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Patterns of DNA methylation across the Leptin core promoter in four diverse Asian and North American populations.

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ABSTRACT

DNA methylation is the most widely studied of epigenetic mechanisms, with environmental effects recorded through patterned attachments of methyl groups along the DNA which are capable of modifying gene expression without altering the DNA sequencing. The degree to which these patterns of DNA methylation are heritable, the expected range of normality across populations, and the phenotypic relevance of pattern variation remain unclear. Genes regulating metabolic pathways appear to be vulnerable to ongoing nutritional programming over the life course, as dietary nutrients are significant environmental determinants of DNA methylation, supplying both the methyl groups and energy to generate the methylation process.

Here we examine methylation patterns along a region of the metabolic gene leptin (*LEP*). *LEP*'s putative function includes regulation of energy homeostasis, with its signals affecting energy intake and expenditure, adipogenesis and energy storage, lipid and glucose metabolism, bone metabolism and

reproductive endocrine function. A pattern of differential methylation across CpG sites of the *LEP* core promoter has been previously identified; however, any consistency of pattern or its phenotypic significance is not fully elucidated among populations. Using DNA extracted from unfractionated white blood cells of peripheral blood samples, our pilot study, divided into two parts, examines the significance of variation in DNA methylation patterns along the leptin core promoter in four populations and uses biomarkers reflecting leptin's functional process in two of those populations to investigate the relevance of the ethnic variation identified in the DNA methylation. Those two populations are the Western Buryats of Siberia and the Mennonites of Central Kansas.

LEP's core promoter region contains both the C/EBP α transcription binding site (TBS) which tempers the final step in adipocyte maturity and capacity to synthesize leptin and the TATA motif controlling leptin synthesis. Previous studies report that increased methylation in this region is correlated to decreased gene expression, with others suggesting tissue-specific methylation variation at this region. We hypothesize that evidence of nutritional epigenetic programming would be identified through variation in patterns of DNA methylation and that functional relevance of that variation among populations would be identified through biomarkers which reflect leptin's metabolic signals: serum leptin levels, lipoproteins of the lipid transport system and anthropometric measures.

A distinct and consistent overall pattern of differential DNA methylation across seven CpG sites of *LEP* core promoter is documented in all ethnicities and both sexes in our combined analyses of 313 individuals. This pattern replicates those identified in previous studies, thus suggesting a conserved core promoter region across populations. The second analyses in two of the four populations (n=239), examines the correlation of methylation at the C/EBP α TBS site with metabolic and anthropometric biomarkers reflecting *LEP* roles. Stature, which reflects bone growth and remodeling, is significantly and inversely correlated with the percentage of DNA methylation at this site in both sexes. We suggest that

variation in DNA methylation along the *LEP* core promoter plays a substantial role in energy signals affecting both adipogenesis and bone metabolism.

INTRODUCTION:

Dietary intake and its nutrient content is a dynamic environmental factor influencing human gene expression and physiological variation, growth and development, and ultimately adaptation and evolution. Nutritional phenotypes, in part represented by the sum accumulation of gene- by- environment interactions, reflect both parental and the individual's own responses to the environment. These phenotypes are determined through biomarkers of metabolism, adiposity, hormone signals, and genotypes (Mosher 2012; Zeisel et al, 2005). Epigenetics are now believed to archive environmental alternations of gene expression, however, little is known about the effects of variation in epigenetic mechanisms on metabolic biomarkers in normal individuals among diverse populations.

Current epigenetic research emphasizes the mechanisms of DNA methylation, histone modification, and noncoding RNA as an intricate system of ongoing intra and intercellular signaling and feedback loops required to maintain a stable pattern of cell differentiation, human growth and development during increasingly complex environmental challenges (Jablonka and Lamm, 2012; Ptashne, 2013). DNA methylation is the most widely studied of these mechanisms. It is interpreted as a method by which effects from environmental factors are registered along the DNA and appear to modify gene expression without altering the DNA sequences. This implies a mediating level of adaptation on gene expression which influences developmental plasticity and phenotypic variation by more rapid, flexible and even reversible means than the effects of natural selection on DNA sequences (Duncan et al, 2014; Donohue, 2013; Gluckman and Hanson, 2007). The process of methylation begins *in utero*, and its regulation relies heavily on the availability of folate, the one-carbon metabolic pathway and energy supply (Crider et al, 2012; Stover, 2009; Hamid et al, 2009). The stability of epigenetic marks across

generations in humans is unclear, yet appears to be related to their specific role in managing the integrity of the genome and epigenome (Godfrey et al, 2015). Although vulnerable windows during fetal development and early neonatal life are recognized as the primary opportunities for environmental effects on DNA methylation variation (Baie, 2015; Bartolomei and Ferguson-Smith, 2015; Weaver et al, 2009), patterns of methylation continue under dietary influence throughout a lifetime (Waterland and Jirtle, 2004) and are therefore potentially reversible (Vickers, 2014; Sebert et al, 2011; Langley-Evans 2009).

Increasing literature documents the importance of metabolic changes driven by novel human dietary choices. Studies now indicate variation in gene regulation between humans and non-human primates (Blekhman et al, 2014), and identify positive selection in humans for distinct gene promoter regions in glucose metabolism (Haygood et al, 2007) due to the high energy demands of the increased brain function. Hancock et al. (2010) examines genetic variation of adaptive phenotypes in a variety of historically distinct dietary environments, and identifies folate biosynthesis pathways as one distinct area exhibiting allelic variation between populations. The influence on epigenetic mechanisms in these instances, however, is not known. We question whether epigenetic mechanisms also archive adaptive responses to ancestral diets in a recognizable and heritable pattern in humans. The candidate gene we select to examine is leptin, which produces a protein signal of the cytokine family (Frühbeck, 2006) and is a primary hormonal signal in a complex neural circuitry mediating energy homeostasis (Friedman and Halaas, 1998). Stöger (2006) identifies the leptin core promoter to be differentially methylated and hypothesizes that this variation might also be tissue-specific. Heijmans et al (2008) study of the Dutch Hunger Winter demonstrates in humans that methylation of the leptin gene is subject to nutritional programming during fetal development and carries sex-specific long-term effects on offspring health. Yajnik (2014) shows a nutritional programming in genes modulating metabolic responses during the life course, including leptin. We examine the relevance of variation in DNA methylation across the leptin's

(*LEP*) core promoter in diverse populations, each with a shared ancestral and environmental history, yet from dissimilar ethnicities, climates, lifestyles and nutritional intake, all of which influence energy balance. We originally hypothesize that significant population differences in patterns of DNA methylation will be evident along the seven CpG sites of the *LEP* core promoter, which contains the C/EPB α TBS associated with a final step in adipogenesis for energy storage, and two sites proximal to the TATA motif which impact expression of the leptin signal (Mason et al, 1998).

We first reported variation in DNA methylation of the *LEP* core promoter in four populations (n=88); maternal/ offspring dyads from three immigrant populations of Mexican, Ethiopian, and Vietnamese (Mosher and Schanfield, 2013a) and a non-immigrant reference group of Colorado residents reporting northern European ancestry on the last analyses (Mosher and Schanfield, 2015b). The immigrant populations include the mothers who originated in their country of origin and the offspring, reportedly born in the United States. While exact ages were not specified, most of the children were infants and toddlers at the time of DNA testing. Analyses of this maternal/offspring data suggest the existence of both sex-specific variation and generational differences in the DNA methylation of the leptin core promoter (Mosher and Schanfield, 2013a). These immigrant populations experience rapid and dramatic changes which occur during modern migration. Such precipitous modifications create dietary change derived from abrupt changes in food chain and simulate dietary insults from feast/famine fluctuations and even seasonality in food availability. However, despite the statistically significant variation in degree of DNA methylation at several sites (Mosher et al, 2013), the overall pattern across the seven sites in all the populations remains remarkably stable (Mosher and Schanfield, 2013a; and 2015c). We then added paternal data to accompany the maternal/offspring dyads in the Vietnamese and US reference groups. The investigation of those parental/offspring trios of Vietnamese immigrants and a reference group of non-immigrant parental/offspring of Northern European ancestry shows similar outcome in the overall pattern of DNA methylation (Mosher and Schanfield, 2015b)

despite their dissimilar ethnic and environmental backgrounds. This study was expanded the study to include two stable populations with wider age range of participants, whose ancestors remained in their environment over several generations. We hypothesize that the distinct patterns of methylation across the promoter region will reflect adaptation to energy supplies which may be interpreted through biomarkers reflecting leptin's physiological roles. The first phase of this present study compares DNA methylation of the leptin core promoter from the parental/offspring trio data of the Vietnamese and Colorado populations with data obtained from population studies of the Siberian Buryat Western Buryat and the Kansas Mennonite. The second phase examines biomarkers of plasma leptin, lipids, and anthropometrics with DNA methylation patterns to identify phenotypic relevance in the Buryat and the Mennonite.

STUDY POPULATIONS:

Pilot Phase 1: Vietnamese and Colorado Reference Group of Northern European Ancestry

DNA, extracted from unfractionated leukocytes of peripheral blood, from two ethnicities of fifteen family trios (n=89) was obtained from George Washington University, Forensic Sciences Laboratory. The samples consist of trios of Vietnamese immigrant families (parents born in their country of origin and offspring, mostly toddlers and younger, believed to be born in the US or immigrated to the US soon after birth. The US reference group consists of fifteen trios of northern European ancestry from Colorado, with offspring mostly of similar age to the Vietnamese offspring. These data, collected in between 1995 and 2003, came with no biomarkers beyond DNA. Little is known regarding the individuals' phenotypes of the original populations, as the DNA is accessed through the paternity studies completed on immigrant and non-immigrant families. Under these circumstances, no identifiers other than reported ethnicity by the mothers, DNA matches to the fathers, generation and

sex is available. DNA methylation percentages along the seven CpG sites of both populations are listed in **Table 1**. The use of these data was cleared by IRBs at both George Washington University and Western Washington University.

Pilot Phase 2: Buryat and Mennonite

Phase 2 of the pilots examines the DNA methylation profiles of two additional populations, the Siberian Western Buryat and the central Kansas Mennonite. These two populations previously participated in extensive studies through the Lab of Biological Anthropology at the University of Kansas. The studies examine genes, energetics and nutrition and result in similar phenotypic data of fasting plasma leptin and lipid profiles, apolipoproteins, anthropometrics and dietary profiles. Therefore, to examine the significance of variation in DNA methylation, we compare DNA methylation profiles and biomarkers of these two diverse populations who have not experienced recent migration. Revised IRB approval was obtained from Western Washington University for this study of the leptin core promoter in both populations.

The Western Buryat peoples, an admixed group of Mongol and Siberian populations formed in the 13th Century, are members of the largest indigenous group in Siberia. No longer transhumant, they now live west of Lake Baikal in the national district of Ust'—Ordynsky where they practice sheep and cattle herding, along with some agriculture. Some foods, including flour, juices, alcohol, and hard candy, are trucked in from Irkutsk. The temperature range of this region is reported from -27⁰ C in winter to summers above 30⁰ C (Mosher, 2002). We obtained data of 77 participants from this Siberian study, ranging in ages from 15 to 74 yrs. Genetic studies identify Buryat exhibiting a lower level of population differentiation than many of the Siberian indigenous populations, although the Ust Orda population had a moderate level of heterozygosity suggesting some gene flow into the area (Novoradovsky et al, 1993).

DNA samples in this study came from Gakhani, an isolated village within the Okrug. This population experiences high rates of poverty, and the severe economic hardship suggests an out-migration in that village due to the lack of employment. There is a paucity of medical and health records in this area, however the primary health risk is reported to be essential hypertension. Their population mean in plasma lipid profiles are some of the lowest population levels reported, while accounts of morbidity from cardiovascular disease is approximately 28%. (Spitsyn, 2001, personal communication reported in Mosher, 2002).

The central Kansas Mennonite are well-defined Anabaptist group originating in the 1600s of Dutch, Swiss, and northern German populations. Their common migration history brought them through the Ukraine, and finally to central Kansas in the United States in 1874, where they now practice an agricultural lifestyle (Crawford, 2000). The sample size for this study is 152, with ages ranging from 20 – 94. A genetics of biological aging study began in 1979 at the University of Kansas (KU). The Mennonite report longevity 8-10 years above their Kansas neighbors (Crawford, 2000), with mortality trends on the 1980 study population published by Melton et al, 2006. In 2003-2005, researchers from KU revisited the population to complete a more in-depth nutrition study, the Kansas Nutrition Project (KNP) and collect blood samples for DNA plasma levels of leptin and lipids, along with anthropometrics. Mean plasma lipid levels of this Mennonite population, previously found to be slightly higher than the average United States levels in the 1980s (Crawford, 2000), now approximate the mean of the United States population (Mosher, 2004). Demarchi et al (2005) suggest the possibility of a selection pressures on a polymorphism in apolipoprotein B (Xbal). This apolipoprotein is the primary protein found in low density lipoprotein, the lipoprotein associated with high delivery of cholesterol around the system. Mennonite frequency of this polymorphism is 0.752 in our sample, while only 0.182 in the Buryat sample (Demarchi et al, 2005; Mosher, 2002). The Xbal polymorphism is recently documented as not

associated with increased heart risks in some European populations (Li, 2014). **Table 2** provides the descriptors and statistical comparison between each sex in both populations.

LABORATORY ANALYSES:

Phenotypes:

Blood samples obtained in the Buryat Study (1998) and the Mennonite Study (2003-2005) for plasma leptin and lipid levels were fasting (8-12 hours) draws, acquired by antecubital venipuncture using EDTA vacutainers. The leptin levels of both populations were analyzed at the Southwest Foundation of Biomedical Research (now the Texas Biomedical Research Institute). Plasma leptin levels were determined by radioimmunoassay (RIA) in 1998 and Luminex (2003-2005), both supplied by Linco Research, Inc. Coefficient of variation (CV) in RIA was reported as 7.2% (Rainwater et al, 1997). Significant differences between the two populations have been identified, with the Siberian Buryat exhibiting lower levels of plasma leptin and plasma lipids than the Mennonite.

Lipid panels, including TC (total cholesterol), HDL (high density lipoprotein), and TG (triglycerides) for the Buryat were determined at the University of Kansas Medical Center using EKTACHEM system, with high density lipoprotein (HDL) measured after precipitation of B-lipoproteins via dextran and magnesium (Leonard et al, 1994). Triglycerides were measured after phospholipids were removed. Both VLDL and LDL were determined through the Friedwald's equation: $LDL = TC - HDL - TG/5$. $VLDL = TG/5$ (Rifai et al, 2008). Texas Biomedical Research Institute measured lipids for the Mennonite and determined all by their laboratory protocols (Mahaney et al, 2003). This included enzyme assay by Gilford SBA-300 technology and using Boehringer-Mannheim Diagnostics reagents for TC and Stanbio for TG. Procedure for HDL in Mennonite was similar to the Buryat. During these studies, both facilities were subject to the National Cholesterol Education Program guidelines for lipid analyses, with an

expected CV of 3% from the reference controls in TC (Caudill et al, 1998) and a 3.2% for TG and 5.6% for HDL (Mahaney et al, 2003). As all original samples of both populations were collected in EDTA tubes, all results reported as plasma levels.

Dietary data were collected through prompted 24 hour- dietary recall by Russian translator with the Buryat and then entered in Food Processor software, updated for data of Russian/Siberian foods acquired from WHO (Moshier, 2002). That information restricted this study to macronutrient energy intake only. The Mennonite study collected dietary intake diaries from three non-consecutive days. The dietary diaries were then entered into NutriBase Software™ (CyberSoft, Inc, Phoenix) for nutrient profiles. Both programs use USDA data, and subject to Atwater System for energy calculations (Merrill and Watt, 1973). Percent of macronutrient intake was calculated using the modified 4,4,9 ratio of K/cal per gram of macronutrient (carbohydrate, protein and fat respectively), with the total k/cal intake of each macronutrient divided by the total caloric intake and multiplied by 100.

Epigenotypes

The Leptin (*LEP*) gene, located on chromosome 7q31.3 is the candidate gene for this pilot study, due to its putative role as a mediator of energy homeostasis. We obtained DNA previously extracted from unfractionated leukocytes of peripheral blood of both populations and stored in freezers at the Laboratory of Biological Anthropology, University of Kansas. (DNA extraction protocol documented in Melton et al, 2010). Working with Reinhard Stöger of Stöger Labs University of Nottingham, UK, who had completed previous leptin studies in both mice and humans (Stöger, 2006), seven CpG sites of the leptin (*LEP*) core promoter were selected. These sites include one which is located in the C/EBPα transcription binding site(TBS) at Site 4 and two proximal to the TATA motif, at Site 6 and 7 (Figure 1). Analyses of the methylation patterns of the *LEP* gene were completed at the Functional Genomics Lab at the University of Washington. Using Qiagen protocols, DNA bisulfite treatment converted unmethylated

cytosines to uracil. Genomic DNA (250-300ng) was bisulfite treated, using EpiTect Fast DNA Bisulfite Kit (Qiagen, Valencia, CA). Following clean-up of the converted product, DNA was brought up to a concentration of 10ng/μl and 20ng of converted DNA was run in the Pyromark PCR reaction as per manufacturer's protocol (Qiagen). After visual inspection of the product by agarose gel, 8-10μl of the PCR product was analyzed using the Pyromark Q24 (Qiagen). Those samples which passed the quality control built into the Qiagen system (Qiagen Resources), were used. Nineteen samples were competed as duplicates at all 7 sites and the results were statistically indistinguishable. Duplicate sample means with (SD) at Site 4 for both sets were 53.19 (14.79) and 53.21(14.21), with a t-test $P = 0.998$.

Polymerase chain reaction (PCR) amplification produced the one amplicon, using forward PCR Primer: **GTGTATATTGAGGGTTAGGGTTAGTAG** and Biotinylated Reverse Primer **GGAGGGAGTTGGAGTTAGAAAT**. Pyrosequencing was completed using the "PyroMark Q24" system. This pyrosequencing method, utilizes an enzyme cascade to release pyrophosphate (PPi) and converts it to light, producing the pyrogram denoting the percentage of DNA methylation, the degree, identified in the region of the cytosine of CpG site to adjacent thiamine (Qiagen, 2013). This approach provides accurate, reproducible and cost-effective quantification of cytosine-methylation levels at a given locus, such as the *LEP* promoter, and variation in DNA methylation of CpG islands lying close together has been identified in differential expression of a gene (Tost and Gut, 2007).

STATISTICAL ANALYSES:

The percent of DNA methylation for each CpG site was determined by the percentage of methylation identified in the region of the cytosine of the CpG site to the adjacent thiamine, per Qiagen PyromarkQ24 (2013). Mean differences in DNA methylation, diet and phenotypic traits were determined by Mann Whitney U and ANOVA using SPSS 23. Plasma TG, leptin levels, and stature were natural log transformed (per outcome of Kolmogorov-Smirnov Z). Analyses of traits with DNA

methylation, uses a partial Pearson's correlation coefficients adjusted for batch, age, and body mass index similar to the approach by Houde et al, (2015). A correlation of all individuals was completed, adjusting for batch, age and BMI, followed by a sex-stratified approach to acknowledge sexual dimorphism widely reported in both adiposity, lipid profiles and plasma leptin (Williams et al, 2015; Fuente-Martin et al, 2013, Guerra et al, 2008; Martin et al, 2002; Rosenbaum et al, 2001, Power and Schulkin, 2008; Wang et al, 2011).

Simultaneous regression analyses were completed on sex-stratified data to substantiate Site 4 DNA methylation variation significance with stature, using two models: one without total caloric intake and one with total caloric intake. Adding total caloric data reduced the sample size by 11 males and 20 females, however their ages, plasma leptin levels, and Site 4 DNA methylation remained within the 95% CI of the mean for the main sample. Significance in this study is set at $p < 0.001$. We describe variables with significance < 0.05 but not < 0.001 as trending).

RESULTS:

Phase 1 Results:

The first group contains two sets of parental/offspring trios, one an immigrant population from SE Asia (Vietnam), and one non-immigrant reference group of Colorado residents of Northern European ancestry. The mean percentage of DNA methylation in each of the seven CpG sites and total methylation of the *LEP* core promoter in both ethnicities is shown in **Table 1**. The Trio data carry only descriptors of ethnicity, generation, and sex and the primary analyses examined these three factors only. Using Mann –Whitney U, sex and ethnicity were not significant in DNA methylation at any of the seven CpG sites on sex-stratified data, Pearson correlations, however, sites 4-6 significantly differ between generations with parental levels of DNA methylation greater than offspring. Site 4, the C/EBP α TBS, is most pronounced, and after sex-stratifying data, no methylation difference between

fathers and sons is significant ($r = -0.073$, $p=0.646$). However, between mothers and daughters, the correlation coefficient was $r = -0.589/p<0.001$, with daughters showing lower percentage of methylation in both ethnicities. In **Figure 2**, total DNA methylation across the seven CpG sites of the leptin core promoter did not differ significantly among fathers, mothers and sons, however, daughters of both ethnicities exhibited the lowest total methylation ($-0.609/p<0.001$). Offspring of both Asian and Colorado reference group have a greater variance in site 4 than parents, yet differences remained significant only in the females. These findings are reported elsewhere (Mosher and Schanfield, 2015a).

Analyzing all four of the populations, variation in DNA methylation at site 4, but not total DNA methylation, was significantly different between Buryat, Mennonite and SE Asian males ($p=0.001$) and Buryat and SE Asian females ($p=0.005$).

Phase 2 Results:

Due to the lack of biomarkers available with the Trio data, the second phase pilot examines only the Buryat and Mennonite samples, which are accompanied by both biological and anthropometric measures. (see **Table 2**). The mean age of Buryat is considerably less than the Mennonite in both males and females. Mennonite males and females were taller and heavier than Buryat, however, BMI was significantly different in males only. Plasma leptin levels differ, with females having greater levels than males and the Mennonite levels greater than Buryat levels. Plasma lipid profiles in this study included total cholesterol (TC), low density lipoprotein (LDL), triglycerides (TG) and high density lipoproteins (HDL). Plasma lipid levels differ by ethnicity but not by sex in both TG and LDL levels, HDL differed by both sex and by ethnicity. HDL is inversely correlated to site 4 degree of DNA methylation after adjusting for ln BMI ($r = -0.287/p<0.001$), with greater levels of HDL correlating with decreased percentage of DNA methylation in females only.

Ethnic differences in degree of DNA methylation >5% are evident only at Site 4 appears in both males and females. Because of the biological significance of Site 4, and the significant ethnic variation documented in these pilot populations, we report the phenotype- by- epigenotype results only with Site 4 and the total DNA methylation for the seven sites. Using the combined data for all participants, and after adjusting for batch, age, and BMI, Site 4 was inversely correlated to stature ($r = -0.309/p < 0.001$) and trended inversely with plasma leptin levels ($r = -0.146/p = 0.031$). The total DNA methylation across these seven sites was similar, showing negative correlation with stature ($r = -0.245/p < 0.001$) and trending inversely with plasma leptin ($r = -0.122/p = 0.071$). After sex-stratification of data, and adjusting for age and body mass index (and batch), Site 4 DNA methylation percentage is inversely correlated with stature ($r = 0.278/p = 0.009$) and trending inversely with plasma leptin ($r = -0.229/p = 0.032$) in males. In females, site 4 correlated inversely stature ($r = -0.354/p < 0.001$), with plasma leptin ($r = -0.268/p = 0.002$), and with HDL ($r = -0.265/p = 0.003$). Stature correlates with plasma leptin levels in both males and females ($r = 0.323$ and $r = 0.319$ respectively with $p < 0.002$). Neither LDL nor TG were significantly correlated with Site 4, or total methylation in either males or females. HDL correlates with stature in females only ($r = 0.259/p = 0.003$). Following identical procedures for total methylation of the seven sites, this factor trends negatively with both stature and plasma leptin in males ($r = -0.181$ and $r = -0.195$ /both NS slightly above 0.05) and negatively correlated with both stature and plasma leptin levels in females ($r = -0.354$ and $r = -0.268$ respectively/ $p < 0.002$). Total DNA methylation was not significantly correlated with lipid profiles in either sex, however.

A simultaneous regression analyses is completed on stature, with factors of age, BMI, plasma leptin, and methylation of at Site 4. When all four variables are included in the model, only leptin is significant (Beta $0.419/t = 3.306/p = 0.003$) with Site 4 degree of DNA methylation in males and trends inversely with stature (Beta $-0.235/t = -2.428/p = 0.017$). In females both leptin and Site 4 DNA methylation are significant (Beta $0.314/t = 2.855/p = 0.005$ and Beta $-0.304/t = -3.602/p < 0.001$ respectively). When the

additional factor of total calories and smoking is introduced to the model, reducing the sample size, in males' leptin (Beta .256/t2.002/p=0.049), and smoking negatively correlate with stature (Beta -.322/t-2.48a/p=0.016). In females, both total caloric intake and Site Four become significant with stature (Beta .319/t3.3400/p=0.001 for total caloric intake and Beta -.388/t—4.093/p<0.001 for Site 4 DNA methylation.). Nineteen Buryat males and eight Buryat females report smoking, while only 1 Mennonite male and 3 Mennonite females report smoking.

DISCUSSION:

This is a pilot study to determine expected normal percentages of DNA methylation across seven CpG sites of the *Lep* core promoter among human populations from different continents and diverse food chains. Additionally, it examines phenotypic relevance of significant variation in DNA methylation at specific CpG sites. Leptin's core promoter region is identified as differentially methylated (Stöger, 2006). It contains both the C/EBPα TBS (at Site 4) and the TATA motif (Site 7), key promoter elements minimally required for initiating transcription of its protein signal leptin. (Kadonaga JT (2012).

Here we analyze parental/offspring trios of both Vietnamese immigrants and a US reference group of nonimmigrants reporting northern European ancestry and two semi-isolate groups who experience vastly different life styles: the Siberia Buryat, a mixed ancestry of Mongolian tribes and Siberian indigenous groups, and central Kansas Mennonite of Swiss, German, and Dutch ancestry. We replicated an overall pattern of DNA methylation variation previously identified in this region, with C/EBPαTBS consistently showing the higher percentage of methylation than adjacent CpG sites (Marchi et al, 2011) The pattern is similar to a Canadian study which reports a similar pattern of methylation variation across this region in samples of blood and two fatty tissues, subcutaneous and visceral. The authors report similar overall pattern, but a lower degree of DNA methylation in adipose tissue than blood (Houde et al, 2015). These findings support the hypothesis that conservation of methylation

patterns across core promoters of primary regulatory regions would be expected among diverse populations (Jiang et al, 2014). Additionally, these findings indicate that the heterogeneity of leukocytes across populations does not result in dramatic variation of methylation findings, as our Site 4 expressed only 5- 6 percent variation, the greatest percent of variation in among all the sites. The constancy of this epigenetic marker has been shown to be relatively stable across a short time frame in a variety of candidate regions. Leptin resembles the construction of some cytokines including IL-6, shown to have a minimal intra-individual change in methylation after adjusting for percent of blood granulocytes, age, and smoking (Byun et al, 2012).

We also identify that DNA methylation variation at Site 4 the C/EBP α TBS variation correlates with height (stature) in both males and females. Here we interpret stature as a surrogate measure representing both developmental growth and bone remodeling. Such findings reflect a more extensive function of Site 4 beyond the accepted action on the maturation of adipocytes for energy storage, as bone growth and remodeling are substantial energy-consuming processes. Leptin's putative role is that of regulating energy homeostasis, which balances energy intake, storage and expenditure through both peripheral and central signaling managed through a group of leptin receptors, extensive hormonal signaling cascades, and neuronal control (reviewed in Allison and Myers, 2014; Ferron et al, 2013; Karsenty and Oury, 2012; Scheller et al, 2010; Mitchell et al, 2009; Frühbeck, 2006). Central signaling is accomplished through the hypothalamus and brain stem neural pathways, while peripheral signaling appears affected by nutritional status in human blood. Leptin circulates in two states, free and bound to a soluble receptor. The receptor levels are determined by sex hormones, adiposity and leptin itself, as well as a circadian influence (Chan et al, 2002). Free leptin reflects body fat mass, while bound leptin appears to have a different regulatory role in energy balance (Brabant et al, 2000). Females exhibit higher free leptin and lower leptin binding protein than males (McConway et al, 2000). Here, the sexual dimorphism of fat depots and muscle mass cannot be ignored. Determining bound versus free leptin

was not possible in this study. However, our findings can be viewed through an integrative and thrifty physiological model of energy mediation responsive to environmental constraints of energy availability and the individual energy demands related age, sex, and growth and development.

The findings in this pilot study support the hypothesis that the leptin core promoter is a region reflecting a conserved pattern of DNA methylation which produces pleiotropic effects (Faggioni et al, 2001). The ultimate fate of multipotent mesenchymal stem cells first located in bone marrow are potentially adipogenic, osteogenic, and even myogenic (Mo et al, 2016), and contain highly functional leptin receptors (Hess et al, 2005). HDL, traditionally inversely linked to adipose tissue and BMI, is also shown to correlate with non-adipose components of the body such as skeletal mass (Pietrobelli et al, 1999.). An animal study reports that HDL promotes proliferation of mesenchymal stems cells derived from bone (Xu et al, 2012). In this study, HDL correlated with stature in females only. C/EBP α is documented in animal studies to control lineage commitment of osteoclasts (Chen et al 2013). The inverse correlation of DNA methylation variation with stature in both males and females at Site 4 suggests that leptin plays a role in the activation of bone remodeling, a very energy consuming process. Recent research now supports the concept that bone is also an endocrine organ similar to adipose tissue and plays an active role in energy homeostasis (Karsenty and Oury, 2012), specifically through a reciprocal hormonal relationship of osteocalcin and leptin regulating insulin and glucose homeostasis (Kanazawa, 2015; Schwetz et al, 2012; Ng, 2011; Ferron et al, 2010; Kim et al, 2010; Hinoi et al, 2010; Lee and Karsenty, 2008;). Further study identifies the negative effect of leptin on osteocalcin, which is believed to inhibit insulin and reduce visceral fat (Ferron et al, 2013). The precise relationship plasma leptin effects on bone remodeling may in fact be dose -dependent and bimodal, producing opposite effects of stimulation and suppression dependent upon its levels (Kim et al, 2010; Motyl and Rosen 2012). Effects are also suggested to be sex-specific (Dalskov et al, 2015) Much work is needed with

studies designed to address the intricacies of this relationship in humans from diverse environments and ancestries.

While stature is believed an example of a classic polygenic trait with high heritability, a recent GWAS study on the genetics underlying this trait reports a low percentage (~3-5%) of effect from most of the common variants (Weeden and Frayling, 2008) leaving some authors to recommend the study of diverse ethnicities and redefine the phenotype to that of height velocity (Lettre, 2011). Recent findings correlate variation in lipid profiles and bone loss, suggesting that these two functions are mutually constrained in some way, however, studies are inconclusive with some authors pointing at a paucity of studies examining ethnic variation (Tian L, Yu X, 2015). We have identified here the ethnic variation degree of DNA methylation and in plasma lipid profiles, and report that HDL in females only appears related to stature. Further research to determine the lipoprotein role, specifically that of triglyceride transport, is vital.

The interrelationship of these variables is not fully elucidated in humans, yet results of this study suggests their connection is through a system of energy regulation and adaptive tradeoffs. Cell signaling is well beyond the scope of this pilot study. As all data were obtained through past studies not specifically designed for our question, we are subject to the constraints of models and limitations of past technology. Confounding variables which could not be evaluated in this study include white blood cell heterogeneity which may affect methylation measures (Aldalsteinsson et al, 2012), a more precise evaluation of smoking history as well as the effects of maternal smoking (Yousefi et al, 2013), and most importantly, the role of DNA methylation of the leptin receptors. Additionally, obtaining accurate assessments of dietary energy intake using the dietary recall method would benefit from repeated measures, as studies find an improvement of total energy reporting over several time-frames (Yunsheng et al, 2009) or use of an active dietary diary, such as used in the Mennonite study, but over a longer time (Willett, 1990).

DNA methylation is a complex process, subject to both genetic and environmental influences, and future studies examining the relevance of methylation variation will need to be addressed using specific models which include genetic architecture underlying the relevant cell signaling of the specific metabolic pathways to be examined. Research in human epigenetic mechanisms is subject to many of the limitations of early genetic studies, including small sample sizes, complexities of tissue-specific variation in expression, and discrepancies between conclusions of epigenome-wide studies and findings of individual candidate regions (Heijmans and Mills, 2012). The results in this study are subject to the smaller samples, yet they also provide a window to focus further research into the regulation of integrated physiological signals providing an adaptation to energy availability in diverse populations.

CONCLUSION:

This study identifies a conserved differentiated pattern of DNA methylation across seven CpG sites of the *LEP* core promoter among populations varying in age, sex, continental origin, ethnic ancestry and diets. This pattern replicates other studies in both humans and animals, supporting a hypothesis that this DNA methylation pattern is conserved to mediate energy effects across systems. Further research must include an integrated approach of both human physiological systems and the environmental factors which impact individual response and population adaptation. Distinguishing epigenetics through an adaptive model offers the framework through which to explain the epigenotype to phenotype relationship. Energy allocation in developmental physiology, their metabolic effects and ultimate phenotypic tradeoffs affecting longevity are primary examples of this concept (Symonds et al, 2009). Our understanding of energy regulation is becoming increasingly complex, as is our technological ability to unravel its architecture.

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TABLE 1: DNA methylation of seven CpG sites of SE Asian immigrants and US reference group of Northern European ancestry.

SE Asian M	NE M	CpG Site	SE Asian Fe	NE Fe
14.02(2.03)	12.65(1.44)	Site 1	12.24(1.39)	12.11(1.12)
11.32(1.71)	11.56(0.96)	Site 2	11.31(1.54)	9.77(1.19)
15.35(1.42)	15.80(1.30)	Site 3	14.07(2.01)	13.80(1.15)
52.39(2.51) *	57.84(2.06) *	Site 4	50.87(4.17)	54.44(2.67)
29.06(1.92)	24.48(1.87)	Site 5	26.02(3.07)	23.95(1.60)
28.57(1.96)	29.00(1.93)	Site 6	26.36(2.90)	34.95(2.09)
33.39(11.06)	39.58(1.93)	Site 7	34.95(2.92)	34.37(1.99)
184.11(10.22)	190.92(9.28)	Total Methyl	175.81(17.00)	173.83(9.82)

TABLE 1 Mean (SD) descriptors of S E Asian immigrant (n= 44) and non-immigrant reference group of northern European Ancestry (n=45). Using Mann-Whitney U, no site was significantly different between ethnicities. Site 4 (C/EBPαTBS) >5% ethnic differences and also showed significant differences in sex-by-generation (p=0.002), with differences between mothers and daughters similar to pattern of total DNA methylation differences in **Figure 1**. (Mosher and Schanfield, 2015b).

TABLE 2: Descriptors of Male and Female Buryat and Mennonite samples

Buryat M	Meno M	Trait	Buryat Fe	Meno Fe
33.0(13) *	49.0(16) *	AGE	32.0(14) *	57.0(16) *
23.7(4.5) *	26.7(3.5) *	BMI	24.6(5.9)	25.1(4.2)
168.63(5.78) *	178.88(6.38) *	HT (cm)	159.22(18.54) *	165.56(7.66)*
67.0(14) *	86.0(13) *	WT (kg)	61.0(15)*	68.0(12)*
2.54(3) *	6.4(4.3) *	LEPTIN (ng/ml)	7.3(4.8) *	16.0(8)*
87.98(36.57) *	111.62(29.86) *	LDL(mg/dl)	78.71(25.25) *	111.73(38.01) *
91.0(48)	120.0(70)	TG(mg/dl)	81.0(35)*	120.0(71)*
38.75(11.33)	43.94(14.60)	HDL(mg/dl)	38.87(17.34) *	57.16 (15.39) *

DNA METHYLATION

		CpG Site		
14.91(4.12)	12.54(3.34)	Site 1	15.66(3.52)*	12.82(3.60)*
11.94(3.85)	10.99(3.37)	Site 2	13.02(3.73)	11.24(5.15)
15.06(4.32)	14.67(4.39)	Site 3	16.25(4.25)	15.01(4.25)
61.62(7.51) *	54.41(6.45) *	Site 4	61.65(7.78)*	55.42(7.99)*
28.81(6.54)	26.31(5.93)	Site 5	29.68(5.74)	27.15(9.73)
27.15(9.73)	27.20(5.93)	Site 6	30.86(5.90)	30.86(5.90)
38.02(7.83) *	34.28(6.35) *	Site 7	38.97(7.08) *	35.06(8.38) *
200.22(37.61) *	179.03(32.07) *	Total Methyl	195.98(34.76) *	185.29(44.88) *

TABLE 2 Mean (SD) descriptors of the 77 Buryat and 152 Mennonite participants. Data sex-stratified and analyzed for ethnic variation.

* P<0.005. Site 4 (C/EBPαTBS), in bold print, remains the only site with >5% differences in DNA methylation between ethnicities.

Figure 1:

FIGURE 1: LEP CpG Island with the 7 CpG sites identified. Site 4 documents the C/EBP α binding site and sites 6 and 7 proximal to the TATA motif.

FIGURE 2:

FIGURE 2: Mean DNA methylation across the seven CpG sites of the Leptin Core promoter in SE Asians (Vietnamese) and the US reference group. Daughters only exhibited statistically lower total DNA methylation in both ethnicities.

FIGURE 3:

FIGURE 3: Consistent overall pattern of DNA methylation along seven CpG sites in the leptin core promoter in males and females of four populations: 1-Buryat, 2-Mennonite, both stable populations, 4-SE Asia of immigrants, 5–Colorado northern European reference group. Reference bar set at overall mean of 26.72. This overall pattern replicates studies of this region, with the C/EBP α TBS (Site 4) showing the greater degree of DNA methylation across the seven CpG sites.

FIGURE 4: Global locations of the four populations of this study. Two, the Kansas Mennonite of Central Kansas and the reference group -- Colorado residents of Northern European ancestry -- are located by one circle within the United States. The SE Asians are reportedly Vietnamese, and the Western Buryat of Siberia are located approximately 65 kilometers north of Lake Baikal.

FIGURE 5a and 5b

FIGURES 5a and 5b: Figure 5a (males) and 5b (females) are scatter plots representing the inclusive model of age, BMI, total caloric intake, leptin and site 4 DNA methylation on stature. In this model, for males Site 4 has Beta $-.280/p=0.008$ with no other factors significant. For females, both total caloric intake and Site Four become significant (Beta $.294/t3.343/p=0.001$ for total caloric intake and Beta $-.281/t-3.242/p=0.002$ for Site 4 methylation). Both models show a significant R^2 change for the addition of DNA methylation of 0.072, representing a 7 percent change. (This model excludes the smoking variable.)