8-1-2013

Relationship of Global DNA Methylation with Cardiovascular Fitness and Body Composition

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RELATIONSHIP OF GLOBAL DNA METHYLATION WITH CARDIOVASCULAR FITNESS AND BODY COMPOSITION

by

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Bachelor of Science
Lenoir-Rhyne University
2010

A thesis submitted in partial fulfillment
of the requirements for the

Masters of Science - Exercise Physiology

Department of Kinesiology and Nutrition Sciences
School of Allied Health Sciences
Division of Health Sciences
The Graduate College

University of Nevada, Las Vegas
August 2013

UNLV
UNIVERSITY OF NEVADA LAS VEGAS

THE GRADUATE COLLEGE

We recommend the thesis prepared under our supervision by

Michael Steele Jarrett

entitled

Relationship of Global DNA Methylation with Cardiovascular Fitness and Body Composition

is approved in partial fulfillment of the requirements for the degree of

Master of Science - Exercise Physiology
Department of Kinesiology and Nutrition Sciences

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August 2013

ABSTRACT

Relationship of Global DNA Methylation with Cardiovascular Fitness and Body Composition

by

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Background: Global DNA Methylation (GDM), an epigenomic modification has been linked to the development of Cardiovascular Disease and its risk factors. Purpose: The purpose of this study was to determine if there is a significant correlation between GDM and cardiovascular fitness, as well as, measures of body composition. Methodology: 26 apparently healthy, adults (11 males) completed a physical activity and diet questionnaire, as well as, had a small blood sample (600µL) collected via finger prick for the determination of GDM. Body composition was assessed by means of a Dual-Energy X-ray Absorptiometry (DEXA) scan, while cardiovascular fitness was evaluated by the completion of a maximal exertion, graded exercise test (VO_{2max}) on a treadmill. Peasron's "r" value was used to determine the correlation GDM and various variables, while t-tests were used to determine if any differences between high and low value groups for each variable existed. Results: Body Mass Index was significantly correlated (p-value, r value; 0.031, -.556) with GDM while there was a significant difference between high and low level folate groups (p=0.034) as determined by the diet questionnaire. No significant correlations or differences

were found in males. Conclusion: The results conclude that as BMI increases, GDM decreases in females. In attempts to further investigate the relationships between GDM and these variables, auxiliary research needs to be conducted with larger subject pools containing additional sedentary participants.

ACKNOWLEDGEMENTS

It is with my deepest and most sincere gratitude that I thank my advisor Dr. James Navalta for all of the guidance, advice, and assistance in the endeavor of completing this thesis. I am a better researcher and physiologist for having had the opportunity to work with him.

I would like to thank the participants of my study, without whom this investigation would not have been completed. Additionally, I thank the Graduate and Professional Student Association for their financial support and contributions. Their support aided in the advancement of this study and was greatly appreciated.

Let it be known that my greatest proponent and source of strength on this journey is, and will always be, my wife, Erin Jarrett. Her willingness to not only be my life-long companion, as well as, my co-researcher is a true testament of her awesomeness. My love for her knows no bounds.

In closing, it is by the grace of God that I am here today and am blessed with all of these individuals. Thank you, Lord, for your guidance and granting me the strength, wisdom, and perseverance to complete this task. You are truly amazing.

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CHAPTER 1

INTRODUCTION

The culmination of the Human Genome Project in 2003 caused a giant expansion in the field of genomics research. It is understood that genomics is the study of the 3 billion base pair long chain of genetic information known as the genome. A multitude of research has investigated numerous aspects of the genome and how it relates to the physical outcome or creation of a being. Throughout this research, it has become apparent that other factors must interact with or affect the genome in order to account for the mass variance in genomic expression (Ordovas & Smith, 2010). It is this mindset that contributes to the field of study known as epigenomics.

Epigenomics is the study of heritable alterations in gene expression potential that are not caused by changes in the actual DNA sequence (Waterland, R. A., 2009). These alterations to the genome result in differences to the future outcome or expression of the genetic information, one possible reason why some genes are recessive and others are dominant. To date, there are three main mechanisms that are attributed to epigenomic alterations: 1) DNA methylation, considered the primary measure, 2) histone modification, and 3) autoregulatory proteins that add in modification (Waterland, R. A., 2009).

A plethora of research on the effects of DNA methylation on health issues and outcomes has been conducted since the early 2000's, with the majority of the research focusing on links to cancer (Choi et al., 2009), (Das and Singal, 2004).

Nearly ten times more research has been published concerning DNA methylation and cancer than the relationship of DNA methylation and cardiovascular disease (31,306 articles vs 3,848 articles on PubMed respectively). More recent studies such has the investigation conducted by Kim et al., 2010 are now turning their attention to the connections between DNA methylation and cardiovascular disease (CVD) with the possibility of creating a new biomarker for the detection of CVD. Risk factors for CVD such as obesity, smoking, and nutritional status have been investigated and were found to correlate with DNA methylation (Ordovas and Smith, 2010). Breton et al (2009) noted that children that were exposed to smoking prenatally, had significantly ($p=0.03$) lower methylation levels at the AluYb8 gene. Similarly, Ong, Moreno, and Ross (2012) investigated the multitude of research that examines the relationship between bioactive food components (BFC), those components that have anticancer potential, and global DNA methylation. A unanimous finding of global DNA hypomethylation was noted in those that consumed the anticancer components (Ong, Moreno, and Ross, 2012).

Not all risk factors for CVD have been investigated thoroughly however. The amount of information concerning the relationship between physical inactivity and/or exercise and DNA methylation is considerably less than that of other CVD risk factors. Recent PubMed searches revealed 4,224 articles concerning “DNA methylation and nutrition”, while only 2,764 articles were found dealing with “DNA methylation and obesity” and a mere 1,235 articles that remotely related to “DNA

methylation and exercise". With this in mind, it is imperative that research be conducted on this relationship to determine whether it follows suit with obesity, smoking, and nutritional status. More importantly, the results of this research could aid in providing evidence to use DNA methylation as a biomarker for CVD and its predecessors/risk factors.

Purpose of the Study

The purpose of this study is to determine if there is a correlation between cardiovascular fitness level, as determined by maximal oxygen consumption, and level of global DNA methylation. Additionally, the study will investigate the relationship between percent body fat and Global DNA methylation.

Research Hypotheses

Hypothesis #1

H_{01}: There will be no correlation between cardiovascular performance measures and global DNA methylation level.

H_{A1}: There will be a correlation between cardiovascular performance measures and global DNA methylation level obtained from isolated white blood cells.

Hypothesis #2

H_{02}: There will be no correlation between body composition and global DNA methylation level.

H_{A2}: There will be a correlation between body composition measures and global DNA methylation level obtained from isolated white blood cells.

Significance of the Study

The significance of this study lies in the possibility of finding a link between cardiovascular fitness level, as well as body composition, and the biomarker known as global DNA methylation. Finding a significant connection between these variables will provide evidence and rational for using global DNA methylation as a new quantitative risk factor for cardiovascular disease.

Definition of Terms

The following definitions are given for the purpose of clarification:

Cardiovascular Disease (CVD) – An umbrella term used to describe any health issue concerning the heart, blood vessels, or cardiovascular system as a whole. Includes but not limited to: myocardial infarction, cerebrovascular accident, and hypertension.

CpG Island – The pairing of cytosine and guanine to form a dinucleotide. This dinucleotide is a base pair in the formation of DNA.

DNA – Deoxyribonucleic Acid

DNA Methlyation – The addition of a methyl group to the 5 position of cytosine in a CpG island.

Epigenome – Any factor that effects genetic expression without directly modifying or altering the genome.

Genome – An organism's complete set of DNA or hereditary information.

Genomics – The study of the genome as a whole.

Global DNA Methylation (GDM) – The amount of methylation across the entire genome, expressed as a percentage.

VO_{2max} – The maximal amount of oxygen that an individual and uptake and utilize for energy production.

CHAPTER 2

REVIEW OF RELATED LITERATURE

As previously mentioned, the amount of published research concerning DNA methylation and cardiovascular disease as well as its risk factors is relatively small in size and scarce in quantity. Despite this fact, key relationships and observations have been noted. The remainder of this review of literature will focus on these findings including the different epigenomic markers, the relationship between DNA methylation and cardiovascular disease, the link between DNA methylation and obesity, and the influence of exercise on DNA methylation.

Epigenomic Markers

There are three primary mechanisms that are responsible for epigenetic and epigenomic modifications: 1) DNA methylation, considered the primary measure, 2) histone modification, and 3) autoregulatory proteins that add in modification (Waterland, R. A., 2009). Most of the current literature on epigenomic modification utilizes DNA methylation as the indictor of alteration. The rationale for this is twofold: 1) DNA methylation is the easiest epigenomic modification to test for on both a genetic and genomic level, 2) both histone modification and autoregulatory proteins effect DNA methylations. This section of the literature review will summarize how each epigenomic mechanism functions.

DNA Methylation

DNA methylation is the process in which a methyl group is added to a CpG dinucleotide within the genome (see Figure 2.1).

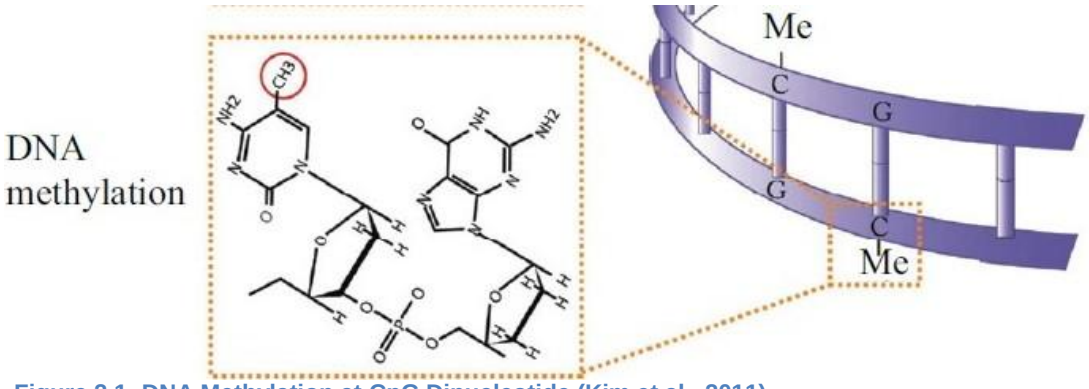

Figure 2.1: DNA Methylation at CpG Dinucleotide (Kim et al., 2011)

This process is outlined in detail by Baccarelli & Ghosh (2012), beginning with the actions of a cohort of enzymes known as the DNA methyl transferases (DNMTS: DNMT1, DNMT3a, and DNMT3b). One of the DNA methyl transferase enzymes interacts with S-adenosylethionine (SAM) and detaches a single methyl group for it, converting SAM into S-adenosylhomocysteine (SAH) (see Figure 2.2). The free methyl group is then attached to the 5th position on a CpG dinucleotide. This CpG dinucleotide is more commonly referred to as a CpG island, and will be referred to as such for the remainder of this paper.

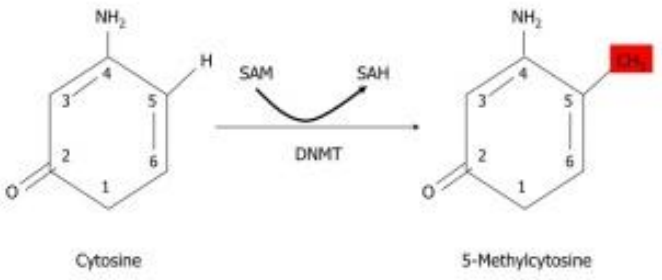

Figure 2.2 CpG Methylation
Courtesy of Dr. Amr Sawlha, University of Michigan

Methylation can occur at any CpG island within the genome. The location of the CpG island determines what effect the methyl group will have on the genome. If the CpG island is located within a coding or promoter region of a gene, then the transcription of that gene will most likely be repressed. This is due to the fact that the methyl group interferes with the binding of transcription factors. It should be noted that only 10% of all methylated CpG islands are located within a coding region (Baccarelli & Ghosh, 2012)

It is unclear at this point in time why this process (methyaltion) takes place, but studies have shown trends and relationships between methylation levels and health complications including atherosclerosis and various forms of cancer (Stauffer & DeSouza, 2010). Additionally, multiple stimuli (i.e. diet and habitual activities) have been linked with abnormal (hyper or hypo) levels of methylation (Stauffer & DeSouza, 2010). These trends and relationships will be discussed in the latter portions of this paper.

Histone Modification

The second epigenomic mechanism is known as histone modification. Histones are spool-like protein formations in which 146 base pairs of DNA are wrapped around. There are comprised of 4 major proteins (H2A, H2B, H3, and H4) and an unstructured N-terminal tail (Mazzio and Soliman, 2012). The main function of a histone is to assist in packaging DNA into chromosomes by the formation of nucleosomes (histones wrapped in DNA). Histones are modified when one of six different actions (methylation, acetylations, phosphorylation, ubiquitination, biotinylation, sumoylation, and proline isomerization) occurs on the N-terminal tail. This modification to the tail is known as a histone mark. The histone mark can cause gene repression or silencing by adjusting how "tightly" the DNA strand is wound around the histone (see Figure 2.3).

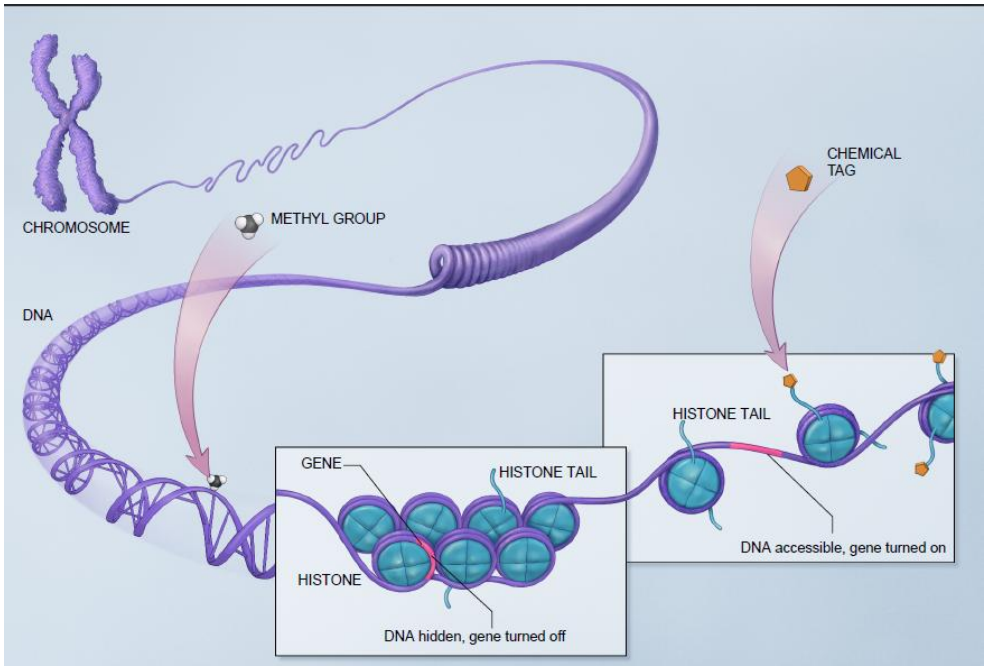

Figure 2.3: Histone Modification Causing Gene Silencing
Courtesy of the National Human Genome Research Institute

Assistant Proteins

The final major mechanism of epigenomics does not directly alter a genes expression per se but does assist in the modification of that gene. There are certain proteins that interact with the chromatins and nucleosoms, causing structural alterations that either allow or deny further DNA methylation or histone modification. There are three primary categories of assistant proteins: 1) chromatin remodeling complexes, 2) effector proteins with various binding modules for different modifications, and 3) insulator proteins (Kim, Samaranayake, & Pradhan, 2009). The complete explanation and discussion of the assistant protein is outside the scope of this paper. The following is a brief explanation of the three primary types of assistant proteins and how their functions contribute to epigenomic modification.

The primary function of chromatin remodeling complexes are to grant access to certain regions of DNA or histones by the use of nucleosomal repositioning, histone-DNA interactions, and histone octamer positioning (Kim, Samaranayake, & Pradhan, 2009). Chromatin remodeling complexes are unique in that they require ATP to accomplish their function. In the diagram below (Figure 2.4, A), a chromatin remodeling complex is altering the position of a nucleosome through an energy-driven reaction. The resulting repositioning allows access to a specific DNA location that was previously hidden to epigenetic modifiers.

Effector proteins can assist chromatin remodeling complexes in the repositioning of histones and chromatins. Additionally, effector proteins can serve as a marker for chromatin-modifying enzymes or remodeling complexes

(Kim, Samaranayake, & Pradhan, 2009). Marking the histone or chromatin in this way can cause additional modification enzymes that would normally not affect the area, to bind to the area and cause alterations. Figure 2.4B demonstrates this mechanism by using the example of effector protein HP1. Normally, the enzymes DNMT and SUV39H1 would not affect the histones, but HP1 has marked the location and is serving as an adaptor to allow DNMT and SUV39H1 to attach. This allows DNMT and SUV39H1 to modify the histones, increasing the amount of methylation.

Insulators are the final category of assistant proteins and are DNA elements that serve as boundary markers on throughout the genome. These markers segregate genes in order to prevent incorrect or unwanted repression or activation of genes. As depicted in Figure 2.4C, the insulator (I) can act as an enhancer blocking, preventing the activation of a promoter by the hindering the access of an enhancer (E). Additionally, insulators (I) can block off entire chromatins and act as a barrier that prevents methylation from neighboring chromatins. (Kim, Samaranayake, & Pradhan, 2009)

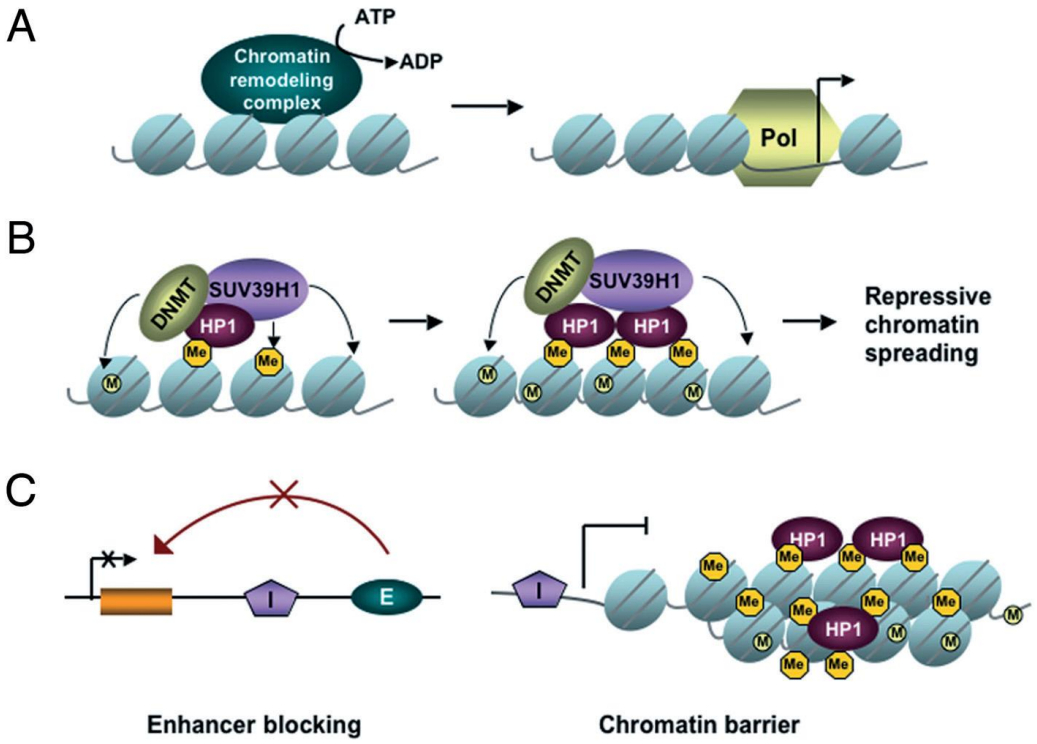

Figure 2.4: Depiction of various assistant proteins. Courtesy of Kim, Samaranayake, & Pradhan (2009)

DNA Methylation and Cardiovascular Disease

With an understanding of the significance of DNA methylation in mind, it is imperative its link with disease be both evaluated and clearly understood. Initially, most of the research in DNA methylation patterns was geared towards cancer and tumor development while more recent research has been focused on DNA methylation and cardiovascular disease risk and development. As with most research, animal models were used initially to determine if research on the topic was necessary. Lund and associates used a rodent model in 2004 to evaluate DNA methylation patterns in mice lacking apolipoprotein E ($apoE^-$) versus their control counterparts (Lund et al., 2004). The $apoE^-$ group was

predisposed to developing atherosclerosis while the control group consisted of normal mice. Both groups were monitored for six months, with blood and aorta tissue sample collected at four weeks and again at six months. All samples were evaluated for global DNA methylation using a DNA fingerprinting technique. At the four week time point, blood analysis did not reveal any significant difference in DNA methylation patterns between the two groups. However, aorta tissue sample in the apoE$^-$ mice were found to have significantly different ($p<0.04$) methylation patterns compared to the control group. These patterns consisted of both hyper and hypomethylation. Samples were taken again after 6 months of observation. Both the blood and aorta tissue samples revealed a significant difference ($p<0.02$, $p<0.003$ respectively) when comparing the apoE$^-$ group and the control group. Again, the revealed patterns were of hyper and hypomethylation levels in the apoE$^-$ group. Additionally, fibrocellular lesions were found in the apoE$^-$ group, suggesting the development of atherosclerosis. While the results were somewhat inconclusive in how atherosclerosis causes a modification in global DNA methylation, they do offer the conclusion that global DNA methylation is significantly altered with the development of atherosclerosis. (Lund et al., 2004)

Similar, yet more conclusive findings were brought about by a study conducted by Stenvinkel et al (2007), in a human model similar to the mouse model used by Lund et al (2004). Stenvinkel and associates investigated the association between DNA methylation and cardiovascular disease development in chronic kidney disease (CKD) patients and disease-free control group. CKD

patients were chosen as the experimental group due to their increased risk of developing cardiovascular disease. Both CKD (n = 155) and control (n = 36) groups were evaluated for global DNA methylation by blood sample as well as signs of cardiovascular disease (inflammation and oxidative stress biomarkers) for 36 months. The global DNA methylation levels of CKD patients (n=62) that developed chronic inflammation (determined by IL-6 and C-reactive protein levels) were compared to those of the control group. It was found that the CKD patients with inflammation had significantly ($p<0.001$) increased levels of methylation. It is noted that the CKD patients that did not present with inflammation had methylation levels similar to the control group. These results indicate that those patients that were developing cardiovascular disease presented elevated global DNA methylation levels, leading the authors to suggest that cardiovascular disease is associated with hypermethylation at the genomic level (Stenvinkel et al., 2007).

While the above mentioned study used a similar model with a control group, Kim and associates published comparable results with a population based study in 2010. Kim et al (2010) evaluated the global DNA methylation and cardiovascular disease status of 286 males and females out of a 63,257 population based cohort in Singapore over 5 years. The subjects presented at baseline for blood draws and cardiovascular disease and disease risk evaluation. Out of the 286 subjects, 101 present with cardiovascular disease at baseline. All subjects reported back for a follow-up visit 5 years later. From the remaining 185 subjects, 52 presented cardiovascular disease at the second visit. All subjects

with cardiovascular disease (n = 153) were compared to remaining disease-free subjects (n = 133) on the basis of global DNA methylation levels. The results did not show a significant difference for the population as a whole but was further separated by gender, revealing significant results. Males that presented with cardiovascular disease had significantly ($p < 0.05$) elevated global DNA methylation levels compared to their disease-free counterparts, while females were not significantly different. These finding were in agreement with Stenvinkel et al (2007). (Kim et al., 2010)

Table 2.1 Summary of DNA Methylation and Disease Research

Article	Subjects	Dependent Variable	Independent Variable/s	Results
Lund et al. (2004)	n=27 mice	GDM	Prevalence of atherosclerosis	↑ GDM
Stenvinkel et al. (2007)	n=191 males & females	GDM	Inflammation measured by IL-6 and C	↑ GDM in patients with inflammation
Kim et al. (2010)	n=286 males & females	GDM	CVD	↑ GDM in CVD patients

GDM – Global DNA Methylation, CVD – Cardiovascular Disease

DNA Methylation and Obesity

At this point in time, global DNA methylation seems to be closely correlated obesity as calculated by body mass index (BMI) or an individual's weight in kilograms divided by their height in meters squared (Kim et al., 2010). Upon the examination of 286 men and women from Singapore, it was found that those with

a BMI value of 24 kg/m^2 or higher had significantly ($p = 0.007$) elevated levels of global DNA methylation compared to those with a BMI value lower than 24 kg/m^2.

These results are in agreement with data collected by both Wang et al (2010) and Milagro et al (2012) in which the methylation status of individual genes were examined. Wang et al (2010) found obese (BMI > 30 kg/m^2) individuals displayed significantly ($p = 0.008$) higher methylation levels in the Ubiquitin-Associated and SH3 domain-containing protein A (UBASH3A) gene compared to non obese individuals. Likewise, Milagro et al (2012) found that overweight/obese individual had elevated methylation levels of the circadian clock system (internal clock) gene, CLOCK 1, compared to lean individuals (17.4% compared to 12.4% respectively).

Though evidence displayed above gives insight to the relationship between obesity and DNA methylation levels, it does not take individual's percentage of fat mass into consideration. All three of the aforementioned studies used BMI as their stratification tool for obesity, there by not actually measuring the amount of fat mass or fat-free mass. One study conducted by Fermin et al (2011) did go a step further than BMI and measured body composition by means of bioelectric impedance analysis. Fermin et al (2011) set out to determine whether or not methylation levels of nine obesity-related genes (AQP9, ATP10A, CD44, IFNG, MEG3, NTF3, POR, TNFRS9, and WT1) could be used to predict weight loss and body composition responses to a hypocaloric diet. The study was conducted on twenty-five (n=25) overweight or obese (BMI M (SD): 30.5 (0.45) kg/m^2)) men. Anthropomorphic measurements (Height, weight, age, percent body fat, and

waist girth) and blood samples were taken prior to and following an eight week hypocaloric diet. DNA methylation levels were measured in peripheral blood mononuclear cells obtained by the blood samples. Two (ATP10A and CD44) out of the nine genes studied were found to be significantly related with the loss of fat mass, the amount of weight lost and changes in waist girth. Figure 2.5 presents the relative data on the relationship between the methylation levels of CpG islands within the ATP10A gene and weight loss, changes in fat mass, and changes in waist girth. Similarly, Figure 2.6 discusses the significance changes of the same factors in relation to methylation levels at CpG islands within the CD44 gene. These results allude to two relative concepts: 1) DNA methylation at a genetic level is significantly correlated with changes in fat mass, over-all weight, and changes in waist girth 2) DNA methylation (ATP10A and CD44 genes specifically) could be used to predict an individual's degree of response to a hypocaloric diet.

While Fermin et al (2011) did not specifically investigate GDM alteration with weight or fat loss, their data does coincide with the aforementioned studies (Kim et al., (2010); Wang et al., (2010); and Milagro et al., (2012)) in that it demonstrates a relationship between DNA methylation and obesity. Additionally, it supports the notice of using DNA methylation as a predictor of CVD risk factors and responses to lifestyle alterations.

	CpG 3-4 r^2(p)	CpG 5-10-16 r^2(p)	CpG 18 r^2(p)
Fat mass change (%)	0.040 (p=0.353)	0.078 (p=0.147)	0.375 (p=0.001)
BMI change	0.016 (p=0.540)	0.172 (p=0.038)	0.434 (p<0.001)
Final leptin (ng/ml)	0.336 (p=0.003)	0.010 (p=0.635)	0.377 (p=0.001)
Waist girth change (cm)	0.119 (p=0.087)	0.036 (p=0.363)	0.382 (p=0.001)

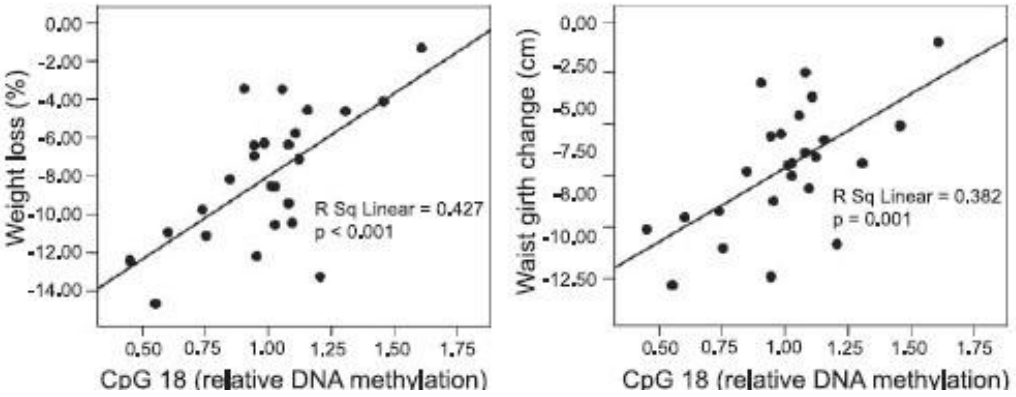

Figure 2.5: ATP10A Results
Relationships between baseline methylation levels of CpG Islands within the ATP10A gene and weight loss, fat mass change, BMI change and waist girth change. (Fermin et al., 2011)

	CpG 9-10 r^2(p)	CpG 14 r^2(p)	CpG 30 r^2(p)
Fat mass change (%)	0.255 (p=0.001)	0.297 (p=0.006)	0.194 (p=0.027)
BMI change	0.364 (p=0.001)	0.304 (p=0.005)	0.299 (p=0.005)
Final leptin (ng/ml)	0.142 (p=0.069)	0.179 (p=0.044)	0.050 (p=0.107)
Waist girth change (cm)	0.246 (p=0.01)	0.181 (p=0.038)	0.120 (p=0.090)

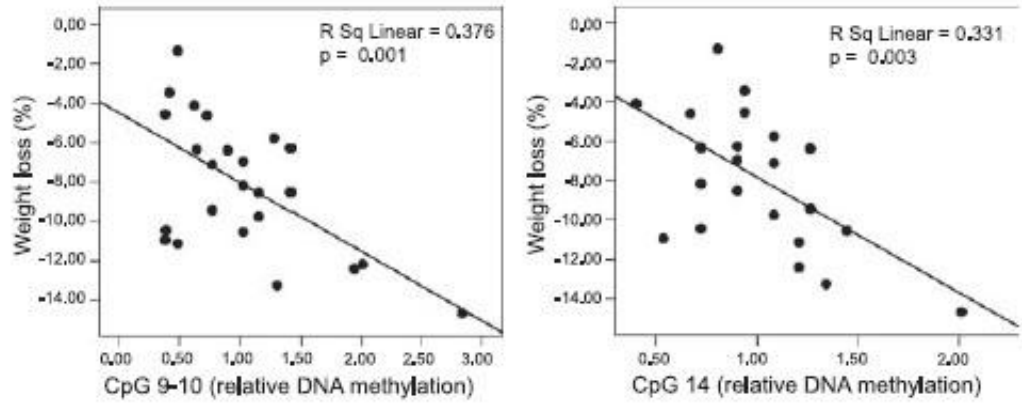

Figure 2.6: CD44 Results
Relationships between baseline methylation levels of CpG Islands within the CD44 gene and weight loss, fat mass change, BMI change and waist girth change. (Fermin et al., 2011)

Table 2.2 Summary of DNA Methylation and Obesity Research

Article	Subjects	Dependent Variable	Independent Variable/s	Results
Kim et al. (2010)	n=286 males & females	GDM	BMI	↑ GDM in obese individuals
Wang et al. (2010)	n=92 males & females	UBASH3A gene methylation	BMI	↑ Methylation in obese individuals
Milagro et al. (2012)	n=60 females	CLOCK 1Gene Methylation	BMI	↑ Methylation in obese individuals
Fermin et al. (2011)	n=25 males	Methylation levels of 9 obesity –related genes	BMI changes with hypocaloric diet	CD44 and ATP10A are related with ↓ in weight/fat mass/waist girth

BMI – Body Mass Index, GDM – Global DNA Methylation

DNA Methlyation and Exercise

The relationship between DNA methlyation and exercise has not been examined in great detail. Recent database searches have yielded minimal findings, resulting in only a hand full of articles pertaining to the topic. Additionally, the available findings are contradictory with reports of both increasing and decreasing in methylation levels.

One study conducted by Zhang et al (2011) examined the relationship between physical activity levels of cancer-free adults (ages 45-75 years old) and

their global DNA methylation level. Participants gave a baseline blood sample at the beginning of the study and wore accelerometers for 4 days (3 weekdays and 1 weekend day) to determine their level of physical activity. It was observed that individuals that were physically active for 26-30 minutes per day had significantly higher levels of global DNA methlyation compared to those who were active for less than 10 minutes per day ($\beta = 2.52$, 95% CI: 0.70, 4.35). Simple linear regression failed to be significant, negating the idea of a dose-response relationship between the two variables. These findings suggest that individuals that achieve the recommended amount of physical activity per day are hypermethylated compared to sedentary individuals. (Zhang et al., 2011)

While the above mentioned study demonstrated an elevated level of methylation on a genomic level, results from Barres and associates revealed differences on a genomic as well as a genetic or per gene basis. Barres et al (2012) examined the acute effects of a single bout of exercise on DNA methylation levels in healthy, sedentary men and women (n=14). Blood samples were taken before and after a peak pulmonary oxygen uptake rate test on a magnetically braked cycle ergometer. The results of a paired t-test showed a significant ($p < 0.05$) decrease in global DNA methylation after the exercise bout. Additionally, individual genes associated with metabolic and structural functions in skeletal muscles (PGC-1a, TFAM, PPAR-d, PDK4, citrate synthase [CS]) were examined and found to have decreased methylation levels following the cycle test. These results seem to contradict the hypermethylation findings of Zhang et al (2011).

To further investigate the effects of acute exercise on DNA methylation, Barres et al (2012) looked at a subgroup (n=8) of the original 14 participants and had them complete two additional exercise sessions in the following days after the VO_{2max} tests. The additional exercise sessions were completed on separate days with at least one week in between the trails. Each exercise session was conducted on the same cycle ergometer and was at varying intensities (40% or 80% of aerobic capacity). Blood samples after each exercise session were evaluated for changes in DNA methylation of the previously mentioned genes. It was found that the low intensity (40% aerobic capacity) exercise did not alter methylation levels compared to resting values, while high intensity (80% aerobic capacity) exercise decreased methylation values ($p < 0.05$). These results are again contradictory to Zhang et al (2011) and suggest that a dose response relationship may exist between exercise and gene methylation levels. (Barres et al., 2012)

Though global DNA methylation was not measured, Nakajima et al (2009) does add to the research compendium by displaying earlier findings of exercise inducing hypermethylation of the ASC (Apoptosis-associated Speck-like protein containing a Caspase recruitment domain) gene in older men. The research group set out to evaluate the effects of chronic exercise on the methylation levels of the ASC gene that is responsible for the production of pro-inflammatory cytokines. A total of 383 older (40-87 years of age) males were divided into either and exercise (n = 230) or a control (n = 153) group. The exercise group completed bouts of high-intensity interval walking (3 minutes of 40% peak

aerobic capacity followed immediately by 3 minutes of walking at 70% peak aerobic capacity) every day for 6 months. Blood samples were taken at the completion of the 6 months and tested for methylation of the ASC gene. The exercise group had significantly ($p<0.01$) higher methylation values for the ASC gene compared to the control group. This lead to the researchers to conclude that exercise induces hypermethylation of the ASC gene in older males, thereby decreasing the production of pro-inflammatory cytokines. (Nakajima et al., 2009)

Table 2.3 Summary of DNA Methylation and Exercise Research

Article	Subjects	Dependent Variable	Independent Variable/s	Results
Zhang et al (2011)	n=161 males females	GDM	4 day physical activity level	↑ GDM
Barres et al (2012)	n=14 males & females	GDM, PGC-1a, TFAM, PPAR-d, PDK4, and citrate synthase methylation	Exercise (peak pulmonary oxygen uptake test)	↓Methylation of all measurements
Barres et al (2012)	n=8 males	PGC-1a, TFAM, PPAR-d, PDK4, and citrate synthase methylation	Excise Intensity (40% and 70% aerobic max)	↓Methylation with ↑exercise intensity
Nakajima et al (2009)	n=383 males	ASC Gene Methylation	6 month walking program	↑ ASC Methylation

GDM – Global DNA Methylation

CHAPTER 3

METHODS

Participant characteristics

Participants were apparently healthy male and female adults between the ages of 21-39 years old (n=26). Apparently healthy was defined as receiving a passing score on the Modified American College of Sports Medicine Health/Fitness Facility Pre-participation Screening Questionnaire.

Table 3.1 Participant Characteristics

	Males	Females
N	11	15
Height (cm)	175.7 ± 7.8	163.9 ± 6.3
Weight (kg)	85.0 ± 15.4	60.0 ± 7.6
Age (years)	25.6 ± 5.2	26.8 ± 4.8
BMI	27.4 ± 3.6	22.3 ± 2.2
Body Fat (%)	20.0 ± 6.2	26.0 ± 7.5

Collection of the Data

Preliminary Data

Prior to the visit, participants were instructed to wear appropriate fitness clothing (i.e. running shoes, lose shirt, and shorts), to be well hydrated and to not consume any substance with calories 2 hour prior to their visit. Additionally, the

participants had been instructed to refrain from consuming alcohol and from exercising for at least 6 and 24 hours prior to their test respectively.

Upon arrival to the Exercise Physiology Laboratory, the participants were fully informed about the purpose, requirements, procedures, risks, and benefits of the study and were asked to sign an informed consent document that had been approved by the UNLV Institutional Review Board (Protocol #: 1209-4268). The participants then completed the Modified American College of Sports Medicine Health/Fitness Facility Pre-participation Screening Questionnaire. Participants were permitted to continue the study only if they received a passing score on the health questionnaire. Afterwards, each participant was assigned a subject identification number and basic anthropomorphic measurements were taken including height, weight, age, and body composition as determined by dual energy x-ray absorptiometry. In order to control for the effects of diet and lifestyle choices on methylation levels, each subject completed a food frequency questionnaire dealing with folate-rich foods as well as a physical activity questionnaire.

Graded Exercise Test

Participants completed a graded exercise test (GXT). The GXT was conducted on a treadmill using two minute stages. The participants walked at a self-selected speed with no incline for two minutes, followed by jogging for two minutes at a self-selected speed with no incline. The incline of the treadmill was increase by 2% grade every two minutes while remaining at the same jogging speed, until volitional fatigue. Before beginning the GXT, the participants were

instructed on how to use the Borg Ratings of Perceived Exertion scale (RPE). The GXT was terminated when the participant could no longer continue or if the participants wished to stop. Heart rate and oxygen uptake were measured continuously throughout the test by a Moxus Metabolic System (AEI Technologies, Pittsburgh, PA). The participant's RPE score was evaluated at the end of every stage. Participants' VO_{2max} values were determined by averaging the two highest, consecutive values in a 30 second rolling average of VO_2 (ml/kg/min). The time period used for the rolling average was defined as the period in which the participant had achieved two of the following criteria: heart rate ≥ 90% of their age predicted max, a respiratory exchange ratio at or above 1.05, an RPE ≥ 19, or their VO_2 (ml/kg/min) value had plateaued despite an increase in exercise intensity.

Blood Sample Collection

Upon completion of the informed consent and health screening questionnaire, a sample of blood (600 µl) was obtained from the participant via finger-stick using antiseptic technique into an anticoagulant tube (Multivette 600 LH, Sarstedt, Fisher Scientific, Pittsburgh, PA). The blood draw site was cleaned with an alcohol swab prior to all blood draws. Samples were labeled with corresponding subject identification numbers before being placed in a sealed and labeled biohazard bag (primary container) inside of a sealed and labeled biohazard cooler (secondary container). Samples were kept in an ice bath until they were analyzed in accordance with the parameters described in the University of Nevada, Las Vegas Institutional Biosafety Manual (Section VIII, page 19).

Standard biological personal safety barriers including latex-free gloves, laboratory coat, and eye safety goggles were worn at all times by research team members. All sharps and biological material were disposed of in a labeled and sealed sharps container and decontamination was conducted in accordance with the University of Nevada, Las Vegas Institutional Biosafety Manual (Section IX, page 23).

Blood Sample Analysis

Each sample was transported to the Exercise Biochemistry Laboratory for DNA isolation and storage. Whole blood (approximately 400 microliters) was added to 1000 microliters of Cell Lysis Solution, thoroughly mixed, and incubated for 10 minutes at room temperature. The sample was then centrifuged, and the supernatant was discarded without disturbing the white blood cell pellet. The cells were then re-suspended via vigorous vortexing and 400 microliters Nuclei Lysis Solution was added and incubated at 37ºC for 15-minutes. After allowing the sample to cool to room temperature, 110 microliters of Protein Precipitation Solution was added, vortexed, and then centrifuged. A dark brown protein pellet was visible at the bottom of the tube, and the remaining supernatant was transferred to a new microcentrifuge tube containing 300 microliters isopropanol. This tube was gently mixed and then centrifuged to pellet the DNA. The supernatant was discarded in a biohazard waste container, and equal volume of 70% ethanol was added in a wash step before centrifugation. The tube was inverted over absorbent paper and allowed to air-dry for 15 minutes. DNA Rehydration Solution (20-100 μL) was added to the tube and allowed to incubate

overnight at room temperature before being stored at 2-8 ºC for subsequent epigenetic analysis. DNA concentration (ng/mL) was determined using an Epoch microplate reader with the Take3 System (Biotek U.S., Winooski, VT)

Once all samples had been collected and purified using the process outlined above, the determination of global DNA methylation was achieved by the use of a commercially available assay kit (MethyFlash Methaylated DNA Quantification Kit, Epigentek Group Inc., Farmingdale, NY). A binding solution, followed by the samples was added to an antibody-infused 96 well plate. Negative and positive controls, as well as diluted positive values were added alongside the samples and tested in order to produce a standard curve for quantification proposes. The samples were allowed to incubate for 90 minutes inside the wells. Afterwards, the samples were removed, the wells were washed with a wash buffer, and a capture antibody was added to each well. The plate then incubated again for 60 minutes, at which time the solution was removed and a washer buffer was used again to clean the wells. Following the second washing, a detection antibody was added and allowed to incubate for 30 minutes. A third wash step took place after the 30 minutes. An enhancer solution was then added to each well and a final 30 minute incubation phase was conducted. A final wash step occurred followed by the addition of the developer solution, which turned the sample a bluish tint depending on the methylation level. Finally, a stop solution was added before the 96 well plate was placed in a plate reader and read at 450 nm. All samples were processed at the same time and global DNA methylation levels

were expressed as an absolute percentage based on the determined standard curve. The formulas used are given below.

$$GDM(ng) = \frac{Sample\ OD - Negitive\ OD}{Slope\ x\ 2}$$

$$GDM\ \% = \frac{GDM(ng)}{S}\ X\ 100$$

S is the concentration of sample DNA used in nano grams.

Data Analysis Methods

A Pearson product-moment correlation coefficient (Pearson's "r") was used to determine the correlations between body composition, fitness measurements, and GDM percentage. A series of t-tests were further conducted to determine any underlying differences. High and low value groups from each variable were constructed and compared for significant differences. The groups will be determined by whether they are higher or lower than the median for each variable. The significance level was set at $\alpha = 0.05$. All statistical analyses were calculated using SPSS Version 20 (IBM Corporation, Armonk, NY).

CHAPTER 4

RESULTS

Correlations

A total of five different measures (percent body fat, body mass index, bone mineral density, VO_{2max} [ml/kg/min], and the control measure of average folate intake) were examined for a relationship with GDM percentage. Correlations were first analyzed as an overall group, followed by a by gender delineation. Total subject group analyses revealed no significant relationships with r values ranging from -0.001 to 0.198 (see Table 4.1). Upon grouping the subject pool by gender, it was determined that BMI was significantly ($r=-.556$, $p=.031$) correlated with GDM percentage for females only. Body fat percentage approached significance in females as well ($r = 0.146$, $p=0.146$). No significant correlations were found in males for any of the variables.

Table 4.1 Correlation Results

		Pearson's r	p-value
Total Group			
	Body Fat	-0.173	0.397
	BMI	-0.198	0.332
	BMD	0.0	0.874
	VO_{2max}	0.0	0.995
	Folate	0.198	0.331
Males			
	Body Fat	0.101	0.767
	BMI	0.258	0.444
	BMD	0.371	0.262
	VO_{2max}	-0.253	0.452
	Folate	0.323	0.333
Females			
	Body Fat	-0.394	0.146
	BMI	-0.556	0.031*
	BMD	0.038	0.894
	VO_{2max}	0.178	0.526
	Folate	0.218	0.434

*BMI was significantly correlated to GDM in Females.

t-tests

To further investigate the possibility of a relationship between GDM percentage and the aforementioned variables, t-tests were conducted for potential differences between high scores verses lower values for any given variable. The median values for percentage of body fat (21.45%), body mass index (24.02 kg/m^2), bone mineral density (1.195g/cm^2), VO_{2max} (52.225 ml/kg/min), and average folate intake were determined (237.2 µg/day) and subjects were grouped according to whether they were higher or lower than the median value for each variable. The t-tests revealed a significant difference in the percentage of GDM between those with higher levels of folate intake

compared to those with lower levels (see Table 4.2). The difference in GDM% between those with higher BMI values and lower BMI values approached significance, while no significant differences were detected between high and low groups for VO_{2max}, bone mineral density, and percentage of body fat.

Table 4.2 t-test Results

	Median	Group	Methylation (%)		p value	Δ%
			Mean	SD		
BF%	21.45%	High	2.835	1.220	0.574	8.95465
		Low	2.602	0.819		
BMI	24.0	High	2.375	0.914	0.088	-22.4512
		Low	3.062	1.052		
BMD	1.1955 (g/cm2)	High	2.692	0.822	0.899	-1.91635
		Low	2.745	1.233		
Folate	237.2 (µg/day)	High	3.139	1.168	0.034*	36.63388
		Low	2.298	0.672		
VO2 Max	52.23 (ml/kg/min)	High	2.647	1.244	0.729	-5.16037
		Low	2.791	0.799		

*The High folate group has significantly increased GDM%

CHAPTER 5

SUMMARY, CONCLUSIONS, AND RECOMMENDATIONS

Discussion of Results

The purpose of this investigation was to determine whether a relationship exists between the percentage of global DNA methylation and measures of aerobic fitness, as well as measures of body composition. It was hypothesized that a significant correlation would exist between GDM% and measures of aerobic fitness and body composition. Regarding the association between VO_{2max} and GDM%, we retain the null hypothesis as no significant correlation was determined. When considering the body composition measure of Body Mass Index, we found that a significant relationship existed with global DNA methylation percentage in the female group only.

Upon classifying the subjects by gender, it was determined that there was a negative correlation with BMI and GDM percentage in females. These results disagree with the findings of Kim et al. (2010) in two ways. First, the association found in the present study suggests that as BMI increases, GDM percentage decreases, while Kim et al. (2010) found the opposite. Secondly, both males and females displayed a significant correlation in the Kim et al. (2010) study while only females did so presently. Possible rationales for these inconsistencies are the facts that the Kim et al. (2010) study was conducted in Singapore, where the obesity rate is much lower than in the United States (12% versus 36%

respectively) and the fact that the present study had a much smaller sample size (n=26 vs n=286 comparatively). This study was also unique in that it took an actual measurement of body composition (DEXA scan), as well as calculating BMI values.

Additionally, Kim et al. (2010) reported that global DNA methylation was significantly associated with an increased risk of cardiovascular disease. Specifically, they reported that males who developed cardiovascular disease (myocardial infarction, stroke) or its predisposing conditions (hypertension and diabetes) over a 5 year span had significantly higher levels of GMD compared to their disease free counterparts ($p=0.03$). As it is well established that physical fitness level and aerobic capacity is inversely related with cardiovascular disease risk (Dencker et al., 2012; Shortreed, Peeters, & Forbes, 2013), it seemed intuitive that a measure of aerobic capacity (VO_{2max}) would also be related to global DNA methylation. However, we found that VO_{2max} was not significantly ($r \leq -0.253$, $p>0.452$) related to GDM percentage in males or females, and there was no significant ($p=0.729$) difference between the high or low VO_{2max} groups. To our knowledge, this is the first investigation to report that there is no association between VO_{2max} and GDM%. As previously addressed, the low number of subjects, specifically sedentary subjects, in the present study could be an explanation for this lack of association. While cardiovascular disease risk is positively impacted by one's aerobic fitness, there are confounding factors that may affect the cardiovascular system, including smoking, dietary habits, and

family history. Further research is necessary to determine whether other measures of aerobic fitness are more sensitive correlates with DNA methylation.

Regarding dietary habits, a measure of average folate intake was estimated in the current study participants. The results of the delineated t-tests demonstrated that nutritional status needs to be accounted for at all times with concerns to methylation levels. The findings are in agreement with the concepts presented by Ong, Moreno, & Ross (2012) in that higher folate intake can influence methylation levels. The article discusses the need for research on using bioactive foods (including those high in folate) as a cancer prevention method. Foods high in folate were chosen due to folate's role in the methylation process as a methyl group donor. The present study demonstrates that individuals with elevated intakes of folate can have GDM percentages higher than those with low intakes of folate by 36.6%. Though this was not the main concern of the present study, folate's influence on GDM levels must be accounted for in future studies in order to limit confounding variables.

Conclusions and Recommendations for Further Study

Most methylation studies, be them GDM or individual gene methylation studies, have subject pools numbering in the hundreds due to the innate variance in individual genomes. As trends were evident with the small number of subjects in the present investigation, continued studies with a more diverse sample are warranted. Sedentary or less physically fit subjects would increase the diversity of the study and more than likely strengthen the correlations.

In addition to the pervious comment, it should be noted that at present time, there is no standard order or procedure for reporting GDM percentages. The current study has an average GDM percentage of 2.7% which is similar to the findings displayed by McGuinness et al. (2012). At the same time, GDM percentages have been published ranging from 0.85% (Liu et al., 2011) to 91% (Bromberg, Bersudsky, & Agam, 2009). This large discrepancy could be due to the fact that various means of DNA isolation and GDM quantification were used by the studies, which has been shown to significantly affect the GDM percentages (Soriano-Tarraga et al., 2013). This lack of standardization does not allow for easy comparisons between studies and can be very misleading when examining multiple studies. Standards need to be established in order for future research to prosper.

To our knowledge, this is the first study to correlate a direct measure of body composition with GDM. While the small number of subjects was a serious limiting factor, a number of interesting findings were present. It was demonstrated that an inverse relationship between GDM and BMI appears to be present in females, while not in males. Additionally, individuals with an increased levels of folate consumption have elevated levels of GDM compared to those that intake decreased amounts of folate. At this time, it does not appear that there is a relationship between cardiovascular fitness, measured by VO_{2max}, and GDM. Additionally, studies with further measures of aerobic fitness and greater numbers of subjects are needed in order to definitively say that this relationship

does not exist. DNA methlyation continues to be a biomarker of great importance in terms of detecting cardiovascular disease risk and prevention.

REFERENCES

Baccarelli, A., Rienstra, M., & Benjamin, E. (2010). Cardiovascular epigenetics: basic concepts and results from animal and human studies. *Circulation: Cardiovascular Genetics*, *3*(6), 567-573.

Barres, R., Yan, J., Egan, B., Treebak, J., Rasmussen, M., Fritz, T., Caidahl, K., & Krook, A. (2012). Acute exercise remodels promoter methylation in human skeletal muscle. *Cell Metabolism*, *15*, 405-411.

Bollati, V., & Baccarelli, A. (2010). Environmental epigenetics. *Heredity*, *105*(1), 105-112.

Breton, C., Byun, H., Wenten, M., Pan, F., Yang, A., & Gilliland, F. (2009). Prenatal tobacco smoke exposure affects global and gene-specific. *American Journal of Respiratory and Critical Care Medicine*, *180*, 462-467.

Choi, J., James, S., Link, P., McCann, S., Hong, C., Davis, W., Nesline, M., & Ambrosone, C. (2009). Association between global dna hypomethylation in leukocytes and risk of breast cancer. *Carcinogensis*, *30*(11), 1889-1897.

Das, P., & Singal, R. (2004). Dna methylation and cancer. *Journal of Clinical Oncology*, *22*(22), 4632-4642.

Dencker, M., Thorsson, O., Karlsson, M., Linden, C., Wollmer, P., & Andersen, L. (2012). Aerobic fitness related to cardiovascular risk factors in young children. *Eurpean Journal of Pediatrics*, *171*, 705-710.

Kim, G., Ryan, J., Gleen, M., & Archer, S. (2011). Epigenetic mechanisms of pulmonary hypertension. *Pulmonary Circulation*, *1*(3), 347-356.

Kim, J., Samaranayake, M., & Pradhan, S. (2008). Epigenetic mechanisms in mammals. *Cellular and Molecular Life Sciences*, *66*, 596-612.

Kim, M., Long, T., Arakawa, K., Wang, R., Yu, M., & Laird, P. (2010). DNA methylation as a biomarker for cardiovascular disease risk. *PLOS ONE*, *5*(3), 1-8.

Lundi, G., Anderson, L., Lauria, M., Lindholm, M., Fraga, M., Villar-Garson, A., Ballestar, E., & Esteller, M. (2004). Dna methylation polymorphisms precede any histological sign or atherosclerosis in mice lacking apolipoprotein e. *The Journal of Biological Chemistry*, *279*(28), 29147-29154.

Mazzio, E., & Soliman, K. (2012). Basic concepts of epigenetics impact of environmental signals on gene expression. *Epigenetics*, *7*(2), 119-130.

Milagro, F., Campion, J., Cordero, P., Goyenechea, E., Gomez-Uriz, A., Abete, I., Zulet, M., & Martinez, A. (2011). A dual epigenomic approach for the search of obesity biomarkers: Dna methylation in relation to diet-induced weight loss. *The Journal of the Federation of American Societies for Experimental Biology*, *25*, 1378-1389.

Milagro, F., Gomex-Abellan, P., Campion, J., Martinez, J., Ordovas, J., & Garaulet, M. (2012). Clock, per2 and bmal1 dna methylation: association with obesity and metabolic syndrome characteristics and monounsaturated fat intake. *Chronobiology International*, *29*(9), 1180-1194.

Nakajima, K., Takeoka, M., Mori, M., Hashimoto, S., Sakurai, A., Higuchi, K., Itano, N., & Shinohara, M. (2010). Exercise effects on methylation of asc gene. *International Journal of Sports Medicine*, *31*, 671-675.

Ong, T., Moreno, F., & Ross, S. (2011). Targeting the epigenome with bioactive food components for cancer prevention. *Journal of Nutrigenetics and Nutrigenomics*, *4*, 275-292.

Ordovas, J., & Smith, C. (2010). Epigenetics and cardiovascular disease. *Nature Reviews of Cardiology*, *7*(9), 510-519.

Shortreed, S., Anna, P., & Forbes, A. (2013). Estimating the effect of long-term physical activity on cardiovascular disease and mortality: evidence from the framingham heart study. *Heart*, *99*(9), 649-654.

Stauffer, B., & Christopher, D. (2010). Epigenetics: an emerging player in health and disease. *Journal of Applied Physiology*, *109*, 230-231.

Stenvinkel, P., Karimi, M., Johansson, S., Axelsson, J., Suliman, M., Lindholm, B., Heimburger, O., & Barany, P. (2007). Impact of inflammation on epigenetic dna methylation - a novel risk factor for cardiovascular disease. *Journal of Internal Medicine*, *261*, 488-499.

Wang, X., Zhu, H., Snieder, H., Su, S., Munn, D., Harshfield, G., Maria, B., & Dong, Y. (2010). Obesity related methylation changes in dna of peripheral blood leukocytes. *Biomed Central Medicine*, *8*(87), 1-8.

Zhang, F., Cardarelli, R., Carroll, J., Zhang, S., Fulda, K., Gonzalez, K., Vishwanatha, J., & Morabia, A. (2011). Physical activity and global genomic dna methylation in a cancer-free population. *Epigenetics*, *6*(3), 293-299.

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