+ T cells between pre to post weight loss intervention. In fact, we identify differences in methylation associated with weight loss only in those overweight and obese participants who began the intervention with the lowest levels of android fat mass (central adiposity). We also identified 448 sites whose DNA methylation levels at the end of the weight loss intervention were associated with the amount of android adipose mass lost over the intervention. The data presented herein

provide information on the DNA methylation profile associated with weight loss in obese and overweight women in CD4+ T cells.

Results

Participant characteristics

Fourteen obese (N=7) and overweight women (N=7) (59.8±1 years old, with an age range of 52 years to 64 years) were selected from a larger sample who were enrolled in a six month weight loss intervention program consisting of both dietary and exercise components (selection details are described in the materials and methods sections). A weight loss goal of at least 10% of baseline body weight was achieved in ten of the fourteen participants, with an average weight loss of 11.99 ± 0.87% for all patients. Participant weight loss ranged from 7.3% to 20%. There was also a successful decrease in BMI, total body fat and android (central) fat over the intervention (**Table 4.1**).

DNA methylation and weight loss

To test associations between DNA methylation and weight loss, we analyzed 451,705 sites of the 485,000 that passed quality control assessment (Teschendorff et al., 2013). A linear regression model was fitted for each site in relation to the weight loss phenotype and its methylation level. M-values ($\log_2 \frac{\beta}{1-\beta}$) were used for the regression analysis as the measure of methylation, as with the small sample size in our study, issues of heteroscedasticity for highly methylated and un-methylated sites is of concern when using standard beta-values (Du et al., 2010). Thus, using the M-values yields better results in regards to both detection rate and true positive rates for those highly methylated and un-methylated sites (Du et al., 2010). Additionally,

as age is known to impact methylation levels (Jung and Pfeifer, 2015), age was added as a covariate in all analysis. Changes in the methylation levels of a site were only considered to be associated with the phenotype if they were still significantly associated ($q<0.05$) after correcting for multiple testing by the FDR method. This stringent criteria allows more certainty that the differences we found are indeed different in our data set, and was applied for all analysis unless otherwise specified. As ß-values are easier to interpret biologically (ß-value of 0 is 0% methylation, ß-value of 1 is 100% methylated), they are used to discuss results determined statistically significant by M-values (Du et al., 2010).

There were no statistically supported differences in DNA methylation from pre- to post-intervention for all participants in the study after correcting for multiple testing ($q<0.05$). We next examined methylome changes as a function of central adiposity, because android fat (central adiposity) is typically associated with an increased risk of metabolic disturbances (Evans et al., 1984; Jensen et al., 1989; Pinnick and Karpe, 2011) and includes visceral adipose tissue (VAT), which is known to contribute to the low grade inflammatory state in obesity and descending diseases (Gerriets and MacIver, 2014; Huh et al., 2014).

For only those participants with levels of baseline android fat per kg body weight in the lowest 25^{th} percentile (N=4), were there changes in DNA methylation from pre- to post-intervention. Eight differentially methylated sites (DMS) had significant differences in DNA methylation ($q<0.05$), yet many sites were trending on significance ($q<0.1$) and we chose to examine all sites with a p-value $< 1x10^{-4}$ ($q<0.09$), yielding 372 DMSs for further analysis. Of these 372 sites, 107 had at least a 10% change in methylation from pre- to post-intervention (**Figure 4.1A**), with 105 sites decreasing methylation levels with weight loss. These 372 sites were associated with 242 named genes, and nine of these genes were associated with multiple

DMS (**Table 4.2**). The *PRDM16* gene which is known to be involved in the development of beige and brown adipocytes, contained four DMSs. *AGAP1* which is involved in membrane trafficking and the cytoskeleton dynamics, and *GAS7* a growth arrest protein, each had three differentially methylated sites (**Figure 4.2**). Two of the DMS in *AGAP1* were located 2 base pairs apart. 143 of these sites were associated with enhancer regions, 17 with promoter regions, and 16 with DMRs (differentially methylation regions). When these sites were examined in context to their relation to a CGI (CG island) (**Figure 4.1B**), there was only an enrichment of sites in the regions not associated with CGIs, and an under representation of sites associated with both CGIs and their flanking regions. Functional gene enrichment analysis was performed for those sites with at least a 10% change in methylation or were significant at $q<0.05$ to determine if any biological processes were enriched in the associated gene list (**Figure 4.3**). Biological processes involving signal transduction, cell adhesion, protein transport, regulation of apoptotic processes and cellular calcium ion homeostasis were found to be enriched in our data set among others.

DNA methylation levels after the weight loss intervention of 448 sites were correlated with the amount of android adipose mass lost (normalized to body weight loss) over the intervention ($q<0.05$). This analysis included the data from all 14 individuals. Of these 448 sites, 77 were associated with enhancer regions, 163 were associated with promoter regions, and 21 were associated with known DMRs. The 448 sites were associated with 395 named genes, with five genes (*PIP5K1C, PPT2, SEPT9, ATP6V1B2, KIAA1731*) associated with two sites whose methylation levels correlated to the amount of weight loss (**Table 4.3**). 210 (47%) of these sites had decreasing levels of methylation associated with increasing amount of android fat lost. When the 448 sites with methylation levels correlating with the amount of android fat lost were

analyzed by relation to CGI's, there was an enrichment of sites located in the island and its south shelf relative to the distribution of probes included on the whole array (**Figure 4.4**). Functional gene enrichment analysis (**Figure 4.5**) showed that the genes associated with these 448 sites were predominantly involved in biological processes including regulation of transcription, cytokinesis, ubiquitination and cell division. Biological processes related to CD4+ T cells were also enriched and included toll-like receptor signaling pathways and response to interleukin-1.

DNA methylation and regulation of gene expression

It seemed reasonable to make use of the potentially rapid turnover of DNA methylation in CD4+ T cells that has been reported for other cell types (Yamagata et al., 2012; Meagher, 2014) to determine if DNA methylation is involved in the regulation of a sample of the DMS containing genes. Methylcytosine turns over biochemically by first oxidation by TETs (ten-eleven translocation methylcytosine dioxygenases), and then base removal by GADD45s (growth arrest and DNA-damage-inducible protein 45) and TDG (thymine DNA glycosylase) and finally remethylaiton by DNMTs (DNA methyltransferases). Even though there is evidence for this rapid turnover (Yamagata et al., 2012; Meagher, 2014), the steady state levels of DNA methylation remain constant, which is primarily due to efficient maintenance methylation of hemi-methylated CG sites by DNMT1. Thus, we treated primary WBCs (non-stimulated, non-dividing) with or without 2μM 5-azacytidine (5azaC), an inhibitor of all DNMTs, for 24 hours, isolated CD4+ T cells, and assessed gene expression of seven genes which had DMSs identified in our analysis (**Table 4.4**). Only one gene, *CACNA1G*, had increased gene expression following 5azaC treatment, suggesting that DNA methylation likely plays a role in regulating this gene in CD4+ T cells (**Table 4.4**. **Supplemental Figure 4.S1**). Another gene, *AGAP1*, had decreased gene expression following 5azaC treatment that was trending on significance ($p \approx 0.1$),

suggesting DNA methylation may also be involved in its regulation in CD4+ T cells, albeit, indirectly.

Discussion

Regardless of the largely successful weight loss intervention in which all participants reduced their body weight (>7% body weight lost) by levels shown to improve metabolic parameters (Goldstein, 1992; Case et al., 2002; Grundy et al., 2005; Phelan et al., 2007), we did not observe statistically supported changes in DNA methylation of any loci from pre- to post-intervention. There were no changes linked simply to their change in BMI, or percent change in body weight or percent body fat. However, we did observe changes in DNA methylation following weight loss in only the participants with the lowest quartile of baseline android fat. We also identified methylation levels of specific loci after the intervention that correlated with the amount of android adipose tissue lost over the intervention.

One possible explanation why we did not observe changes in DNA methylation from pre- to post-weight loss may involve retention of changes to molecular memory of CD4+ T cells in the overweight and obese individuals. DNA methylation has been suggested to be involved in molecular memory by altering the methylation status of specific loci in response to different stimuli that are retained once the stimuli is removed (Riggs, 1989; Thomassin et al., 2001; Yu et al., 2011; Leung et al., 2016). This has been demonstrated previously in CD4+ T cells, where genes activated in the immune response in memory T cells have been shown to be under methylation control (Komori et al., 2015). It is suggested that this regulation of immune response genes by DNA methylation is involved in 'priming' the memory T cells to respond quickly upon future stimulation, making a memory of previous stimuli encountered (Komori et al., 2015). It is known that there are many biological adaptations favoring weight regain once weight is lost,

including changes in endocrine signaling and energy expenditure (Rosenbaum et al., 2008; Maclean et al., 2011). It has been suggested that these adaptations arise from reprograming during the time which the body considers overweight or obese its new normal (Leung et al., 2016). These biological 'memories' of the obese state may be partially implemented at the level of DNA methylation in CD4+ T cells. Reprograming may be maintained with weight loss, as the body's molecular memory 'remembers' it's normal state is obese, working to reestablish the obese state through weight regain.

This premise fits well with our analysis of DNA methylation following weight loss when we examined this relationship for those with different baseline characteristics of android fat. Only the participants who had the lowest amounts of baseline android fat (normalized to body weight) had changes in their DNA methylome from pre- to post-intervention. Central adiposity, has been shown to be associated with a more negative metabolic profile, where increasing levels are associated with an increased risk for obesity related comorbidities (Evans et al., 1984; Jensen et al., 1989). Additionally, this collection of adipose tissue depots also includes VAT which is known to be intimately involved in the development of the inflammatory response associated with obesity (Gerriets and MacIver, 2014; Huh et al., 2014; Pecht et al., 2014). As central adiposity is more metabolically unhealthy, perhaps those with less of this fat depot have not had this 'reprograming' of DNA methylation in CD4 + T cells in the obese state, as those with higher amounts do. Without this 'reprograming' their methylomes are still responsive to weight loss, while those with higher levels of central adiposity have a methylome that is no longer responsive to weight loss.

Further evidence of this persistent DNA methylation reprograming has been shown in response to dietary change. For example, in one study examining the effect of short term

overfeeding in humans there were many changes in DNA methylation levels in skeletal muscle (Jacobsen et al., 2012). Yet, after six weeks without overfeeding, the methylation changes had not returned to that of baseline (Jacobsen et al., 2012). In mice, it appears that the persistent epigenetic reprograming from a high fat diet depends on genetic background. In C57BL/6J mice, after switching to a normal chow diet from a high fat diet, the changes in chromatin structure from the high fat diet remained (Leung et al., 2016). However, in A/J mice, the chromatin changes were transient, and returned to normal following the return to a chow diet (Leung et al., 2016). It is well established that C57BL/6J mice are useful for studies of obesity, and is interesting that reprograming of the methylome was retained once the high fat diet was removed. This difficulty of reversing DNA methylation memory appears to be relevant to obesity as well. A study examining DNA methylation in subcutaneous adipose tissue in obese, post-obese (2 years), and never obese women, identified DNA methylation levels that are similar between obese and post obese women and different from women who were never obese (Dahlman et al., 2015). Interestingly, gene expression analysis showed that even though the DNA methylation profile was similar between the obese and post obese, the expression of the related genes was similar between the post obese and the never obese (Dahlman et al., 2015). This data suggests that the DNA methylation 'reprograming' that occurred with obesity persists even with weight loss, and may be acting to 'prime' the genes for altered expression in response to an obesogenic environment.

Other studies examining DNA methylation in response to weight loss in blood cells have had mixed results. Studies looking at specific loci or genes DNA methylation profile have identified changes in methylation in response to weight loss in *SCD1* promoter methylation (Martin-Nunez et al., 2014), *IL-6* and *SEPRINE-1* (Nicoletti et al., 2015) in *BMAL1* and *NR1D1*

(Samblas et al., 2016) in peripheral blood, and in *WTI* and *ATP10A* in PBMCs (Milagro et al., 2011). No common genes with differential methylation were identified in any two studies, or in our analysis. All of these studies have examined methylation in mixed blood cell types, which may confound the data. To our knowledge no studies examining DNA methylation and weight loss have been performed in CD4+ T cells, and only two studies have examined DNA methylation and obesity in CD4+ T cells. Global methylation levels in CD4+ T cells did not differ between obese individuals and normal weight controls (Simar et al., 2014), but differences in gene sequence specific loci have been identified in CD4+ T cells of obese individuals (Aslibekyan et al., 2015). We did not observe any baseline differences in DNA methylation that were association with BMI (data not shown), as were identified by Aslibekyan et al. (2015). Additionally, none of the eight sites they identified to have methylation levels associated with BMI, or the five they found associated with waist circumference in CD4+ T cells (Aslibekyan et al., 2015), were found to be associated with android fat lost, or predictive of android fat loss in our study.

Although we did not identify any sites with methylation changes over the weight loss intervention, we did identify 448 sites whose methylation levels after the intervention correlated with the amount of android adipose mass lost. Again, we only observed this association of DNA methylation after the intervention in regards to the android fat mass outcome of weight loss, which is associated with more negative metabolic and health status with increasing adiposity (Evans et al., 1984; Jensen et al., 1989). Perhaps the level of this depot is important to the DNA methylation changes or 'reprograming' that occurs in obese or overweight states. Considering we only observed changes in DNA methylation with weight loss in those with the lowest starting amounts of this depot, this rationale seems probable. None of the sites whose methylation levels

after the intervention correlated with the amount of android adipose mass lost were previously identified in any of the studies examining DNA methylation and weight loss, yet none looked in CD4+ T cells alone.

Finally, the interest in studying DNA methylation roots from its ability to respond to the environment and alter gene expression. A limitation to our study was that we did not have mRNA from our participants to study concurrent changes in gene expression. To circumvent this limitation, we choose to carry out a primary cell culture experiment in CD4+ T cells examining the effect of a DNMT inhibitor on gene expression of a few genes where DNA methylation differences were observed. Inhibiting DNMTs in cell culture for two hours, yields a 10% decrease in methylation due to the inhibition of DNMT1 (Yamagata et al., 2012), suggesting this experiment may produce changes in gene expression due to the inhibition of maintenance methylation. For one of the seven genes tested (**Table 4.4**) we observed a significant increase in expression of *CACNA1G* following 5azaC treatment. We observed two sites in this gene where DNA methylation levels changed with weight loss in the participants with the lowest amount of baseline android fat. When this gene is knocked out in mice, they are resistant to high fat diet induced weight gain (Uebele et al., 2009), and its expression has been shown to be regulated by methylation in a colon cancer cell line (Toyota et al., 1999). This data suggests that DNA methylation is involved in the regulation of the transcription of *CACNA1G* in CD4+ T cells, its methylation may be altered in response to weight loss, and it plays some role in weight gain or loss.

In summary, we have identified sites whose methylation levels after weight loss were correlated with the amount of android adipose mass lost over the intervention. We also showed that changes in DNA methylation associated with weight loss were only observed in those with

the lowest baseline levels of android fat. As all our results were only associated measures of central adiposity, it suggests a relationship between central adiposity and DNA methylation with weight loss in CD4+ T cells that warrants further investigation.

Materials and Methods

Participant Recruitment

Twenty five women aged 50-64 years old were recruited form the Athens, GA area by paid newspaper advertisement, flyers, emails to listservs, and by word of mouth. Exclusion criteria included a BMI $\leq$25 kg/m^2, smoking, dietary restrictions, weight loss surgery, use of weight loss, steroid, or anti-inflammatory medications, and a current diagnosis or history of unstable cardiovascular disease, chronic obstructive pulmonary disease, lung disease, severe asthma, cancer (active or treatment within five years), balance disorders, severe arthritis, physical limitations, dementia, and psychological conditions that would affect adherence. Additionally, to be included in the study, participants must self-identified as postmenopausal (defined by the absence of a menstrual cycle for 12 months). All included women were weight stable (within 2 kg over 6 moths), community dwelling and sedentary (<1 hour physical activity per week), obtained physician clearance, and self-identified as Caucasian (to eliminate variation in DNA methylation due to race differences). From the twenty five women enrolled in the weight loss intervention, seventeen participants had adequate blood samples (10 ml) at both baseline and the end of the intervention to be included for methylation analysis. Fourteen of the seventeen women were selected for methylation analysis due to limited availability of spaces on the HumanMethylaiton450 BeadChip. To select the fourteen participants, the three with the lowest percent weight loss and BMI points lost were eliminated, and the fourteen participants

with the highest percent weight loss and BMI points lost were included. Many of the included women in this study were on medications or had medical conditions. This data is presented in **supplemental table S4.2**. As DNA methylation can be impacted my different medications (Csoka and Szyf, 2009; Szyf, 2009), it is important to consider this may be a confounding factor in the DNA methylation analysis performed in this study. All studies were approved by the University of Georgia IRB.

Weight loss intervention

All participants were enrolled in a six-month weight loss intervention with both exercise and dietary components. The exercise intervention consisted of 75 minute sessions three times per week that were multi-modal involving cardiorespiratory, resistance, balance and flexibility training. All exercise interventions were participant tailored and compliance was set to completion of 80% of training sessions. The dietary intervention consisted of both energy restriction and macronutrient goals to achieve 10% weight loss. The energy restriction goal was 500 kcal less than each participants calculated needs calculated by the Mifflin St. Jeor equation, with a minimum possible energy goal of 1200 kcal/day. Macronutrient goals were given as percent of energy with 30% from protein, 40% from carbohydrate, and 30% from fat. To achieve the higher protein diet, participants were instructed to consume one serving of lean beef daily. To facilitate the dietary intervention participants had individual counseling by an RDN or trained graduate student. This consisted of at least two 60 min sessions at the start of the intervention to instruct participants to reach their energy restrictions, meet their macronutrient goals, use self-monitoring techniques (myfitnesspal.com and the exchange system as needed), and to consume lean beef. There was also a midpoint individual session with all participants, and other individual sessions as needed. Participants were also enrolled in weekly group classes that were 45 to 60

min and consisted of 2-15 women per group. Group sessions were based on the Social Cognitive Theory and covered topics covering nutrition education and behavior modification. Participants were also provided with a multivitamin mineral supplement (Centrum Silver Women) and TUMS for any additional calcium needs based on participants food intake. Anthropometrics (weight, height, dual-energy X-ray absorptiometry (DXA)) were measured at baseline and at 6 months following standard protocol. 10 ml of venous blood was collected from fasting participants at baseline and 6 months in EDTA containing tubes and immediately frozen at -80°C until processing. To determine differences from pre- to post-intervention, a two-tailed t-test was performed with significance set at $p<0.05$.

Cell isolation and methylation analysis

Blood samples were thawed on ice and CD4+ T cells were isolated as previously described in Hohos et al. (2016). Isolated cells were stored at -80ºC in 200 µl of PBS (Phosphate Buffered Saline) until genomic DNA extraction with the DNeasy Kit (Cat # 69506, QIAGEN). The extracted DNA was then quantified using Quant-iT PicoGreen dsDNA assay kit (Cat #P7589, Life Technologies) following manufacture protocol.

In total 28 samples of genomic DNA (~1.6 µg; N=14 pre-intervention samples and N=14 post-intervention samples) were loaded onto the Illumina-provided, midi deep well, barcoded plate and sent to Illumina for processing of the HumanMethylation450 BeadChip to interrogate >485,000 independent CG sites throughout the genome with 99% coverage of RefSeq genes following Illumina's instructions (Illumina, San Diego, CA). CpGassoc was used for implementation of quality control parameter (Barfield et al., 2012). Samples with probe detection call rates < 90% were excluded, as were those with an average intensity value of either < 50% of the experiment-wide sample mean or < 2,000 arbitrary units. Data points with detection p-values

> 0.001 were set to missing, and probes that cross hybridize between autosomes and sex chromosomes were excluded (Chen et al., 2013). No individual subject samples were excluded. BMIQ was then used to normalize the probe distributions and background signals (Teschendorff et al., 2013). Estimated DNA methylation proportions (the ration of methylated signal to total signal) or β-values were then computed for each CpG site. A total of 451,705 CG sites were included for analysis.

MethLAB (Kilaru et al., 2012) was used to test for association with time point (pre- or post-intervention) in CD4+ T cells via linear regressions that modeled the M-values $\log_2 \frac{ß}{1-ß}$ as the outcome and the time point as a categorical independent variable. This analysis was also performed for the participants in the bottom quartile and top quartile of baseline android fat (normalized to body weight). We also tested the association of post-intervention methylation levels and the amount of android fat lost (normalized to body weight lost) over the intervention following the same analysis as the prior test, however with the amount of android fat lost as a continuous variable. Age was added as a covariate in all regression analysis. To control for false positives due to multiple testing, associated sites were considered significant after controlling the false discovery rate with a q-value < 0.05. Functional enrichment analysis were performed using GeneCodis (Carmona-Saez et al., 2007; Nogales-Cadenas et al., 2009; Tabas-Madrid et al., 2012) to find any enriched biological processes for genes associated with methylation differences. Enriched GO (gene ontology) terms for biological processes were included that reached a corrected p-value < 0.05 by FDR method and contained ≥3 genes.

For a validation assay to determine if DNA methylation is involved in the regulation of gene expression of genes with identified differences in DNA methylation from pre- to post-

weight loss or with the methylation levels after the intervention associated with the amount of android adipose mass lost, we assessed the effect of 5azaC on gene expression in CD4+ T cells. Venous blood samples were collected in EDTA tubes in the morning from a nonfasted healthy 65-year-old male volunteer free of cardiovascular and other diseases with a BMI of 29. These studies were approved by the Institutional Review Board at the University of Georgia. White blood cells were immediately isolated as previously described (Hohos et al., 2016). Following isolation cells were washed 1X with 10 ml of DMEM (at 37°C) at 100 x g for 10 min. Cells were resuspended in DMEM+ (DMEM, 1% penicillin-streptomycin, 10% FBS) and plated in a volume of 2 ml (~500,000 WBCs) in a 6 well culture dish. 2 µM 5azaC in DMEM was added to drug treated samples. N=6 control (no drug) and N=6 treatment (5azaC) were incubated in a 37°C incubator for 24 hours. Cells were then washed with 1X PBSBE (phosphate buffered saline, 1% BSA, 2mM EDTA, pH7.4) and CD4+ T cells were isolated as previously described (Hohos et al., 2016). RNA was extracted from the isolated CD4+ T cells with RNeasy mini kit (Cat#74101, QIAGEN) following manufactures protocol. RNA was quantified with Quibit RNA assay kit (Cat # Q32855, Life Technologies) and 400 ng of RNA was used for cDNA synthesis with qScript cDNA synthesis supermix (Quanta Biosciences, Gaitthersburg, MD, USA Cat# 95148-100). Oligonucleotide primer sequences (**Supplemental Table 4.S1**) were synthesized by Integrated DNA Technologies (Coralville, IA, USA). Two primer sets were tested for each of the target genes and those having a single sharp dissociation peak, ensuring the specific gene target was being amplified, and the lowest CT values were selected for subsequent use. A 25 µl reaction using SYBR green master mix (Life Technologies, Grand Island, NY, USA Cat# 43677659) and 4 ng of cDNA was used for analysis of the gene panel. All reactions were repeated in triplicate. All data was normalized to the endogenous control 18s mRNA and then the

relative expression to the control samples was calculated with the ddCT method. A two-tailed t test was performed to determine if there was a change in gene expression with the 5azaC treatment, with significance set to $p<0.05$.

Table 4.1. Participant characteristics

	Age	Weight (kg)	BMI (kg/m^2)	% Body Fat	Android Fat (g)	Android Fat (g/kg body weight)
Pre-intervention	59.8±1	80.3±3.5	30.6±1.4	45.9±0.9	3470.4±245.6	42.7±1.4
Post-intervention		70.4±2.6	26.8±1	39.5±1.1	2404.3±245.6	33.8±1.4
p-value		0.039	0.040	0.00025	0.0020	0.00018

Biometrics of study participants are shown for both pre and post intervention. Data is presented as mean ± standard error of the mean. A two-tailed t test was performed to determine differences from pre to post intervention and significance was set to $p<0.05$, with the p-value for each test in displayed on the table.

Table 4.2. Genes containing multiple sites with methylation changes associated with weight loss

Gene Name	CG sites	Relation to android fat loss	CGI location
CACA1G	cg17319889	Decrease (-10.7%)	S-Shelf
	cg20204921	Decrease (-6.9%)	Island
CYP11B1	cg21298978	Decrease (-9.5%)	
	cg20073007	Decrease (-10.4%)	
MYT1L	cg07910488	Decrease (-5.9%)	
	cg23861841	Decrease (-7.0%)	
NCKAP5	cg17081914	Decrease (-10.2%)	
	cg00827382	Decrease (-9.85)	
PXDN	cg27160524	Decrease (-7.5%)	
	cg09996777	Decrease (-5.6%)	
MC3R	cg19226099	Decrease (-5.9%)	N_Shore*
	cg24298684	Decrease (-9.7%)	N_Shore*
AGAP1	cg15068641	Decrease (-7%)	
	cg21672829	Decrease (-8.8%)	
	cg14406878	Decrease (-11.5%)	
GAS7	cg22596049	Decrease (-6.4%)	
	cg18657751	Decrease (-11.4%)	
	cg07049421	Decrease (-7.5%)	
PRDM16	cg14169886	Decrease (-11%)	Island
	cg26153353	Decrease (-8.5%)	
	cg24878051	Decrease (-16.1%)	N_Shore
	cg17861161	Decrease (-7.4%)	N_Shore

Nine genes were identified to have multiple sites with DNA methylation changes associated with weight loss in the women with the lowest quartile of baseline android fat (N=4). These nine genes and relevant information including the CG site with methylation change, the direction of the change for each, the average change in methylation with weight loss of that site, and the sites

relation to any region of a CGI are provided. Sites not associated with a CGI are left blank. *Two sites were located in the same region of the same island.

Table 4.3. Genes associated with multiple sites whose methylation levels after the intervention were associated with android weight loss

Gene Name	CG sites	Relation to android fat loss	CGI location
PIP5K1C	cg03228408	Inverse	Island
	cg13995193	Inverse	N_Shore
PPT2	cg13836183	Inverse	N_shelf
	cg09599399	Direct	Island
Sept9	cg12985929	Direct	S_Shore
	cg15803122	Direct	N_Shore
ATP6V1B2	cg11856918	Direct	Island*
	cg17637107	Direct	Island*
KIAA1731 (CEP295)	cg04583011	Direct	Island*
	cg05338497	Direct	Island*

The five genes with multiple sites with methylation levels after weight loss associated with the amount of android mass loss are shown. The relation of the DNA methylation level to the amount of android fat loss is shown as well as each sites relation to a CGI. *Two sites were located in the same region of the same island.

Table 4.4. Effect of 5azaC on gene expression in CD4+T cells

Gene Name	Number of DMS associated with phenotype	q-value	Sites gene-region location	Direction of methylation change	Effect of 5azaC on gene expression	p-value for 5azaC treatment
KLKB1	1	0.049	TSS1500	Decreases with greater android fat loss	No effect	0.21
PRDM16	4	0.077 0.078 0.078 0.077	Body Body Body Body	Decreases with weight loss	No effect	0.31
CACNA1G	2	0.087 0.087	Body Body/3'UTR	Decreases with weight loss	Increased	**0.0079**
MC3R	2	0.089 0.077	1st exon/ 5'UTR 1st exon	Decreases with weight loss	No effect	0.50
AGAP1	3	0.087 0.078 0.076	Body Body Body	Decreases with weight loss	Decreased	0.11
CCL15	1	0.081	TSS1500	Decreases with weight loss	No effect	0.44
CD4	1	0.087	TSS200	Decreases with weight loss	No effect	0.58

The seven genes chosen to see if DNA methylation is involved in the regulation of their expression in CD4+ T cells are listed along with the details of this experiment. A two-tailed t-test was performed between control and 5azaC samples at 24 hours. Significance was set to $p<0.05$ (bold), and the p-values for this analysis are listed.

Supplemental Table 4.S1. Oligonucleotide sequences

Oligonucleotide Name	Oligonucleotide sequence
18s	CACGGACAGGATTGACAGATT GCCAGAGTCTCGTTCGTTATC
KLKB1	TGCCACGCAAACATTTCAC CCACGTTACTCAGCACCTTTA
PRDM16	CCACCCAAGATCCCTCAATTAT ACAGAGGAGGGAAGGAAGAT
CACNA1G	TGCTCTGCTTCTTCGTCTTC GAGGCTGAAATTCTCAGGTAGG
MC3R	TCAGCCAACACTGCCTAATG GATGAAGACCTGCTCACAGAAG
AGAP1	CTGCAAGTCGCTACCTAATTCT GCTTAAACTCCCACCTCCATTA
CCL15	ACTCCTATCTCAGGCTTAGAGG CCCTGTGGATTTCCCGAATTA
CD4	ACCTTTGCCTCCTTGTTCTC CTCCAGAAAAATTTGACCTGTG AG

Oligonucleotide pairs for each assayed transcript are listed in order of sense (S) followed by antisense (A) oligonucleotides.

Supplemental Table 4.S2. Medications and Medical Conditions

Participant	Number of Medications	Classes of Medications	Number of Medical Conditions
1	1	Hypothyroid	0
2	3	OTCNSAID, RxNSAID, ProtonPumpInhibitor	0
3	2	Migrane, OTCNSAID	1
4	1	Statin	0
5	0		2
6	4	HRTEst, ACEInhibitor, Migrane, Hypothyroid	3
7	0		0
8	7	Allergy (2), SSRI/NE, ProtonPumpInhibitor, OTCNSAID, Statin, Hypothyroid	3
9	0		0
10	1	SSRI/NE	1
11	1	HRTEst, ACEInhibitor, Migrane, Hypothyroid	
12	0		0
13	2	HRTEst (2)	1
14	1	OtherBP	2

The total number of medications taken by each participant are listed along with their drug classification. The total number of medical conditions as defined by the diagnosis of cardiovascular disease, heart rhythm disorder, hypertension, peripheral vascular disease, pulmonary disease, obstructive sleep apnea, epilepsy, arthritis, diabetes, cancer, and hyper/hypothyroidism.

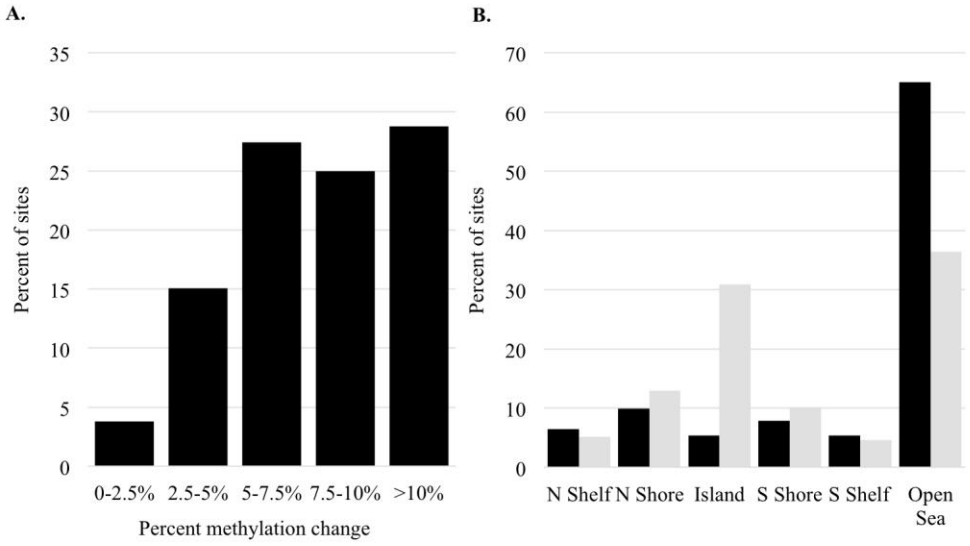


Figure 4.1. Distribution of methylation change with weight loss in participants in the lowest 25th percentile of baseline android fat. The individuals with the lowest quartile of baseline android fat per kg body weight (N=4) were included in this analysis to determine if there were DNA methylation changes in those with the lowest amount of central adiposity following weight loss. **A.** The absolute change in the methylation levels of the 372 sites with methylation differences associated with weight loss were determined. The percent of sites with methylation differences falling in each of the five categories of percent methylation change are presented in the bar graph. **B.** The 372 sites with DNA methylation levels changing with weight loss were classified by their relation to a CGI. All sites on the array were also classified based on their relation to a CGI. The percent of sites are shown for each of the regions of the CGI (island, shores, and shelves), or in the open sea (not associated with a CGI), for both those with changes associated with weight loss (black) and the whole array (grey; 451,705 sites). The regions of the CGI are defined as previously reported in (Bibikova et al., 2011; Ronn et al., 2013).

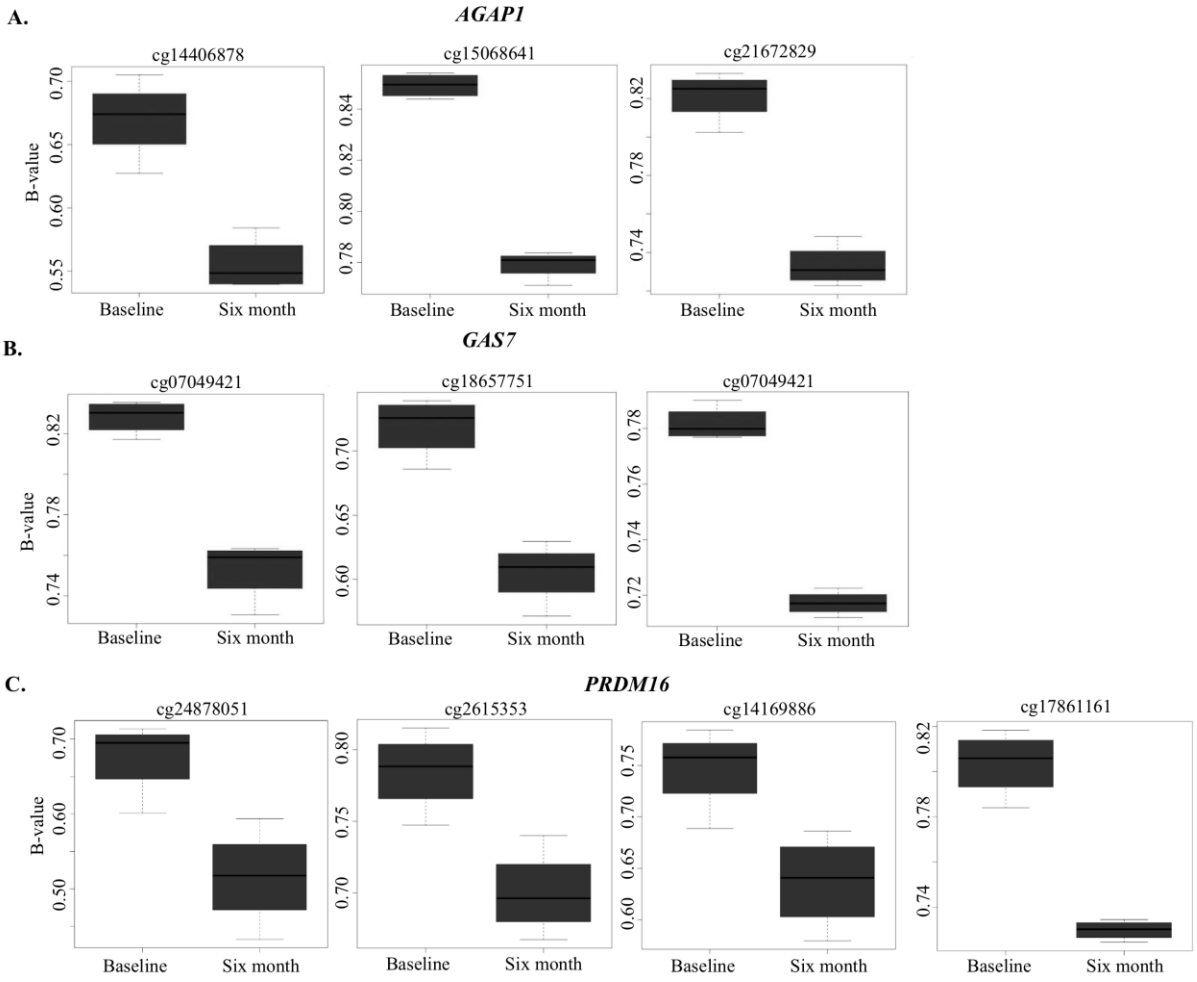
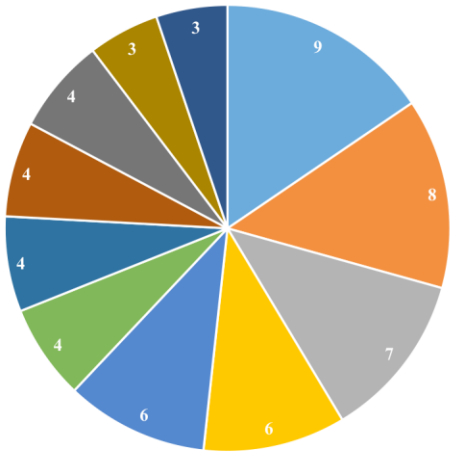


Figure 4.2. DNA methylation change in response to weight loss in participants with low central adiposity. The genes which had at least three sites with methylation changes associated with weight loss for the participants with the lowest quartile of baseline android fat (N=4) are shown. **A.** The three sites with methylation changes associated with *AGAP1*. **B.** The three sites with methylation changes associated with *GAS7.* **C.** The four sites with methylation changes associated with *PRDM16.* The DNA methylation levels are presented as ß-values, where a ß-value of 0 is considered 0% methylated, and 1 is considered 100% methylated. The data is presented as a box plot, with the average methylation level depicted by the black line in each box.

A.



B.

GO Term	p-value*	Genes
GO:0007165 signal transduction	0.0445377	*CD4,UNC5A,KALRN,CCL15,RPS6KA2,EGFR, RGL1,CASP1,DOCK1*
GO:0007275 multicellular organismal development	0.0387332	*HECA,UNC5A,GAS7,PLCZ1,ATOH8,PDGFD, EGFR,ITGA8*
GO:0007399 nervous system development	0.0071491	*KALRN,GAS7,ATOH8,CRIM1,KCNQ2,ITGA8, LSAMP*
GO:0007155 cell adhesion	0.0397535	*OPCML,CD4,AJAP1,ITGA8,LSAMP,CD300A*
GO:0007411 axon guidance	0.00664218	*UNC5A,CACNA1G,RPS6KA2,KCNQ2,EGFR, DOCK1*
GO:0007264 small GTPase mediated signal transduction	0.0446717	*KALRN,AGAP1,RGL1,DOCK1*
GO:0015031 protein transport	0.0481351	*AGAP1,CHMP4C,RAB11FIP1,CADPS*
GO:0007268 synaptic transmission	0.0455465	*CACNA1G,RPS6KA2,KCNQ2,NPY2R*
GO:0042981 regulation of apoptotic process	0.0376182	*UNC5A,BCL2A1,CARD17,CASP1*
GO:0006874 cellular calcium ion homeostasis	0.0273144	*CCL15,STIM2,PYGM*
GO:0007507 heart development	0.0442306	*HDAC9,RPS6KA2,PLCE1*

Figure 4.3. Functional enrichment analysis of DMS associated with weight loss in participants in the lowest 25th percentile of baseline android fat. Functional enrichment analysis for GO: Biological Processes was performed on only the sites with at least a 10% change in methylation, or were significant after FDR correction (N=114). **A.** The enriched terms are presented in a pie chart, the number in each wedge signifies the number of genes enriched in that term. **B.** The enriched terms, the p-value of enrichment, and the genes enriched in each terms are provided. *A hypergeometric corrected p-value based on the FDR correction is listed for each enriched GO term.

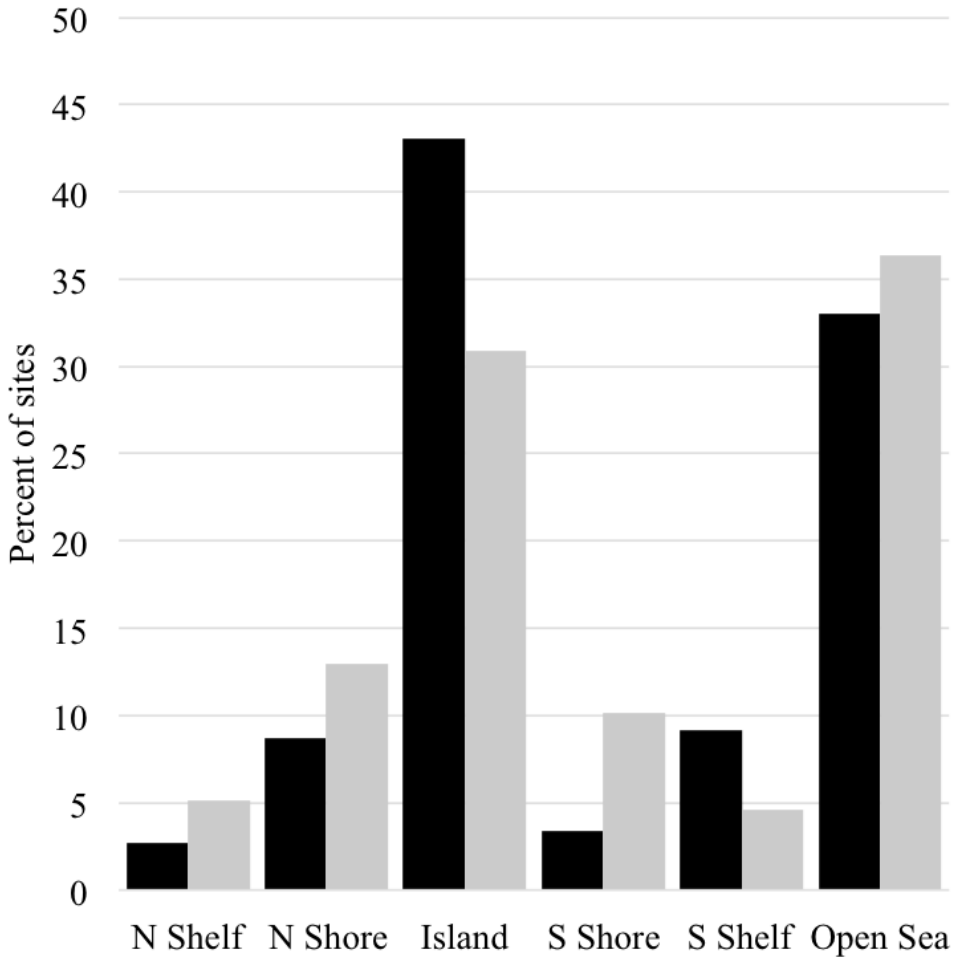
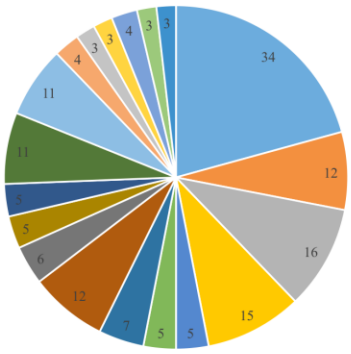


Figure 4.4. Distribution of sites with methylation levels after weight loss associated with central adiposity loss in relation to CGI regions. The 448 sites after the intervention with methylation levels associated with android fat loss (N=14) were classified by their relation to a CGI. All sites on the array that were included in this study (451,705 sites) were also classified based on their relation to a CGI. The percent of sites are shown for each of the regions of the CGI (island, shores, and shelves), or in the open sea (not associated with a CGI), for both those predictive of weight loss (black) and the whole array (grey). The regions of the CGI are defined as previously reported in (Bibikova et al., 2011; Ronn et al., 2013)**.**

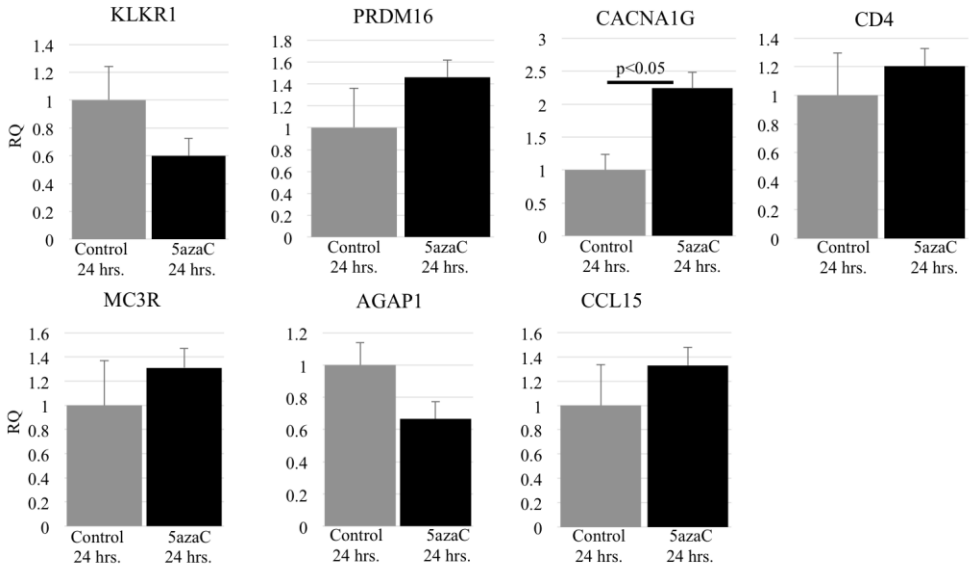
A.



B.

GO Term	p-value*	Genes
GO:0006355 regulation of transcription, DNA-dependent	0.0231075	ZFP36L1,C14orf43,SFMBT2,JRKL,DAPK3,PHF10,C11orf30, GTF2H5,ZMYM2,ARID1B,PPP1R13L,SAFB,PRR13,DPY30, ATAD2,NME2,KEAP1,ZNF384,YLPM1,ZNF200,CRTC3,SAFB2, NFATC2IP,VAX2,TERF1,TGS1,ZNF460,SAP3,AFF1,BATF3, ZNF566,TMEM189-UBE2V1,RREB1,NDN
GO:0006357 regulation of transcription from RNA polymerase II promoter	0.0124385	CREG1,SMARCC1,CUX1,MED21,WDR77,KLF12,RHOA,AES, THRA,ELF2,MED9,VEZF1
GO:0000122 negative regulation of transcription from RNA polymerase II promoter	0.0108431	CTBP1,MDM2,KDM6B,PPP1R13L,JARID2,CUX1,HDAC4, KLF12,PRDM16,VAX2,AES,TCF25,SKI,HIPK2,SAP30,SORBS3
GO:0045893 positive regulation of transcription, DNA-dependent	0.0290788	MAPK1,SMARCC1,TESC,HDAC4,PRMT2,PRDM16,EAF2, HIPK2,AFF1,FOXJ3,TMEM189,UBE2V1,ELF2,TFAP4,RREB1, ZP3
GO:0000910 cytokinesis	0.0282252	DAPK3,RAB35,RASA1,MYH9,BIN3
GO:0042787 protein ubiquitination involved in ubiquitin-dependent protein catabolic process	0.0277268	PARK2,MDM2,MAP3K1,HUWE1,HECTD1
GO:0000209 protein polyubiquitination	0.0236537	PARK2,MAP3K1,PSMC6,TNKS2,HUWE1, TMEM189-UBE2V1,LRSAM1
GO:0051301 cell division	0.0345386	CCND1,SENP5,ARPP19,SPAG5,CEP63,RHOA,MAD1L1,TERF1, CCND2,KIAA1009,NEK1,ANAPC13
GO:0002755 MyD88-dependent toll-like receptor signaling pathway	0.0219915	MAPK1,MAP3K1,NOD2,RPS6KA2,IRAK3,TMEM189-UBE2V1
GO:0034130 toll-like receptor 1 signaling pathway	0.0481502	MAPK1,MAP3K1,NOD2,RPS6KA2,TMEM189-UBE2V1
GO:0035556 intracellular signal transduction	0.0400958	MAPK1,TMEM68,NOD2,RASA1,RPS6KA2,RAC3,SPSB3, PRKCA,DFFB,ASB7,ZP3
GO:0016070 RNA metabolic process	0.0241445	ZFP36L1,RPLP1,EXOSC6,UPF2,PSMC6,WDR77,PAIP1,TGS1, PRKCA,NOM1,SNRPG
GO:0050885 neuromuscular process controlling balance	0.0462738	MYH10,APP,APLP2,RAC3
GO:0010506 regulation of autophagy	0.0247871	PARK2,DAPK3,MAPT
GO:0042221 response to chemical stimulus	0.0375076	KCNQ1,CHRNA7,AGPS
GO:0070555 response to interleukin-1	0.0284583	HDAC4,IRAK3,AES,PRKCA
GO:0042325 regulation of phosphorylation	0.0499988	LIMK2,CCND1,MCM7
GO:0006241 CTP biosynthetic process	0.0247871	NME1-NME2,NME2,CTPS

Figure 4.5. Functional enrichment analysis of genes with methylation levels after weight loss that were associated with central adiposity loss. Functional enrichment analysis for GO: Biological Processes of the genes associated with the 448 sites that had methylation levels after the intervention that were associated with the amount of android adipose mass lost. **A.** The enriched terms are presented in a pie chart, the number in each wedge signifies the number of genes enriched in that term. **B.** The enriched terms, the p-value of enrichment, and the genes enriched in each terms are provided. *A hypergeometric corrected p-value based on the FDR correction is listed for each enriched GO term.



Supplemental Figure 4.S1. Gene expression following 5azaC treatment in CD4+ T cells. The relative expression of transcripts as determined by qRT-PCR for the seven genes with DMS associated with weight loss in CD4+ T cells. The relative expression of each gene with 5azaC treatment (black) in CD4+ T cells in comparison to CD4+ T cells with no drug treatment (grey) is shown. All genes consist of the average of six independent drug treatments or control preparations. Error bars represent the standard error of the mean.

CHAPTER 5

CONCLUSIONS

The work presented in this dissertation focused on the leukocyte type specific changes in DNA methylation associated with obesity. The majority of the previously published studies examining the relationship between DNA methylation and obesity have been conducted on samples derived from mixed cell types. As DNA methylation profiles have been shown to be different in different cell and tissue types, the data from these studies is the weighted average of the methylation data of all included cell types. Thus, cell type specific differences in DNA methylation associated with obesity can be lost in the average. In order to provide information on single cell type DNA methylation associated with obesity, the studies of this dissertation examined this relationship in different leukocyte types.

Chapter 2 of this dissertation provides a reiterative isolation method for rapidly isolating the seven main leukocyte types from fresh or frozen peripheral blood. Additionally in this chapter, the potential of each of the seven leukocytes to respond to physiological cues via their methylomes were assessed. 5-hydroxymethylcytosine (5hmC) levels, the first product in the de-methylation of 5-methylcytosine (5mC), were evaluated in each of the leukocyte types. The CD4+ T cells were found to have the highest levels of 5hmC (3.67% of CG sites), followed by CD14+ monocytes (2.69% of CG sites), while CD8+ T cells had the lowest levels of 5hmC (1.91% of CG sites). Transcripts of the genes encoding the enzymes involved in the establishment (DNMTs) and removal (TETs, GADD45) of 5mC were also assessed with the CD4+ and CD8+ T cells having the highest levels of these factors. Together this data suggests

that the CD4+ T cells, CD8+ T cells, and CD14+ monocytes are the most poised to respond to physiological cues via DNA methylation.

Chapter 3 of this dissertation provides evidence of cell type specific DNA methylation differences associated with obesity. Genome wide DNA methylation was assessed in CD4+ T cells, CD8+ T cells, and CD16+ neutrophils in a group of obese and normal weight women. The two T cell types were selected for analysis based on their high potential to respond via DNA methylation as determined in Chapter 2. The neutrophils were selected even though they were not identified to be highly potentiated to respond via their methylome as they are the predominant leukocyte type in blood and are functionally altered in obesity. DNA methylation differences were only identified in obese women in the CD4+ and CD8+ T cells. 19 sites were differentially methylated in the obese women in the CD4+ T cells and 16 in the CD8+ T cells ($q<0.05$). No differences in methylation in the obese women overlapped between the two T cell types. Further, the amount of visceral adipose tissue was associated with the methylation levels of 79 sites in CD4+ T cells ($q<0.05$). One gene, *CLSTN1*, had four differentially methylated sites associated with it ($q<0.05$) and its expression in CD4+ T cells was shown to be effected by the inhibition of the DNMTs, suggesting that this gene is under methylation control in CD4+ T cells. However, we were not able to assess the expression levels of *CLSTN1* in the obese and normal weight women, so it is yet to be determined if there are corresponding alterations in expression with the methylation changes observed.

Chapter 4 of this dissertation provides information on DNA methylation and weight loss in a group of overweight and obese women in CD4+ T cells. CD4+ T cells were selected for this analysis based on the high number of associations between DNA methylation and obesity determined in Chapter 3. DNA methylation levels only changed with weight loss in the women

who started the intervention with the lowest levels of android fat. Additionally, the DNA methylation levels of 448 sites after the intervention were associated with the amount of android fat lost over the intervention ($q<0.05$). Interestingly in this study, DNA methylation differences were only identified in relation to measures of android fat. As central adiposity is known to be a more harmful fat depot, this suggests that there may be reprograming of the DNA methylome in CD4+ T cells in relation to the amount of central adiposity.

Collectively the data presented in Chapters 2 to 4 provides insight into the molecular modifications that occur in obesity at the level of DNA methylation. The inter-individual differences that are observed in obesity, weight loss, and weight loss maintenance suggest that there is a genetic component to this disease. Epigenetic mechanisms, including DNA methylation can provide a link between changes in the environment and the genome and may be in part facilitating these individual differences. The data presented in this dissertation provides evidence that there are leukocyte type specific differences of the DNA methylome in obesity. Additionally, it appears that the DNA methylation difference in obesity are not altered with weight loss, unless there are low baseline levels of central adiposity.

Future work needs to further characterize the role of DNA methylation in obesity and weight loss in other leukocyte types, as well as study the relationship with changes in gene expression. Understanding the DNA methylation profile in obesity will help learn more about this complex disease.

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