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Relationship of DNA Methylation with Cardiovascular Fitness and Body Composition

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RELATIONSHIP OF DNA METHYLATION WITH CARDIOVASCULAR FITNESS AND BODY COMPOSITION

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ABSTRACT

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Global DNA Methylation (GDM), an epigenomic modification has been linked to the development of cardiovascular disease and its risk factors. Our focus was to identify potential relationships between epigenetic alterations and both cardiovascular fitness and body composition measures. **Purpose:** As increases in aerobic fitness have a beneficial effect on cardiovascular disease, one purpose was to determine if a relationship was evident between global DNA methylation and VO2max. A secondary purpose was to determine if the relationship extends to body composition measured via Dual X-ray Absorbtometry (DXA). **Methods**: Fifty-two (male n=25, female n=27) subjects provided a blood sample for DNA isolation, underwent a DXA scan, and completed a maximal exertion exercise test on a treadmill for the determination of maximal aerobic capacity (VO2max). Global DNA methylation (GDM) (%) was evaluated utilizing a commercially available ELISA kit, and correlated with BMI, percent body fat (%BF), and VO_2max using Pearson's correlation coefficient with significance accepted at $p \leq 0.05$. Participants were divided into high and low groups according to median score and differences were determined by the independent t-test. **Results:** When the overall group was considered, GDM was not significantly correlated with any measure (BMI r=-0.15, p=0.27; %BF r=-0.20, p=0.14; VO_{2max} r=0.24, p=0.09). When separated by gender, males displayed no significant correlations for any variable. In females, GDM was significantly correlated with BMI (r=-0.38, p=0.05), % BF (r=-0.43, p=0.02), and VO_2max (r=0.39, p=0.04). Also, when the t-test was preformed there were no differences in the whole group or in the female group, but there were differences in the male group for %BF (p=0.007) and BMI (p=0.028). **Conclusion:** The results provide evidence that as BMI and % body fat increases, GDM decreases in females when they were tested for a relationship. In

addition, epigenetic modifications appear to be associated with aerobic fitness in women. Future research should be directed toward identifying the gender difference observed from this data. The men in this study had significantly lower body fat (21±9 vs 29±8%) and greater VO_2max (54±10 vs 46±9 ml/kg/min) compared to the women. Upon examining the data via the t-test in men, our results show that greater %GDM are found in the lower groups for %BF and BMI. It is possible that epigenomic effects are associated with a threshold of body fatness, and future studies should investigate this possibility.

INTRODUCTION

Cardiovascular diseases (CVD) is one of the most prevalent deaths in the U.S. as well as worldwide. In the U.S. alone 179.1 deaths were reported in 2010 per 100,000 population (5). This is one-third of all U.S deaths (5). Financially, CVD impacts the U.S. by approximately $444 billion. This number is expected to increase since the U.S. population ages (5). Global DNA Methylation (GDM), an epigenomic modification has been linked to the development of cardiovascular disease and its risk factors. Thus, it seems appropriate to determine if there is a relationship between cardiovascular fitness level and the level of Global DNA Methylation (GDM). GDM is an epigenetic modification that occurs in the human genome and it is essential for regulatory and developmental processes (28). The indicator for the cardiovascular fitness level used is the maximal oxygen consumption.

The field that studies the 3 billion base pair long chain of genetic information is known as genomics. The human genome is incredibly complex and was first elucidated in 2003. Since then this field has expanded tremendously. The field of epigenomics explores the external factors that humans choose to do that affect the great variety phenotypes in the human genome (19, 21).

Epigenomics is the study of heritable genome alterations in gene expression potential that are not caused by changes in the actual DNA sequence (23). These alterations to the genome result in differences to the future outcome or expression of the genetic information. Today there are three main mechanisms that contribute to epigenomic alterations: 1) DNA methylation, considered the primary measure, 2) histone modification, and 3) autoregulatory proteins that add in modification (23).

A great deal of research has been conducted on the effects of DNA methylation on health issues since the early 2000s, however most of this research has been focused on the links to cancer (7, 8). The more recent research such as the study published by Kim et al. (7) has tried to find a biomarker such as the DNA methylation to identify the CVD. So far CVD risks have been shown to link with DNA methylation: obesity, smoking, and nutritional status (15). Furthermore, Breton et al.

(3) observed that children who were prenatally exposed to smoking, had significantly ($p=0.03$) lower methylation levels at the AluYb8 gene. Methylation of this gene is correlated with birth weight percentile therefore lower levels of methylation are not desirable because these children may be more prone to CVD.

Not all risk factors for CVD have been investigated thoroughly so far. The least investigated is the relationship between physical inactivity and/or exercise and DNA methylation. A simple search on PubMed revealed that 1,235 articles have been published on DNA methylation and exercise, whereas the number of published articles concerning DNA methylation and obesity, or DNA methylation and nutrition are two to four times more. Considering this, it is a good approach to research the relationship between obesity, smoking, and nutritional status. The results of this analysis could provide evidence that the DNA methylation biomarker can be considered for CVD and its risk factors.

This study will contribute to the field of genomics if it reveals a connection between cardiovascular fitness level, body mass composition, and the biomarker GDM. If significant connections are found between the variables chosen then the GDM biomarker would be a valuable identifier of the quantitative risk factor for cardiovascular diseases.

As previously mentioned, PubMed search results show the number of publications that discuss DNA methylation and its relationship with cardiovascular disease is very limited. However, there is some evidence in regards to the relationships between the epigenomic markers in relationship with cardiovascular disease. The rest of this paper discusses these findings including the different types of epigenomic markers, the correlation between DNA methylation and CVD, the relationship between DNA methylation and obesity, and the influence of exercise on DNA methylation.

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.

LITERATURE REVIEW:

Epigenomic Markers

DNA methylation, histone modification, and autoregulatory proteins that cause modification are the three mechanisms responsible for epigenetic and epigenomic modifications (25). DNA methylation has been utilized as an indicator in the most up to date literature. The reason for using it is because DNA methylation is a commonly used tool that can test both at the genetic and genomic level, and because DNA methylation is effected by histone modification and autoregulation. The following information illustrates briefly how these epigenomic mechanism works.

DNA Methylation

As illustrated in Figure 1, the process of DNA methylation involves the addition of a methyl group to a CpG dinucleotide within the genome.

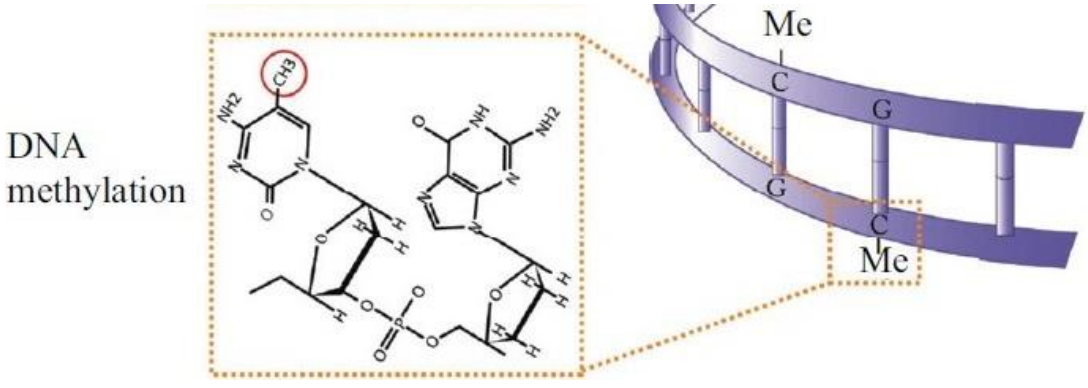

Figure 1: DNA Methylation at CpG Dinucleotide (7)

Baccarelli & Ghosh (1) describe the whole DNA methylation process, which begins with the action of four enzymes collectively known as DNA methyl transferase. One of the DNA methyl transferase enzymes interacts with S-adenosylethionine (SAM) and accepts a single methyl group from SAM, converting SAM into S-adenosylhomocysteine (SAH) (see Figure 2). Next, the free methyl group is attached to the 5th position on a CpG dinucleotide, hence the red color coded CH_3 (CpG island).

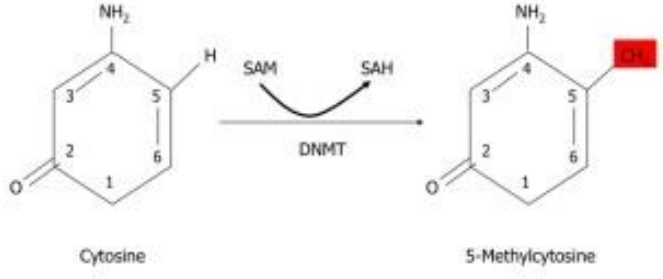

Figure 2: CpG Methylation (10)

The position where the process of methylation occurs in the genome is very important because it can occur anywhere within the CpG island. The position controls the effect of the methyl group in the genome. For instance, the transcription of a gene can be repressed if the CpG island is located within the coding or promoter region. However, this process occurs very rarely, only 10% of all methylated CpG islands are found within a coding region (1).

Why the process of methylation occurs is not well understood, however some research has shown a correlation between methylation levels and health complications including atherosclerosis and certain cancers (8). Diet and exercise habits have been correlated hyper or hypo levels of methylation (8). The trends and relationships of hyper and hypo levels of methylation is discussed below.

Histone Modification

Another epigenetic marker used is histone modification. Histones are proteins that are surrounded by 146 base pairs of DNA. Four main proteins make up the histone (H2A, H2B, H3, and H4) and an unstructured N-terminal tail (13). Histones help DNA package into nucleosomes that can form a chromosome. In order for the histone to be modified one of the following processes needs to occur at the N-terminal tail: methylation, acetylation, phosphorylation, ubiquitination, biotinylation, sumoylation, and proline isomerization. These alterations at the N-terminal tail are known as the histone mark. Alterations at the histone mark can cause gene repression or silencing by adjusting expression of that part of the DNA strand around the histone (see Figure 3).

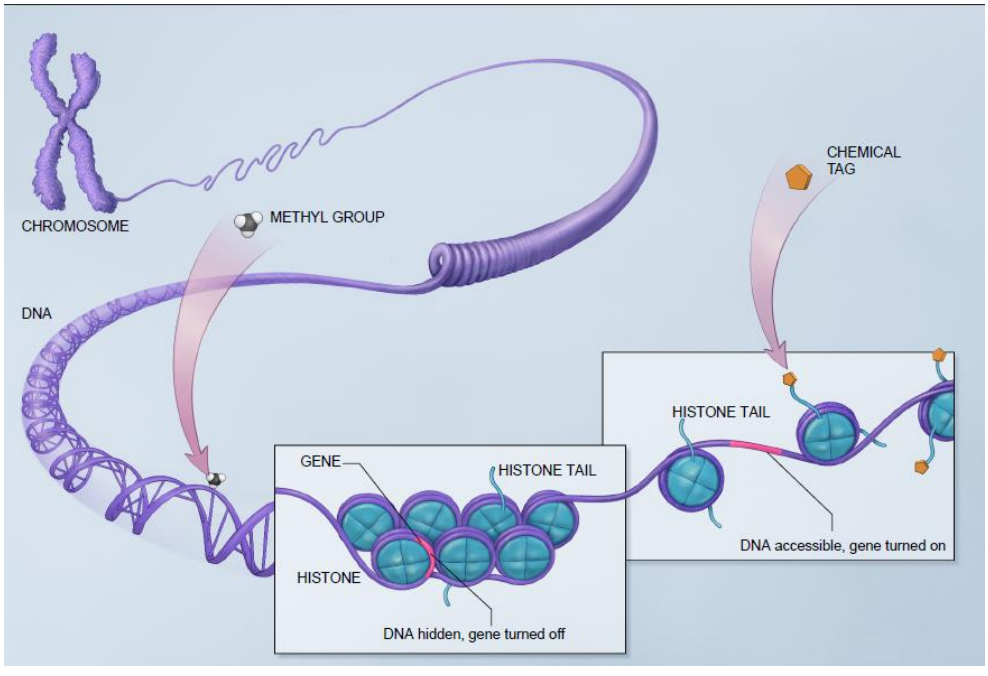

Figure 3: Histone Modification Causing Gene Silencing

Courtesy of the National Human Genome Research Institute

DNA Methylation and Cardiovascular Disease

DNA methylation is important especially when correlated with diseases. Historically, DNA methylation was primarily used in cancer and tumor development, but recently it has been used in cardiovascular diseases to indicate the risks and the stages of development. These relationships were first identified in animal studies. Lundi et al. (2004) conducted a study in mice to elucidate the DNA methylation patterns in mice without apolipoprotein E (apoE) versus a control group (12). The results showed that the apoE group was more likely to develop atherosclerosis whereas the control group contained the unaffected/normal mice. These two groups were observed for a period of six months. A DNA fingerprinting procedure was used to analyze the level of DNA methylation in these two groups. At four weeks blood analysis did not show any significant difference in DNA methylation patterns between the two groups. However, aorta tissue samples from 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. However, when samples were collected after 6 months, both the blood and aorta tissue samples indicated a significant difference between the apoE and control group: ($p<0.02$, $p<0.003$ respectively). These patterns were the hyper and hypomethylation levels in the apoE group. Furthermore, in the apoE group it was the fibrocellular lesions which indicate the development of atherosclerosis. Although the results were somewhat inconclusive as to how atherosclerosis causes modifications in GDM, they do show that GDM is significantly altered with the development of atherosclerosis (12).

In 2007, Stevinkel et al. (23), came up with a similar approach as Lundi et al. (12) but their work was done on human subjects. Stevinkel et al. (23) identified the link between DNA methylation and cardiovascular disease development in chronic kidney disease (CKD) patients who have a higher risk of cardiovascular disease, and a control group. The control and the CKD group were monitored for the elevated GDM levels (by collecting blood samples) and the symptoms of cardiovascular diseases (by analyzing the inflammation and oxidative stress biomarkers for a period of 36 months. The results revealed that the patients with CKD and inflammation had higher levels of methylation ($p< 0.001$) and those who did not have inflammation had results similar to those in the control group. This study concluded that patients that were developing cardiovascular diseases presented elevated levels of methylation or hypermethylation (23).

Another study came out three years later showing important aspects of the levels of methylation in a population based study. Kim et al. (9) used a similar approach as Stevinkel et al. (23). The levels of DNA methylation and the cardiovascular disease level were assessed in 286 males and females out of a population (N=63,257) based cohort in Singapore over a five year period. At the beginning of the study, 101 subjects were identified with cardiovascular disease. Then, at the second meeting, another 52 subjects of the remaining 185 were identified with cardiovascular disease. Subjects with (n=153) and without (n=133) cardiovascular had their DNA methylation levels analyzed. Although, the results obtained did not indicate significant difference for this population, the

results were further analyzed after the samples analyzed based on differences in gender. A higher level of GDM ($p<0.05$) was identified in males with cardiovascular disease versus those who did not have cardiovascular disease. However, in females the results were not significantly different. These results were consistent with Stenvinkel et al. (23). Table 1 below shows the results.

Table 1. Summary of DNA Methylation and Disease Research

Article	Subjects	Dependent Variable	Independent Variable/s	Results
Lundi 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
- IL-6=Interleukin 6
- C=C-reactive protein

DNA Methylation and Obesity

Recently, it has been shown that GDM may be closely correlated with obesity via the body mass index indicator (9). The researchers looked at 286 men and women from Singapore and discovered that those with a body mass index (BMI) value of 24 kg/m^2 or higher had significantly (p

= 0.007) elevated levels of GDM than those with a BMI value lower than 24 kg/m^2. Furthermore, Wang et al. (28) and Milagro et al. (16) found consistent results with Kim et al. (9) in which the methylation status of individual genes were examined. Similarly, Milagro et al. (16) found that overweight/obese individuals 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 looked at whether methylation levels could be used to indicate weight loss and body composition to a hypocaloric diet measured body composition with the bioelectric impedance analysis and not by BMI (16). Milagro et al. (16) set out to determine whether methylation levels of nine obesity-related genes (AQP9, ATP10A, CD44, IFNG, MEG3, NTF3, POR, TNFRS9, and WT1) in order to determine the weight loss and body composition responses to a hypocaloric diet. The study of twenty-five (n=25) overweight or obese (BMI M (SD): 30.5 (0.45) kg/m^2)) men tracked height, weight, age, percent body fat, waist girth, and blood levels prior to and following an eight week hypocaloric diet. The DNA methylation levels were assessed by peripheral blood mononuclear cells. Their results help us consider 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, and 2) DNA methylation (ATP10A and CD44 genes specifically) can be used to predict an individual's degree of response to a hypocaloric diet.

Although Milagro et al. (16) did not investigate the GDM alteration with weight or fat loss, their data illustrates the correlation between DNA methylation and obesity and it is consistent with the other sources mentioned above (9, 23, 17,19).Moreover, it provides evidence of using DNA methylation as an indicator of CVD risk factors and responses to lifestyle changes.

DNA Methylation and Exercise

The relationship between DNA methylation 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.

Zhang et al. (27) examined the relationship between physical activity levels of cancer-free adults (ages 45-75 years old) and their GDM level. A baseline blood sample was taken from each participant at the beginning of the study and they 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 GDM 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 was not 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 (27).

While the above mentioned study demonstrated an elevated level of methylation on a genomic level, results from Barres and associates (2) revealed differences on a genomic as well as a genetic or per gene basis. Barres et al. (2) 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 GDM after exercise. 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. (27).

To further investigate the effects of acute exercise on DNA methylation, Barres et al. (2) 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 VO_{2max} test measures the volume of oxygen that one individual can consume while exercising at his/her maximum capacity. VO_2 max is the maximum amount of oxygen in millilitres, one can use in one minute per kilogram of body weight. Those who are fit have higher VO_2max values and can exercise more intensely than those who are not as well conditioned). In the study conducted by Barres et al. (2), the additional exercise sessions were completed on separate days with at least one week in between the trials. Each exercise session was conducted on the same cycle ergometer 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 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. (27) and suggest that a dose response relationship may exist between exercise and gene methylation levels (2).

Though GDM was not measured, Nakajima et al. (19) shows that exercise induced 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 an 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 led the researchers to conclude that exercise induces hypermethylation of the ASC gene in older males thereby decreasing the production of pro-inflammatory cytokines (19)**.**

The primary objective of this study is to determine if there is a correlation between body composition measures, cardiovascular performance measures, and GDM level obtained from isolated white blood cells.

METHODOLOGY

Participant characteristics

Based on what participants answered in the Modified American College of Sports Medicine Health/Fitness Facility Pre-participation Screening Questionnaire all subjects were appearing healthy. This was defined after they received a passing score in the questionnaire. The sample size consisted of fifty-two subjects (male n=25, female n=27) with ages ranging from 18 to 44. The height, weight, and age average and standard deviations are shown below.

Table 2. Participant Characteristics

	Males	Females
N	25	27
Height (cm)	179 ± 9.4	163.2 ± 5.7
Weight (kg)	83.9 ± 14.5	59.4 ± 7.4
Age (years)	27.4 ± 7.6	26.8 ± 5.4

Collection of the Data

Preliminary Data

Each subject was informed to come appropriately dressed (running attire) prior to the $VO2_{max}$ testing and dual energy x-ray absorptiometry (DEXA) scan. Subjects were instructed to be very well hydrated and not eat two hours before the test, no alcohol or caffeine nor exercising for at least 6-24 hours prior to being tested.

Before testing, each individual had to sign an informed consent document that explains the purpose, requirements, procedures, risks, and benefits of the study. This document was approved by the UNLV Institutional Review Board (Protocol #: 1209-4268). The subjects also completed the Modified American College of Sports Medicine Health/Fitness Facility Pre-participation Screening Questionnaire. Only those who passed the health questionnaire entered the study. Next, each subject was assigned an identification number and their height, weight, and age was taken. Then, using DEXA, body composition was determined.

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 $\geq$ 90% of their age predicted max, a respiratory exchange ratio at or above 1.05, an RPE $\geq$ 19, or their VO_2 (ml/kg/min) value had plateaued despite an increase in exercise intensity.

Blood Sample Collection

A sample of blood (600 µl) was collected via finger-sick using aseptic technique into an anticoagulant tube (Multivette 600 LH, Sarstedt, Fisher Scientific, Pittsburgh, PA). The anatomical site of the blood draw 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 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 members of the research team. All sharps and biological materials were disposed off in 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 300 µl) was added to 900 µl of Cell Lysis Solution, thoroughly mixed, and incubated for 10 minutes at room temperature. The sample was centrifuged, and the supernatant discarded without disturbing the white blood cell pellet. The cells were then re-suspended via vigorous vortexing and 300 µl Nuclei Lysis Solution was added and it was pipetted 5-6 times to lyse the white blood cells. At this point the solution appeared very viscous. Next, 100 µl 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 µl room temperature 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).

After all samples were collected via the method explained above, the level of GDM was determined. This was done by using the commercially available assay kit (MethyFlash Methylated 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. Then, the samples were incubated for 90 minutes inside the wells. Next, the samples were removed and the wells were washed with a wash buffer and a capture antibody was added to each well. The plate was incubated for 60 minutes. The removal of the solution and the washing procedure with the buffer was repeated for cleaning purposes. After the second wash, a detection antibody was added and the wells were incubated for 30 minutes. An enhancer solution was added to each well and a final 30 minute incubation phase followed. The wells were washed for the last time and the developer solution was added giving the sample a bluish color based on the methylation level. Finally, a stop solution was added to all wells in order to control the reaction (which one??) and have it run for only a specific amount of time. Next, the 96-well microtiter plate was placed in a microplate reader and analyzed at 450 nm. All samples were processed at the same time and GDM levels were expressed as an absolute percentage based on the determined standard curve. The formulas used were given below.

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

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

S stands for the concentration of sample DNA used in nano grams (ng). OD stands for optical density (basically it's an indicator of the amount of color displayed; the darker the blue, the greater the concentration).

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 conducted to determine any underlying differences for the whole group as well as for each gender. High and low value groups from each variable were constructed and compared for significant differences. The groups were determined by whether they are higher or lower than the median for each variable. In case a subject fall on the median, then the value will be compared with the standard for that specific variable and a decision will be made whether that value will be in the lower or higher group. The significance level was set at $\alpha = 0.05$. All statistical analyses were calculated using SPSS Version 20 (IBM Corporation, Armonk, NY).

RESULTS

Correlations

A total of four different measures (percent body fat, body mass index, bone mineral density, VO_{2max} [ml/kg/min]) were examined for a relationship with GDM percentage. The overall group was first analyzed for correlations and then each gender was analyzed separately. When the overall group was considered, GDM was not significantly correlated with any measure (BMI r=-0.15, p=0.29; %BF r=-0.2, p=0.13; VO_{2max} r=0.39, p=0.07).When separated by gender, males displayed no significant correlations for any of the variables. However, in females, GDM was significantly correlated with BMI (r=-0.38, p=0.05), % BF (r=-0.43, p=0.02), and VO_2max (r=0.39, p=0.04).

Table 3. Correlation Results

	Name of Variable	Pearson's r	p-value
Total Group			
	Body Fat	-0.2	0.137
	BMI	-0.38	0.256
	BMD	0.24	0.686
	VO_{2max}	0.39	0.089
Males			
	Body Fat	0.107	0.409
	BMI	-0.166	0.508
	BMD	0.269	0.269
	VO_{2max}	0.18	0.180
Females			
	Body Fat	**0.128**	**0.022***
	BMI	**-0.431**	**0.047***
	BMD	0.394	0.515
	VO_{2max}	**-0.085**	**0.042***

*% BF, BMI, and VO_{2max} were 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 analysis was done for the whole pool of subjects as well as for each gender. When the whole group of subjects was considered, the median values were: percentage of body fat (24.10%), body mass index (23.4 kg/m^2), bone mineral density (1.209 g/cm^2), VO_{2max} (50.1 ml/kg/min); 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 VO_{2max} values compared to those with lower levels (see Table 3.A). The difference in GDM% when compared to the high and low groups for body mass index, bone mineral density, and percentage of body fat revealed no significance. When the groups were divided by gender and analyzed in the same fashion only the males group showed significant results in percent

body fat and body mass index when compared to percentages of GDM (see Table 3.B). The females group showed no difference for any of the variables (see Table 3.C).

Table 3.A t-test results for the whole group							
	Median			Group	Methylation (%)		p value
		Mean	SD		Mean	SD	
BF%	24.10%	32.50%	1.40%	High	1.4	1.4	0.13
		17.60%	4.50%	Low	1.9	1.2	
BMI	23.4	27.4	3.9	High	1.5	1.1	0.29
		21	1.4	Low	1.9	1.4	
BMD	1.209 (g/cm^2)	1.33	0.08	High	1.6	1.2	0.58
		1.15	0.05	Low	1.8	1.4	
VO_{2max}	50.1 (ml/kg/min)	58.4	6.4	High	2.1	1.4	0.07
		41.9	6.1	Low	1.4	1.2	

Table 3.B t-test results for the male group							
	Median			Group	Methylation (%)		p value
		Mean	SD		Mean	SD	
BF%	26.10%	35.20%	6.40%	High	1.2	0.8	**0.007***
		22.10%	2.80%	Low	2.6	1.5	
BMI	21.8	24.4	1.7	High	1.3	1	**0.028***
		20.5	1.1	Low	2.5	1.5	
BMD	158 (g/cm	1.21	0.05	High	2.1	1.6	0.54
		1.11	0.04	Low	1.7	1.2	
VO_{2max}	.9 (ml/kg/n	53.4	7	High	2.4	1.5	0.14
		39.5	6.4	Low	1.6	1.1	
*The % BF has significant differences in % GDM between the high and the low groups							
* The BMI has significant differences in % GDM between the high and the low groups							

Table 3.C t-test results for the female group							
	Median			Group	Methylation (%)		p value
		Mean	SD		Mean	SD	
BF%	18.70%	28.70%	6.60%	High	1.5	1.3	0.78
		14.20%	3.60%	Low	1.3	1.1	
BMI	25.2	29.7	4.2	High	1.73	1.27	0.15
		23.4	1	Low	1.06	1.07	
BMD	1.326 (g/cm^2)	1.4	0.05	High	1.5	1.3	0.8
		1.24	0.05	Low	1.3	1.1	
VO_{2max}	53.7 (ml/kg/min)	61.8	5	High	1.6	1.1	0.76
		46.4	6.2	Low	1.4	1.4	

SUMMARY, CONCLUSIONS, AND RECOMMENDATIONS

Discussion of Results

Since an increase in aerobic fitness have a beneficial effect on cardiovascular disease, the first purpose of this investigation was to determine whether a relationship exists between the percentage of global DNA methylation and measures of aerobic fitness (via VO_{2max}). The second purpose was to determine if a relationship extends to measures of body composition (via Dual X-ray Absorbtometry (DXA)). It was hypothesized that a significant correlation would exist between GDM% and measures of aerobic fitness and body composition. Upon experimentation the association between VO_{2max} and GDM%, was proved as being not significant. Thus, for the overall group, we retain the null hypothesis. When considering the body composition measure, the percentage of body fat measure, and the aerobic fitness measure, we found that a significant relationship existed with global DNA methylation percentage in the female group only. Thus, we accept the alternate hypothesis with regard to BMI, % BF, and VO_{2max}.

Upon classifying the subjects by gender, it was determined that there was a negative correlation with BMI and percent body fat when compared with GDM percentage in females. The

results provide evidence that as BMI and % body fat increases, GDM decreases in females. These results disagree with the findings of Kim et al. (7) in two ways. First, the association found in this study suggests that as BMI increases, GDM percentage decreases, while Kim et al. (7) found the opposite. Secondly, both males and females displayed a significant correlation in the Kim et al. (7) study while only females did so presently. These disagreements are due to the fact that the Kim et al. (7) 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=52 vs n=286 comparatively). The population groups are also different as well as their diets. Furthermore, epigenetic modifications appear to be associated with aerobic fitness in women. Future research should be directed toward identifying the gender difference observed from this data. The men in this study had significantly lower body fat (21±9 vs 29±8%) and greater VO_{2max} (54±10 vs 46±9 ml/kg/min) compared to the women. These data indicate that % body fat is an important variable and it is inversely related with VO_{2max}. It is possible that epigenetic effects are more evident in a population with reduced overall fitness parameters. 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. (7) 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 GDM 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, it seemed intuitive that a measure of aerobic capacity (VO_{2max}) would also be related to global DNA methylation (8, 19). We found that VO_{2max} was significantly ($r =0.20$, $p=0.14$) related to GDM percentage in females but not in males. Since the results provide evidence that as BMI and % body fat increases, GDM decreases in females only. In addition, epigenetic modifications appear to be associated with aerobic fitness in women. Future

research should be directed toward identifying the gender difference observed from this data. The men in this study had significantly lower body fat (21±9 vs 29±8%) and greater VO_{2max} (54±10 vs 46±9 ml/kg/min) compared to the women.

However, when the independent t-test was performed, after the participants were divided into high and low groups according to median score, we observed differences in the male group only for % BF ($p=0.007$) and BMI ($p=0.028$). The % GDM was greater in the lower groups for these two variables. Nonetheless, there was no significant ($p=0.14$) difference between the high or low VO_{2max} groups as we would have thought. However, the males' %BF is normal (35.2% in the high group) and the VO_{2max} is high (53.4 in the high group) compared to the females' %BF that is very low (28.7% in the high group) and the VO_{2max} is very high (61.8 in the high group). It is possible that epigenomic effects are associated with a threshold of body fatness, therefore % body fatness is a key factor and future studies should investigate this possibility.

It is possible that epigenetic effects are more evident in a population with reduced overall fitness parameters. 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.

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. A future study should be considered in evaluation the levels of DNA

methylation in a group with sedentary or less physically fit women subjects versus physically fit, or athletic women. This way we would be able to test the results found in this study and we may see stronger correlations. Furthermore, instead of dividing the groups by their median score, it would be better to partition the groups based on a set threshold level such as a BMI greater than 25.

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. (14). At the same time, GDM percentages have been published ranging from 0.85% (11) to 91% (4). 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 (21). This lack of standardization does not allow for easy comparisons between studies and can be very misleading when examining multiple studies. Standardized measurements 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 and % BF appears to be present in females, while not in males. 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. Also, new studies should be conducted in order to test the gender differences that were noticed in this study since the males in this group had a lower body fat percentage as well as greater VO_{2max} when compared with the females group. DNA methylation continues to be a biomarker of great importance in terms of detecting cardiovascular

disease risk and prevention. Its effects may be more obvious in a pool of subjects with reduced overall fitness parameters.

TABLES

Table 1. Participant Characteristics		
	Males	Females
n	25	27
Height (cm)	179 ± 9.4	163.2 ± 5.7
Weight (kg)	83.9 ± 14.5	59.4 ± 7.4
Age (years)	27.4 ± 7.6	26.8 ± 5.4

Table 2. Correlation Results

	Name of Variable	Pearson's r	p-value
Total Group			
	Body Fat	-0.2	0.137
	BMI	-0.38	0.256
	BMD	0.24	0.686
	VO_{2max}	0.39	0.089
Males			
	Body Fat	0.107	0.409
	BMI	-0.166	0.508
	BMD	0.269	0.269
	VO_{2max}	0.18	0.180
Females			
	Body Fat	**0.128**	**0.022***
	BMI	**-0.431**	**0.047***
	BMD	0.394	0.515
	VO_{2max}	**-0.085**	**0.042***

*% BF, BMI, and VO_{2max} were significantly correlated to GDM in Females.

Table 3.A t-test results for the whole group							
	Median			Group	Methylation (%)		p value
		Mean	SD		Mean	SD	
BF%	24.10%	32.50%	1.40%	High	1.4	1.4	0.13
		17.60%	4.50%	Low	1.9	1.2	
BMI	23.4	27.4	3.9	High	1.5	1.1	0.29
		21	1.4	Low	1.9	1.4	
BMD	1.209 (g/cm^2)	1.33	0.08	High	1.6	1.2	0.58
		1.15	0.05	Low	1.8	1.4	
VO_{2max}	50.1 (ml/kg/min)	58.4	6.4	High	2.1	1.4	0.07
		41.9	6.1	Low	1.4	1.2	

Table 3.B t-test results for the male group							
	Median			Group	Methylation (%)		p value
		Mean	SD		Mean	SD	
BF%	26.10%	35.20%	6.40%	High	1.2	0.8	**0.007***
		22.10%	2.80%	Low	2.6	1.5	
BMI	21.8	24.4	1.7	High	1.3	1	**0.028***
		20.5	1.1	Low	2.5	1.5	
BMD	158 (g/cm	1.21	0.05	High	2.1	1.6	0.54
		1.11	0.04	Low	1.7	1.2	
VO_{2max}	.9 (ml/kg/n	53.4	7	High	2.4	1.5	0.14
		39.5	6.4	Low	1.6	1.1	
*The % BF has significant differences in % GDM between the high and the low groups							
* The BMI has significant differences in % GDM between the high and the low groups							

Table 3.C t-test results for the female group							
	Median			Group	Methylation (%)		p value
		Mean	SD		Mean	SD	
BF%	18.70%	28.70%	6.60%	High	1.5	1.3	0.78
		14.20%	3.60%	Low	1.3	1.1	
BMI	25.2	29.7	4.2	High	1.73	1.27	0.15
		23.4	1	Low	1.06	1.07	
BMD	1.326 (g/cm^2)	1.4	0.05	High	1.5	1.3	0.8
		1.24	0.05	Low	1.3	1.1	
VO_{2max}	53.7 (ml/kg/min)	61.8	5	High	1.6	1.1	0.76
		46.4	6.2	Low	1.4	1.4	

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