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Research Article

Epigenetic Changes in DNA Methylation with Weight Loss and Remission of Prediabetes in Obese, Prediabetic Subjects

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Abstract

Obesity is a disease that has reached epidemic levels over the past two decades. Prediabetes or impaired glucose tolerance (IGT) is often seen in conjunction with obesity and both are associated with increased incidence of various diseases. The purpose of this study was to determine if epigenetic changes occur in patients that convert from prediabetic to normal glucose tolerance (NGT) by determining DNA methylation at baseline and at remission of prediabetes at 6 months (mo).

24 obese, prediabetic adults were randomized to a HP or HC diet for 6 mo with all food provided. The HP diet consisted of 30% protein (P), 30% fat (F), 40% carbohydrates (C) and HC diet consisted of 15%P, 30%F, 55%C distributed by percent of daily kcals derived for each subject's resting metabolic rate. Oral Glucose Tolerance Test (OGTT) was performed at Baseline (BL) and 6 mo to determine IGT/NGT status. The HP diet group had 100% (12/12) conversion to NGT while the HC diet had 33% (4/12) conversion. Both diet groups had weight loss and improvement in insulin sensitivity determined by Matsuda Index [HP (BL 2.3 ± 0.3; 6 mo 6.5 ± 1.1)], [HC (2.3 ± 0.3; 6 mo 3.2 ± 0.4)]. DNA was extracted from OGTT 0-minute blood samples from BL and 6-mo visits. Specific site DNA methylation was determined using Illumina chips for Whole Genome Bisulfite Sequencing. Changes in DNA methylation of numerous genes were observed from BL to 6 mo with remission of prediabetes and weight loss. Some of these genes with altered DNA methylation include the KEGG pathways of: insulin signaling (11 genes), cardiovascular disease (15 genes), inflammation (18 genes), metabolic pathways (32 genes), and cancer pathways (31 associated genes). The changes in DNA methylation of the genes involved in insulin signaling, cardiovascular disease, and inflammation correlate with the improvements in insulin sensitivity, cardiovascular risk factors and inflammation observed in the subjects after 6 months on the diets with the HP diet being most effective with respect to DNA methylation changes and clinical parameters. The DNA methylation changes of specific genes may be important for reduction of certain disease states.

Keywords: *DNA Methylation; Epigenetic Changes; Cardiovascular Risk Factors; Inflammation; Insulin Sensitivity; Remission of Prediabetes; Oxidative Stress; Weight Loss; High Protein Diet; High Carbohydrate Diet*

Background

The expression of a person's genes doesn't just depend on the genes' DNA sequence. It is also affected by so-called epigenetic factors. Epigenetics involves genetic control by factors other than an individual's DNA sequence. Changes in these factors can play a critical role in disease. The external environment effects upon genes can influence disease, and some of these effects can be inherited in humans.

Epigenetic changes can switch genes on or off and determine which proteins are transcribed [1]. Epigenetics is involved in many normal cellular processes. For example, our cells all have the same DNA, but our bodies contain many different types of cells: muscle cells, neurons, liver cells, pancreatic cells, inflammatory cells, and others. Tissues and organs differ because they have certain sets of genes that are "turned on" or expressed, as well as other sets that are "turned off" or inhibited.

Epigenetic silencing is one way to turn genes off, and it can contribute to differential expression. Silencing might also explain, in part, why genetic twins are not phenotypically identical. In addition, epigenetics is important for X-chromosome inactivation in female mammals [2], which is necessary so that females do not have twice the number of X-chromosome gene products as males Thus, the significance of turning genes off via epigenetic changes is readily apparent.

Diet is one of the environmental factors in epigenetic change. The nutrients we extract from food enter metabolic pathways where they are manipulated, modified, and molded into molecules the body can use. One such pathway is responsible for making methyl groups - important epigenetic tags that silence genes. DNA methylation is a chemical process that adds a methyl group to DNA. It is highly specific and always happens in a region in which a cytosine nucleotide is located next to a guanine nucleotide that is linked by a phosphate called a CpG site [3]. Inserting methyl groups changes the appearance and structure of DNA, modifying a gene's interactions within a cell's nucleus that is needed for transcription. DNA methylation is used in some genes to differentiate which gene copy is inherited from the father and which gene copy is inherited from the mother.

Familiar nutrients like folic acid, B vitamins, and SAM-e (S-Adenosyl Methionine) are key components of this methyl-making pathway. Diets high in these methyl-donating nutrients can rapidly alter gene expression, especially during early development when the epigenome is first being established [4].

A mother's diet during pregnancy and the diet of an infant can affect the epigenome in ways that stick with the child into adulthood. Animal studies have shown that a diet with too little methyl-donating folate or choline before or just after birth causes certain regions of the genome to be under-methylated for life [5]. For adults too, a methyl- deficient diet leads to a decrease in DNA methylation, but the changes are generally reversible when methyl is added back to diet [6].

Experiments in mice show just how important a mother's diet is in shaping the epigenome of her offspring. Such an example is the gene called agouti. When a mouse's agouti gene is completely unmethylated, its coat is yellow and it is obese and prone to diabetes and cancer. When the agouti gene is methylated (as it is in normal mice), the coat color is brown and the mouse has a low disease risk. Fat, yellow mice and skinny brown mice are genetically identical. The fat yellow mice are different because they have an epigenetic "mutation." When researchers fed pregnant yellow mice a methyl-rich diet, most of her pups were brown and stayed healthy for life. These results show that the environment in the womb influences adult health. Therefore, our health is not only determined by what we eat, but also what our parents ate [7].

Chemicals that enter our bodies can also affect the epigenome. Bisphenol A (BPA) is a compound used to make polycarbonate plastic. It is in many consumer products, including water bottles and tin cans. Controversial reports questioning the safety of BPA came out in 2008, prompting some manufacturers to stop using the chemical [8]. Some studies showed that BPA caused reduction in the methylation of the agouti gene and when mothers were fed methyl-rich foods the offspring were more likely to be healthy.

The maternal nutrient supplementation had counteracted the negative effects of exposure [9,10].

Obesity is a disease that has reached epidemic levels over the course of the past two decades. Pre-diabetes or impaired glucose tolerance (IGT) is often seen in conjunction with obesity and both are associated with increased incidence of various diseases [11]. Prediabetes progresses to T2DM at ~ 10%/yr [12]. Weight loss has been demonstrated to prevent progression of pre-diabetes to T2DM [13].

Our studies have shown improvements in insulin sensitivity and cardiometabolic risk factors after 6 months on a moderately High Protein (HP) diet compared to a High Carbohydrate (HC) diet in obese non-diabetic women [14]. Our studies have also demonstrated greater remission of prediabetes and improvement in metabolic parameters, cardiovascular risk factor, inflammation and oxidative stress with a HP diet compared to a HC diet in prediabetic obese subjects [15].

The effect of HP and HC diets on epigenetic changes in DNA methylation is not known.

Purpose of the Study

The purpose of this study was to determine if epigenetic changes occur in patients that convert from prediabetic to normal glucose tolerance (NGT) bydetermining DNA methylation at baseline and at remission of prediabetes at 6 months and how these DNA methylation changes are involved in metabolic changes.

Methods

Patients

Prediabetic women and men age 20 - 50 years old with a BMI > 30 kg/m^2 to < 55 kg/m^2 for a total of 24 participants were recruited for this study. Subjects were selected on the basis of inclusion criteria of age, BMI, fasting glucose of < 126 mg/dl, 2 hour glucose level of 140 to 199 mg/dl mg/dl during a single oral glucose tolerance test (OGTT), and HbA1c of 5.7 - 6.4%. They were excluded if they had proteinuria or elevated serum creatinine (> 1.5 mg/dl), surgical or premature menopause, history of liver disease, abnormal liver function tests, diabetes mellitus, on antidiabetic agents or insulin, thyroid disease with abnormal TSH, weight > 350 lbs, triglycerides > 400 mg/dl, LDL cholesterol > 160 mg/dl, SBP > 145 or DBP > 100 mm, use of medications known to effect lipid or glucose metabolism (niacin, steroids, statins, metformin), pregnancy or the desire to become pregnant in the next 6 months, weight loss of more than 5% of body weight in the last 6 months, or history of cancer undergoing active treatment, use of alcohol or smoking.

Study design

The study was a prospective randomized controlled trial of a moderately High Protein-Low Carbohydrate (HP) diet based on 30% Kcals from protein, 40% Kcals from carbohydrate (CHO) and 30% Kcals from fat vs High Carbohydrate-Low Protein (HC) diet comprised of 15% Kcals from protein, 55% Kcals from CHO and 30% Kcals from fat for a period of 6 months. All food was provided for the subjects with pick up on a weekly basis for the 6 month study. The study was approved by the Institutional Review Board of the University of Tennessee Health Science Center (UTHSC).

The study participants underwent a standard OGTT and mixed meal tolerance test (MMT) at baseline and 6 months which were done after an overnight 12 hr fast on the respective diets. If the subject had an elevated temperature or signs of inflammation, the tests were postponed until the subject was free of inflammation.

Glucose was measured at baseline and at 30 minute intervals for 2 hours. At baseline and 6 months, dual energy x-ray absorptiometry (DXA) scan, resting metabolic rate (RMR), chemistry profile, Complete Blood Count (CBC), vitamin D, lipid profile, urinalysis were done. These measurements were done to determine the changes in body weight and body composition (lean and fat mass), insulin sensitivity and glucose response, lipid profile, calcium metabolism and protein breakdown (by urinalysis).

Patients were requested to continue their current level of physical activity throughout the study. After meeting the screening criteria subjects were randomized to either the HP or HC diet using a permuted block randomization method generated by the biostatistician.

Subjects were considered to have remission of their prediabetes if at 6 months they had a fasting glucose of < 100 mg/dl, and a 2 hour glucose level of < 140 mg/dl during a single oral glucose tolerance test. Subjects who did not have remission did not meet any of this criteria. Some of our results of this study of remission of prediabetes have previously been reported [15].

Determination of DNA methylation

Twenty four subjects completed the 6 months study. Baseline (Bl) and 6 month samples on 12 patients on the HP diet that had remission of prediabetes at 6 months and baseline and 6 month samples on 12 patients on the HC diet that did not have remission of prediabetes were analyzed.

DNA extraction from the peripheral blood leukocytes from fasting blood samples at baseline and completion of the study (6 month) was performed using the PreAnalytix Whole Blood DNA extraction kit. DNA quantity was measured using the Quant-iT PicoGreen dsDNA Assay Kit according to the manufacturer's instructions (Life Technologies, Carlsbad CA) and DNA quality was assessed by A260/A280 ratios determined with a Biomate5 dual beam UV spectrophotometer (ThermoFisher, Waltham MA).

Genomic DNA (500 ng) from each sample was treated with sodium bisulfite using the EZ96 DNA methylation kit (Zymo Research, CA, USA) following the manufacturer's standard protocol. Genome-wide DNA methylation was assessed using the Infinium Human Methylation450 BeadChip array (Illumina, San Diego CA) according to manufacturer's instructions. BeadChip arrays were scanned using the Illumina iScan and GenomeStudio Methylation Software Module (v 1.9.0, Illumina) was used to extract the raw signal intensities of each probe (without background correction or normalization).

Quality control was conducted in GenomeStudio software (v2011.1) using the methylation module (v1.9.0) according to the manufacturer's recommendations (Illumina). Briefly, the controls included assessment of DNP and Biotin staining, hybridization, target removal, extension, bisulfite conversion, G/T mismatch, and negative and non-polymorphic controls. The various controls indicated overall good quality of DNA preparations and chip performances.

Statistical analysis

Raw A and B signal values for Infinium type I and type II probes were quantile normalized separately and Infinium type II probe signals were peak-corrected prior to re-integration of the data from both probe types into a single data set for generation of beta-values [16-18]. Beta-values were calculated as the ratio of methylated probe intensity over total intensity (methylated and unmethylated) + 100 for each probe, and range from 0 to 1, corresponding to the percent methylation detected by the probe. Low quality probes (detection p-values ≥ 0.01), probes containing overlapping SNPs at or within 10 bp of the targeted CpG site with reported minor allele frequencies ≥ 0.05,

reported cross-reactive probes, Y chromosome probes (all samples were female) and invariable probes with a mean beta-value = 0.95 or = 0.05 across case (V7) and control (S1) samples were removed from the data set prior to analysis [19,20].

Differentially methylated CpG sites were identified as follows: probes with an absolute beta-value difference ≥ 0.20 (a 20% change in methylation status) between the mean beta-values of cases (V7) and control (S1) were selected for subsequent statistical testing using M-values. M-values were calculated as the log2 ratio of the intensities of methylated probe versus unmethylated probe and give higher resolution than the beta values for extreme methylation levels, whereas at low methylation levels they are co-linear [21]. M-values close to 0 indicate a similar intensity between the methylated and unmethylated probes, indicating the CpG site is roughly half-methylated, whereas positive M-values indicate more molecules are methylated than unmethylated, while negative M-values indicate more molecules are unmethylated.

Beta-value filtered probes with paired t-test p-values and Benjamini and Hochberg [22]. FDR corrected independent t-test p-values < 0.05 were considered to be significant.

Pathway annotation and enrichment analysis was performed using WebGestalt (http://bioinfo.vanderbilt.edu/webgestalt) [23,24]. Significant enrichment of specific pathways or transcription factor target sites was estimated by Benjamini-Hochberg FDR corrected hypergeometric tests with p-values ≤ 0.05. Unsupervised hierarchical clustering and heat map generation were performed in GeneMaths XT (Applied Maths, Belgium) using M-values; probe and sample clustering were performed by Complete Linkage based on Cosine Correlation.

Results and Discussion

Table 1 shows the mean and SE of various parameters monitored on the 12 HP and 12 HC diet subjects from Baseline (Bl) through 6 months and the significant difference of changes of the parameters in the subjects on the HP and HC diets. At Bl, the HP and HC groups were not statistically different. Of significance is the 100% (12/12) remission of pre-diabetes to normal glucose tolerance in all the HP diet group subjects; whereas, there was only a 33% (4/12) remission in the HC group. Subjects on the HP and HChad significant weight loss at 6 months from their Bl weights but not significantly different between the HP and HC groups at 6 months. HbA1c and insulin sensitivity (HOMA IR and ISI) were all significantly improved at 6 months from Bl in the HP and HC diets; however, the HP group had significantly greater improvement in these parameters compared with the HC group at 6 months. The CVR factors (BP, cholesterol, triglycerides, LDL, FFA, PCSK9,CRP, E-Selectin, and BNP), inflammation markers (TNFα, IL-6, Il-1β) and Oxidative Stress markers (DCF, MDA) were significantly decreased in both diet groups; however, the HP diet resulted in significantly greater reduction in the CVR factors (triglycerides, LDL, FFA, PCSK9, CRP, E-Selectin, and BNP), inflammation, and oxidative stress markers compared with the HC diet at 6 months. This reduction in TNFα, IL-6 and IL-1β demonstrates a better anti-inflammatory effect of the HP diet compared with the HC diet. This study also demonstrates a greater improvement in insulin sensitivity and decreased oxidative stress effects of the HP diet compared with the HC diet.

The heat map in figure 1 shows the changes in DNA methylation from Baseline (S1) and at 6 months (V7) on the 6 HP subjects that had remission of prediabetes.

Table 2 shows the some of the various KEGG pathways and number of genes in the pathways that had changes in DNA methylation from baseline to 6 months. The changes in DNA methylation of genes involved in the Metabolic pathways are largely those involved in

	HP (n = 12)			HC (n=12)			
Parameters	**Baseline**	**6 months**	**p***	**Baseline**	**6 months**	**p***	**p****
% Remission		100			33.3		0.001
Age	43.1 ± 1.3			41.1 ± 1.7			0.96
Ethnicity AA/C	10/2			9/3			
Female/Male	9/3			10/2			
% Weight Loss		9.8 ± 1.4	<0.001		11.3 ± 1.8	<0.001	0.692
BMI (kg/m^2)	40.5 ± 1.8	37.3 ± 1.9	<0.001	37.4 ± 1.7	33.8 ± 1.6	0.002	0.391
Insulin Sensitivity							
HbA1c %	6.0 ± 0.015	5.46 ± 0.12	.0005	5.93 ± 0.12	5.73 ± 0.17	0.005	<0.0001
HOMA IR	4.79 ± 0.71	1.58 ± 0.38	0.0005	4.74 ± 0.72	3.34 ± 0.78	0.005	<0.0001
B-cell Function	3.74 ± 0.3	11.24 ± 2.1	0.0005	3.79 ± 0.3	5.68 ± 0.6	0.005	0.001
Myonectin (ng/ml)	74.4 ± 9.8	48.3 ± 8.2	0.001	75.3 ± 9.2	67 ± 7.9	0.05	0.001
Cardiovascular Risk Factors							
BP (SBP/DBP)	130/81 ± 3/2	116/72 ± 2/2	.01/.01	126/81 ± 3/2	118/74 ± 3/3	.01/.01	0.73/0.7
TG (mg/dl)	106.9 ± 10	69.4 ± 6.7	0.001	110.1 ± 11	98.7 ± 9.1	0.002	0.04
LDL (mg/dl)	105.9 ± 4.4	82.4 ± 3.4	0.0005	106.2 ± 5.6	101.9 ± 6.2	0.096	0.037
Cholesterol (mg/dl)	165.3 ± 5.7	151.8 ± 5.3	0.0005	167.9 ± 6.1	161.7 ± 6.3	0.02	0.42
HDL (mg/dl)	44.9 ± 1.7	46.3 ± 1.4	0.10	45.8 ± 2.6	46.2 ± 2.5	0.69	0.85
FFAs (mmol/L)	0.60 ± 0.04	0.47 ± 0.03	0.002	0.61 ± 0.03	0.78 ± 0.06	0.038	0.001
hCRP (mg/L)	9.1 ± 0.4	4.0 ± 0.3	0.0001	8.8 ± 0.3	6.4 ± 0.2	0.005	0.0003
PCSK 9 (ng/ml)	13.7 ± 0.5	8.5 ± 0.3	0.001	13.5 ± 0.4	11.2 ± 0.3	0.02	0.001
E-Selectin (ng/ml)	53.7 ± 1.5	35.0 ± 1.1	0.0005	53.4 ± 1.6	44.6 ± 1.7	0.005	0.0005
BNP (pg/ml)	149.2 ± 2.3	55.9 ± 2.8	0.003	148.6 ± 2. 9	89.6 ± 3.1	0.02	0.001
Proinflammatory Cytokines							
TNF-α (pg/ml)	12.8 ± 0.4	3.8 ± 0.2	0.0005	12.5 ± 0.4	9.6 ± 0.3	0.005	<0.0001
IL-6 (pg/ml)	8.57 ± 0.34	4.55 ± 0.13	0.0005	8.43 ± 0.22	6.8 ± 0.11	0.0005	<0.0001
IL-1β (pg/ml)	10.8 ± 0.7	2.9 ± 0.4	0.001	10.9 ± 0.08	7.4 ± 0.07	0.01	0.002
Oxidative Stress							
ROS (DCF) (μM)	3.6 ± 0.3	2.3 ± 0.3	0.0001	3.5 ± 0.3	3.1 ± 0.2	0.01	<0.0001
Lipid peroxidation MDA (μM)	1.5 ± 0.07	0.6 ± 0.04	0.0008	1.5 ± 0.08	1.2 ± 0.04	0.02	0.0004

Table 1: *Effect of HP or HC diets on remission of prediabetes, weight loss, insulin sensitivity, cardiovascular risk factors, inflammation and oxidative stress.*

P indicates Wilcoxon Signed Rank Test for comparison of Baseline to 6 months; and P** indicates Wilcoxon Rank Sum Test for 6 months comparison of HP vs HC.*

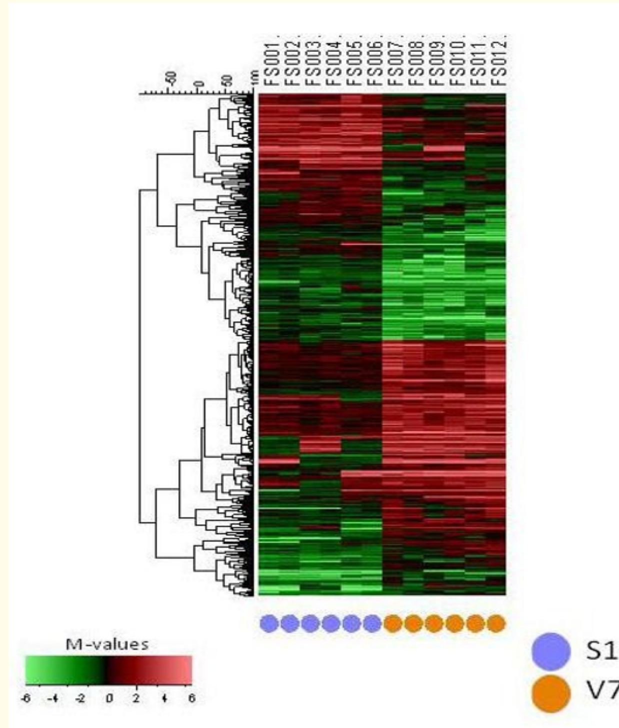

***Figure 1:** Heat map of changes in DNA methylation.*

Clustered heat map of the M-values in the DMR List

DNA 450K probes and samples independently underwent unsupervised hierarchical clustering by the Complete Linkage algorithm based on Cosine Correlation coefficients.

Red = positive M-values, Green = negative M-values, Black = 0.

Insulin Signaling, Cardiovascular Diseases and Inflammation. The genes involved in these three KEGG pathways are listed in table 3-5 and discussed with each table regarding the effects of the changes in DNA methylation of these genes. The DNA methylation changes of genes involved in Cancer Pathways will be discussed with respect to obesity and weight loss at another time.

KEGG Pathway Name	Number of Genes with DNA Methylation Changes
Insulin Signaling	11
Cardiovascular Diseases	15
Inflammation	18
Metabolic Pathways	32
Cancer Pathways	31

Table 2

Insulin signaling genes that have changes in DNA methylation from Baseline to 6 months on the HP and HC diets are shown in the Insulin Signaling (Table 3). The up arrows indicate an increase in DNA methylation and the down arrows indicate a decrease in DNA methylation after 6 months on the HP or HC diets. The double up or down arrows indicate a greater increase or decrease compared to the HC diet at 6 months. Changes in DNA methylation of these genes can be responsible for some of the improvements in insulin sensitivity and other metabolic factors which are discussed below.

Gene Symbol	HP Diet	HC Diet	Gene Name
CTRP15	↑↑	↑	Myonectin
TGFB2	↓↓	↓	TGFB2
PRKCH	↑↑	↑	Protein kinase C eta
IRS2	↓↓	↓	Insulin receptor substrate 2
PTEN	↑↑	↑	Phosphatase and tensin homolog
SHC3	↑↑	↑	SHC (Src homology 2 domain containing)
CYTH3	↓	–	Transforming protein 3 cytohesin 3
MAP4K4	↑↑	↑	Mitogen-activated protein kinase
PIK3R4	↓↓	↓	Kinase kinase kinase 4 phosphoinositide-3-kinase
GRB10	↑↑	↑	Regulatory subunit 4 growth factor receptor-bound protein 10
REG1A	↓↓	↓	Regenerating islet-derived 1 alpha
↑ Indicates increase in DNA methylation; ↓ Indicates increase in DNA methylation			

Table 3: *DNA methylation changes of genes involved in insulin signaling.*

CTRP15 encodes for myonectin, a myokine that is predominately expressed in skeletal muscle and regulates fatty acid metabolism [25]. Increased methylation would decrease the level of myonectin expressed and would improve insulin sensitivity and decrease inflammatory consequences.

TGFB2 encodes TGFB-2 which regulates proliferation, differentiation and apoptosis. TGFB-2 can induces β cell proliferation and promotes insulin release [26]. Decreased methylation would increase the TGFB-2 expressed and would be expected to increase β cell insulin release.

PRKCH encodes for protein kinase C eta which is a modulator of insulin action. PKCeta inhibits IGF-1 phosphorylation of AKT due to insulin stimulation [27]. Increased methylation would be expected to decrease PKC eta expressed levels which would increase insulin sensitivity as the insulin signaling pathway would progress.

IRS2 encodes for a signaling protein that mediates effects through the insulin receptor. Once activated IRS2 leads to activation of various pathways in insulin signaling and increase insulin sensitivity, and also increase Beta cell development [28]. Decreased methylation of IRS2 would increase expression of IRS2 and be expected to increase insulin sensitivity.

PTEN encodes a phosphatase that dephosphorylates phosphoinositide substrates. In insulin signaling, it acts as a negative regulator of insulin/PI3K signaling [29].

Hypermethylation would have a protective effects against insulin resistance and diabetes.

SHC3 encodes for a protein that regulates insulin signaling and involved in cardiac development. SHC has downstream signaling effects on the MAPK system in insulin signaling. SHC is also associated with oxidative stress and modulation of mitochondrial Reactive Oxygen Species (ROS) through cytochrome c [30]. Increased methylation results in decreased expression of SHC and would be associated with a decrease in insulin resistance and cardiac defects.

CYTH3 encodes for PSCD which is involved in guanine- nucleotide exchange protein activity, phospholipids. In insulin signaling it modulates the PI3K/AKT pathway [31].

Decreased methylation of the CYTH3 gene would be expected to result in improved lipogenesis and decrease in triglycerides.

K4 encodes for the kinase involved in activation of the MAPK8/JNK pathway which involves the TNF-a activity (MAP/MKK) pathways. In insulin signaling MAP4K4 protein is a negative regulator of insulin signaling and sensitivity [32]. Hypermethylation of the gene would result in less MAP4K4 protein express and be expected to result in improvement in insulin sensitivity.

PIK3R4 encodes for an enzyme involved in cellular growth, proliferation and differentiation. The enzyme is active in the PI3K/AKT/mTor pathway. AKT activation requires PI3K, so lack of signaling would inhibit the pathway for proceeding [33].

Decreased methylation of the PIK3R4 gene would result in increased enzyme and greater insulin sensitivity.

GRB10 encodes for a protein involved in growth factor signaling in insulin signaling. GRB10 protein negatively regulates insulin signaling and degradation of the insulin receptor [34]. Hypermethylation of the GRB10 gene would decrease the GRB10 protein and be expected to improve insulin sensitivity.

REG1A encodes for a protein involved in islet cell regeneration and diabetogenesis. REG1A protein signals to neighboring islet cells to regenerate [35]. Decreased methylation of this gene would lead to increased expression of the REG1A protein and result in increased insulin sensitivity.

Genes involved in Cardiovascular disease (CVD) that have changes in DNA methylation from Baseline to 6 months on the HP and HC diets are shown in table 4. The up arrows indicate an increase in DNA methylation and the down arrows indicate a decrease in DNA methylation after 6 months on the HP or HC diets as discussed for table 3.

LDLR encodes for a low-density lipoprotein receptor. Hypermethylation of the gene will lead to decreased expression of LDLR in the liver, muscle, heart, and adipose tissue. This decreased expression will lead to an increase in CVD risk due to reduced uptake of LDL, hypercholesterolemia, and accelerated atherosclerosis [36]. The decreased methylation observed in this study would decrease CVD risk.

PPARA encodes for a transcription factor which is active in fasting conditions which will in turn lead to ketogenesis. PPARA regulate various genes, including: APOA1/2/5, LPL, and PLTP [37]. Thus, the decreased methylation observed could be responsible for lower triglycerides and increased HDL cholesterol observed in these patients.

Gene Symbol	HP Diet	HC Diet	Gene Name
LDLR	↓↓	↓	Low density lipoprotein receptor
PPARA	↓↓	↓	Peroxisome proliferator-activated receptor alpha
IRX4	↑	↑	Iroquois homeobox 4
CD14	↑↑	↑	CD14 molecule
FBN2	↓↓	↓	Fibrillin 2
EDNRA	↑↑	↑	Endothelin receptor type A
ZFHX3	↓↓	↓	Zinc finger homeobox 3
AGTR1	↑↑	↑	Angiotensin II receptor, type 1
MMP2	↑	–	Matrix metallopeptidase 2
P2RY12	↑↑	↑	Purinergic receptor P2Y, G-protein coupled, 12
OR13G1	↓	↓	Olfactory receptor, family 13, subfamily G, member 1
COL4A1	↑↑	↑	Collagen, type IV, alpha 1
TBX20	↓↓	↓	T-box 20
ANGPT2	↑↑	↑	Angiopoietin 2
RNLS	↓↓	–	Renalase, FAD-dependent amine oxidase

Table 4: *DNA methylation changes of genes involved in cardiovascular disease.*

IRX4 encodes for a protein that plays a role in the formation of the heart during embryogenesis. Irx4 expression is restricted to the ventricle. Increased expression of the IRX4 gene would lead to aberrant growth in the ventricles, hypertrophic cardiomyopathy, and resulting aberrant conduction [38]. The increased methylation observed will decrease expression of the IRX4 gene and decrease CVD risks.

CD14 encodes for a protein in the innate immune system. CD14 binds to PAMPs from bacteria and acts as a coreceptor with toll-like receptor and bone marrow differentiation protein-2, leading to activation of various pathways and production of inflammatory markers. Due to this pro-inflammatory role, over activation, or de-methylation would lead to an increased risk in CVD and CAD [39]. The increase in methylation observed in this study will decrease CVD risk.

FBN2 encodes for fibrillin, which serves as a structural component in elastic and nonelastic tissues. Reduction in fibrillin can lead to various sequelae. It can lead to a reduction in elastic fibers in the vasculature and an increased risk of degeneration and abnormal conductivity due to increased inflammation and less deposition of extracellular matrix [40]. Thus, a decrease in methylation can led to an increase in fibrillin and decrease of vascular disorders and cardiovascular events.

EDNRA ETA receptors are in smooth muscle surrounding vasculature. Binding of endothelin promotes vasoconstriction, retention of sodium, and increased blood pressure. Binding stimulates Ca^{2+} release from the SR, PKC, and MAPK signaling to cause vasoconstriction and cellular proliferation [41]. Over-activation, or de-methylation will lead to hypertension and hypertension-related effects on the cardiovascular system. The increased methylation observed in this study should reduce hypertension.

ZFHX3 encodes a transcription factor involved in suppression of alpha-fetal protein and c-Myc in cardiac tissues. ZFHX3 acts as a tumor suppressor gene, and variants are also implicated in atrial fibrillation predisposition. Hypermethylation of ZFHX3 would lead to an increased risk for atrial fibrillation and cancer [42,43]. This study showed a decrease in methylation which would decrease risk of atrial fibrillation.

AGTR1 encodes for AT1 which regulates aldosterone secretion, blood pressure, and volume status via the RAAS pathway. De-methylation of AGTR1 is associated with an increased risk for essential hypertension (HTN) [44]. Increased methylation of AGTR1 can have a protective effect on HTN.

MMP2 encodes for the matrix metalloproteinase-2 protein (gelatinase A, 72kDa gelatinase,72kDa type IV collagenase). It is universally expressed in cardiac cells, and it degrades collagen and other cellular substrates. It can be used as a marker in heart failure (HF) patients. High levels of MMP predict LV remodeling post-MI and is associated with mortality in patients with HF [45]. Thus, increased methylation of MMP2 can be protective for patients and decrease risk of mortality in HF patients.

P2RY12 encodes a receptor for ADP, belonging to Gi receptor class. Gi signaling inhibits cAMP-dependent pathways by inhibiting adenylate cyclase. P2RY12 is also involved in the activation of platelet aggregation and clotting via platelet binding to fibrin. Inhibitors of P2Y12 are used in acute coronary syndromes and STEMIs [46]. Thus, de- methylation would be expected to predispose patients to excessive clotting and cardiovascular events such as MI and stroke. This study showed an increase in methylation which should prevent MI and stroke.

OR13G1 encodes olfactory receptors which interact with odorants within the nose. OR13G1 is a g-protein coupled receptor. A study found a relationship between SNPs in OR13G1 with an increased risk for MI and an association with diabetes. Inactivation of this gene or hypermethylation would increase risk for MI [47]. This study showed a decrease in methylation which should decrease risk of MI.

COL4A1 is implicated in the production of fibrosis in atherosclerosis. Type IV collagen is the primary component of basement membranes. By binding to integrin, COL4A1 controls the formation of capillaries. Also, it inhibits MAPK signaling to regulate HIF-1A and VEGF expression. Collagen IV deposition in the arterial wall prevents smooth muscle proliferation. Thus, coronary artery disease could be regulated by decreased methylation of COL4A1 gene [48].

TBX20 encodes a transcription factor that is essential in early heart development. It is a positive regulator for the Wnt pathway. Functioning TBX20 is important in cardiomyocyte proliferation regulation via TBx2 and N-myc1. Studies have shown SNPs predispose patients to longer QRS duration [49]. Thus, decreased methylation would improve patients risk to cardiovascular disease.

ANGPT2 encodes for a growth factor involved in vascular maturation and stability. This growth factor binds to Tie2 and integrins in the setting of hypoxia. This will disrupt endothelial integrity. Decreased methylation of ANGPT2 leads to an increase in this growth factor. This further destabilizes tissues in the presence of infarct and can exacerbate heart remodeling after an MI [50]. The increased methylation observed in this study would be beneficial for heart tissue.

RNLS encodes for renalase, which is a flavin dinucleotide-dependent amine oxidase which metabolizes catecholamines. Renalase release is related to reduction in blood pressure, heart rate, and vascular tone by degrading catecholamines [51]. Decreased methylation of the gene may lead to a decreased risk for essential hypertension.

Genes involved in inflammation that have changes in DNA methylation from Baseline to 6 months on the HP and HC diets are shown in table 5. The up arrows indicate an increase in DNA methylation and the down arrows indicate a decrease in DNA methylation after 6 months on the HP or HC diets as discussed for table 3.

Inflammation			
Gene Symbol	**HP Diet**	**HC Diet**	**Gene Name**
IFNG	↑↑	↑	Interferon, gamma
PF4V1	↓↓	↓	Platelet factor 4 variant 1
S100A7	↑↑	↑	S100 calcium binding protein A7
ALOX12	↑↑	↑	Arachidonate 12-lipoxygenase
HIF1A	↑↑	↑	Hypoxia inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)
NR1H4	↓↓	↓	Nuclear receptor subfamily 1, group H, member 4
MMP2	↑↑	↑	Matrix metallopeptidase 2 (gelatinase, 72KDa gelatinase, 72 kDa type IV collagenase)
IRAK3	↓↓	↓	Interleukin-1 receptor-associated kinase 3
PPARA	↓↓	↓	Peroxisome proliferator-activated receptor alpha
CD14	↑↑	↑	CD14 molecule
CXCR6	↑↑	↑	Chemokine (C-X-C motif) receptor 6
NGF	↑↑	↑	Nerve growth factor (beta polypeptide)
ADAM33	↑↑	↑	ADAM metallopeptidase domain 33
RORA	↓↓	↓	RAR-related orphan receptor A
CCR5	↑↑	↑	Chemokine (C-C motif) receptor 5 (gene/pseudogene)
ANGPT2	↑↑	↑	Angiopoietin 2
SAA4	↑↑	↑	Serum amyloid A4, constitutive
MIR146A	↓↓	↓	MicroRNA 146a

Table 5: *DNA methylation changes of genes involved in inflammation.*

DNA methylation changes involved in inflammation are discussed below.

IFNG encodes for interferon gamma which is an important mediator of inflammation. It is an activator of macrophages and MHC class II expression. It also directly inhibits viral replication. IFNG binding to a receptor will activate the JAK-STAT pathway. Increased methylation of the IFNG gene would decrease interferon gamma and decrease inflammation [52].

PF4V1 encodes for platelet factor 4 variant 1 which is a chemokine that regulates angiogenesis and tumor growth. PF4V1 is regulated by chemokine receptor 3 and is active in the chemokine-mediated signaling pathway. When active, PF4V1 downregulates inflammatory markers such as: HIF-1a, VEGF, TNF-a and caspase-3. In diabetes patients, PF4V1 presence protects against blood-retinal barrier breakdown.

Decreased methylation would lead an increase in platelet factor 4 and a protective effect on the retina in diabetes and tumorigenesis [53].

S100A7 is a member of S100 proteins which regulate a wide variety of cellular processes. It has been implicated in hyperproliferative skin diseases and induction of immunomodulatory diseases. It is a calcium binding protein that is induced by IL-17 and IL-22. Once activated, S100A7 leads to activation of pathways that upregulate Th1, Th17 and alpha-secretase. Increased methylation of the gene would be expected to decrease inflammatory diseases [54].

ALOX12 encodes for an enzyme that acts on PUFA to generate eicosanoids and lipoxins. These products and the enzyme itself regulates platelet function. Elevated expression, or de-methylation is associated with diabetic patients. The enzyme is an important producer of ROS, inflammation, and atherosclerosis, and increased methylation of the ALOX12 gene would decrease ROS, inflammation and atherosclerosis [55].

HIF1A encodes for the alpha subunit of HIF-1 which is a master regulator of cellular and homeostatic responses to hypoxia. It activates transcription of various genes that will upregulate metabolism, angiogenesis, and apoptosis to facilitate oxygen delivery. It is important in the pathophysiology of ischemic disease. It is a downstream effector of P13K/mTor. Overactivation or demethylation would predispose patients to worse outcomes following periods of hypoxia, and high levels of HIF1A is a marker of poor prognosis in diabetic nephropathy [56,57].

NR1H4 encodes for a transcription factor involved in bile acid synthesis and transport. The receptor binds to bile acids and regulates synthesis and transport via regulation of CBP/p300/SRC-1 and upregulation of NR1H4 to form a dimer with RXR. It is an important regulator of hepatic triglyceride levels. NR1H4 activation limits lipogenesis and free fatty oxidation by activating PPARa. Decreased methylation of NR1H4 would be expected to prevent fatty liver and improve insulin sensitivity [58].

MMP2 encodes for the matrix metalloproteinase-2 protein. It is universally expressed in cardiac cells, and it degrades collagen and other cellular substrates. Increased expression of MMPs can be observed in nearly all inflammatory diseases. MMPs lead to activation of inflammatory cytokines: TNF-a and IL-1 and inflammatory chemokines.

MMP2 is implicated in lung inflammation, TNF-a induced cardiomyopathy, colitis, and immune-mediated arthritis [59]. Thus, increased methylation of MMP2 can be protective for patients and decrease all inflammatory diseases.

IRAK3 encodes an enzyme and is essential in repairing the endothelial barrier in the presence of an endotoxin. The enzyme is a part of the Toll/IL-R signaling pathway and is found within monocytes, macrophages and acts as a negative regulator of the toll-like receptor signaling [60]. Decreased methylation of the IRAK3 gene would lead to a decrease in toll-like receptor signaling and decreased inflammation.

PPARA encodes a peroxisome proliferator activated receptor which is responsible for targeting genes in cell proliferation, cell differentiation, and immune/inflammation response. Lower levels of PPARA are associated with endothelial dysfunction and obesity in metabolic syndrome [37,61]. Thus, decreased methylation of the PPARA gene in diabetes patients would be expected to lead to improved outcomes in terms of weight control and vascular permeability. This can also be attributed to decreased levels of triglycerides as a result of increased fatty acid oxidation.

CD14 encodes a protein that is expressed as a cell surface antigen on monocytes and macrophages. It mediates the innate immune response once bound to LPS. In conjunction with TLR, it activates the MAPK/MEK/ERK pathway and other downstream regulators like NF-kB to induce inflammatory responses [62]. Increased methylation of this gene would lead to decreased inflammatory responses and improvement in T2DM due to improvement in inflammatory insulin resistance.

CXCR6 encodes a chemokine receptor which can bind CXCR1 on neutrophils. It is a GPCR that activates JAK/STAT, IP3/DAG, and P13K/AKt/NF-kB. The activation of these pathways lead to chemotaxis, gene transcription, and cellular proliferation [63]. Increased methylation of the CXCR6 gene decreases expression of the chemokine receptor and decreases angiogenesis and inflammatory responses.

NGF encodes for nerve growth factor which stimulates the growth and differentiation of sympathetic and sensory neurons. Dysregulation of the gene is implicated in neuropathy and allergic rhinitis. NGF is expressed by structural and inflammatory cells in bronchial and conjunctival mucosa [64]. Increased methylation of the NGF gene would be expected to decrease inflammatory response in patients, especially in bronchiolar and conjunctival mucosa.

ADAM33 encodes for a disintegrin/metalloprotease and is involved in cell-cell and cell- matrix interactions like fertilization, muscle development and neurogenesis. It is implicated in asthma and bronchial hyperresponsiveness via the activation of pathways that lead to remodeling of bronchial walls due to its presence in smooth muscle, fibroblasts, and myofibroblasts [65]. Demethylation of ADAM33 would be expected to predispose patients to hyperinflammatory states.

RORA encodes for a nuclear hormone receptor. It interacts with kinases that involve organogenesis and tumor suppressor products. It also interacts with and controls macrophages. It has been shown to regulate levels of TNF, IL-1B and IL-6 [66].

Decreased methylation of the RORA gene would be expected to lead to a decreased inflammatory state due to increased regulation of macrophages and macrophage mediating cytokines.

CCR5 encodes a chemokine receptor expressed by T cells and macrophages which is a target by various viruses. MCP-2, MIP-1a, MIP-1b, and RANTES are ligands for this specific receptor. It is a mediator of granulocyte proliferation and differentiation [67].

Inactivation of the gene, or increased methylation is associated with protective effects on atherosclerotic disease processes.

ANGPT2 encodes for a protein that is an antagonist for Ang-1. Ang-1 and Ang-2 are both ligands for Tie2, which will upregulate angiogenesis during embryogenesis and tumorigenesis. ANGPT2 prevents vascular remodeling of Ang-1 and induces endothelial cell apoptosis [68]. Decreased methylation of ANGPT2 would be expected to lead to a decrease in inflammatory response and decreasing atherosclerosis due to regulation of Ang-1.

SAA4 encodes serum amyloid A4, an apolipoprotein associated with HDL. It is an acute phase marker that is similar to CRP in that SAA responds to inflammatory stimuli, and it can be used as a serum marker in inflammatory disease states, such as arthritis [69]. Increased methylation of the gene would be expected to result in decreased inflammation.

MIR146A encodes a microRNA that is involved in post-transcriptional regulation by affecting stability and translation of mRNA. miRNA targets TNF, IL-1 receptor kinase 1, IL-1b, TNF receptor associated factor 6, and complement factor H. MIR146a is a noted as a negative of

inflammation and oxidative stress in chronic T2DM induced by hyperglycemia in mice studies. miR-146a down-regulated inflammatory mediators like Cox-2, TNF-a, IL-6, and IL-1b [70]. Decreased methylation of MIR146A would be expected to result in a decreased inflammatory state, leading to improvement of T2DM oxidative stress.

Conclusion

The HP diet was more effective in inducing conversion from prediabetes to NGT than the HC diet. Subjects on both diets lost a similar amount of weight, however, the HP subjects had an increase in the lean mass along with a decrease in fat mass while the HC subjects lost both lean and fat mass. The HP diet was more effective in improving insulin sensitivity, decreasing cardiovascular risk factors, inflammatory markers and oxidative stress than the HC diet.

Numerous alterations in DNA methylation occurred with weight loss and remission of prediabetes with the HP diet. Additionally, differences in DNA methylation were observed in the non-remission HC diet compared to the HP diet group. The changes in DNA methylation of the genes involved in insulin signaling (CTRP15, TGFB2, PRKCH, IRS2, PTEN, SHC3, CYTH3, MAP4K4, PIK3R4, GRB10, REG1A) after 6 months on the HP and HC diets would lead to improvement in insulin sensitivity with greater improvements with the HP diet. DNA methylation changes of genes involved in the Cardiovascular disease pathway (LDLR, PPARA, IRX4, CD14, FBN2, EDNRA, ZFHX3, AGTR1, MMP2, P2RY12, OR13G1, COL4A1, TBX20, ANGPT2, RNLS) could be responsible for improvements in cardiovascular risk factors with greater improvement observed with the HP diet subjects at 6 months. DNA methylation changes of genes involved in the inflammation pathway (IFNG, PF4V1, S100A7, ALOX12, HIF1A, NR1H4 MMP2, IRAK3, PPARA, CD14, CXCR6, NGF, ADAM33, RORA, CCR5, ANGPT2, SAA4, MIR146A) could be responsible for decreased inflammation markers with a greater decrease observed with the HP diet subjects at 6 months. As observed in the table 3-5 various gene have increased or decreased DNA methylation which would lead to increased or decreased translation to proteins. For example, the ANGPT2 gene has increased DNA methylation at 6 months and a decrease in the PCSK9 enzyme in table 1 which is involved in lipid metabolism. The CTRP15 increase in DNA methylation leads to a decrease in the expression and translation to myonectin, as shown in table 1, which is involved in insulin sensitivity and increased levels have been shown to be a marker of Type 2 Diabetes [25]. The decrease in DNA methylation of the IRS2 gene leads to increased expression and translation of the insulin receptor substrate 2 involved in insulin signaling. Thus, changes in DNA methylation can increase or decrease the transcription and translation to the specific protein which can be involved in metabolic processes depending on the site of interaction in the pathway.

Gene expression is regulated by epigenetic factors such as DNA methylation as other studies have shown. The changes in gene expression from DNA methylation leads to changes in transcription and translation to protein. Studies have shown that in the Beta cells that the insulin gene expression is regulated by DNA methylation, demonstrating a decrease in methylation of the insulin promoter gene for increased expression of the insulin gene [71]. A study determining some of the changes in DNA methylation post Roux-En Y gastric surgery and weight loss also found 51 promotor regions of DNA had changes in methylation at 6 months post surgery [72].

A study has shown that dietary modification and weight loss affect global DNA methylation in breast cancer survivors [73]. Global DNA methylation has also been associated with higher fruit/vegetable intake and higher physical activity [74-76].

Although the weight loss was similar between HP and HC diet subjects in our study, the muscle mass was not lost in the HP diet subjects while it was lost in the HC diet subjects. This fact may explain some of the reasons for greater DNA methylation changes and more significant improvement in insulin sensitivity, decreased cardiovascular risk factors, inflammation and oxidative stress markers after 6 months on the HP diet compared to the HC diet.

To our knowledge this is the first paper reporting the changes in DNA methylation with remission of prediabetes with weight loss on a HP or HC diet in obese prediabetic subjects. As we better understand the connections between diet and the epigenome, the opportunity arises for clinical applications. Just as mapping our gene variations gives us a window into our personalized medical needs, so might a profile of one's unique epigenome. Formed through a lifetime of experiences beginning in the womb, our epigenome may provide a wealth of information about how to eat better. This could lead to the future field of nutrigenomics, where nutritionists take a look at a subjects methylation pattern and design a personalized nutrition plan.

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