DNA methylation signatures in peripheral blood mononuclear cells from a lifestyle intervention for women at midlife: A pilot RCT

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<tr>
<th>Journal:</th>
<th><em>Applied Physiology, Nutrition, and Metabolism</em></th>
</tr>
</thead>
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<td>Manuscript ID</td>
<td>apnm-2017-0436.R2</td>
</tr>
<tr>
<td>Manuscript Type:</td>
<td>Article</td>
</tr>
<tr>
<td>Date Submitted by the Author:</td>
<td>03-Oct-2017</td>
</tr>
<tr>
<td>Complete List of Authors:</td>
<td>McEwen, Lisa; Centre for Molecular Medicine and Therapeutics, BC Children's Hospital Research Institute Gatev, Evan; Centre for Molecular Medicine and Therapeutics, BC Children's Hospital Research Institute; Simon Fraser University - Vancouver, Beedie School of Business Jones, Meaghan; Centre for Molecular Medicine and Therapeutics, BC Children's Hospital Research Institute MacIsaac, Julia; Centre for Molecular Medicine and Therapeutics, BC Children's Hospital Research Institute McAllister, Megan; University of British Columbia, Family Practice; Centre for Hip Health and Mobility Goulding, Rebecca; University of British Columbia, Family Practice Madden, Kenneth; University of British Columbia, Medicine Dawes, Martin; University of British Columbia, Family Practice Kobor, Michael; Centre for Molecular Medicine and Therapeutics, BC Children's Hospital Research Institute Ashe, Maureen; University of British Columbia, Family Practice; Centre for Hip Health and Mobility</td>
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<tr>
<td>Is the invited manuscript for consideration in a Special Issue?:</td>
<td></td>
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<tr>
<td>Keyword:</td>
<td>Epigenetics, DNA methylation, physical activity &lt; exercise, weight loss, randomized controlled trial</td>
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https://mc06.manuscriptcentral.com/apnm-pubs
DNA methylation signatures in peripheral blood mononuclear cells from a lifestyle intervention for women at midlife: A pilot RCT

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Abstract

Objectives: Physical activity confers many health benefits, but the underlying mechanisms require further exploration. In this pilot randomized controlled trial we tested the association between longitudinal measures of DNA methylation and changes in objective measures including physical activity, weight loss, and C-reactive protein levels in community-dwelling women aged 55 to 70 years.

Methods: We assessed DNA methylation from 20 healthy post-menopausal women (55-70 years of age), who did not have a mobility disability and allocated them to a group-based intervention, Everyday Activity Supports You (EASY), or a control group (monthly group-based health-related education sessions). The original randomized controlled trial was six months in duration and consisted of nine sessions of two-hour focused on reducing sedentary behavior for the intervention group, or six one-hour sessions on other topics for the control group. We collected peripheral blood mononuclear cells, both at baseline and six months later. Samples were processed using the Illumina 450k Methylation array to quantify DNA methylation at >485,000 CpG sites in the genome.

Results: There were no significant associations between DNA methylation and physical activity, but we did observe alterations at epigenetic modifications that correlated with change in percent body weight over a six-month period at 12 genomic loci, two of which are located near the previously reported weight-associated genes RUNX3 and NAMPT. We also generated a potential epigenetic predictor of weight loss using baseline DNA methylation at five CpG sites.

Conclusions: These exploratory findings suggest a potential biological link between body weight changes and epigenetic processes.

Key words: Epigenetics, physical activity, weight loss, DNA methylation, intervention, randomized controlled trial
Introduction

Physical activity is a fundamental aspect of healthy living contributing to the prevention of obesity, type II diabetes, cardiovascular disease, and other lifestyle-related diseases (Warburton et al. 2006). Benefits of an active lifestyle include reductions in stress, risk for developing frailty, and premature mortality (Nocon et al. 2008, Barlow et al. 2014). It is also associated with normalizing blood pressure, triglycerides, and cholesterol levels (Nunan et al. 2013, Mann et al. 2014). Although the association between physical activity and health is well established, research investigating the molecular pathways, particularly epigenetic processes, underlying the response to physical activity remain limited. Characterizing such molecular changes will contribute to understanding the biological processes between physical activity and health outcomes.

The field of epigenetics focuses on studying modifications to DNA and DNA packaging that have the potential to influence gene expression without altering the genetic sequence (Bird 2007). In human populations, the most widely studied and characterized epigenetic modification is DNA methylation (DNAm): the chemical process of adding a methyl group onto DNA; predominantly occurring cytosine DNA nucleotides followed by guanine bases, connected by a phosphate bond (referred to as a ‘CpG’). CpGs are enriched in regions called CpG islands, areas of >200bp densely packed with CpGs (Hatchwell and Greally 2007, Illingworth and Bird 2009). Approximately 60% of promoters, regions that determine the activity of genes, are associated with a CpG island, emphasizing the role of CpG methylation in controlling gene expression (turning a gene “off” or “on”) (Jones 2012).

An important role of DNAm is to orchestrate cell type differentiation during embryogenesis, given that all cell types have identical DNA sequences, the epigenome in general and DNAm in
particular allows for the specification of cell type identity. Thus not surprisingly, the primary source of DNAm variation is tissue type. Cellular differences in DNAm are larger than inter-individual variation, such that DNAm profiles of two different tissues obtained from one individual are less alike than the DNAm of the same tissue from two different people (Farré et al. 2015). Though having a smaller effect than tissue or cell type, underlying genetic makeup at certain sites has also been shown to substantially affect DNAm of nearby CpGs (Banovich et al. 2014).

Additionally, DNAm is a dynamic mark that can act as a mediator between environmental influences and genomic regulation. With that, DNAm is variable over the life-course and can be altered in response to certain environmental/lifestyle exposures. Specific exposures such as cigarette smoking, diesel exhaust, and physical activity have recently been associated with DNAm signatures (Joubert et al. 2012, Brown 2015, Clifford et al. 2016). As most research has been focused on cross-sectional study designs, the majority of the findings have merely identified associations. Therefore, intervention studies are crucial in distinguishing the specific effects of environmental or lifestyle influences on the methylome.

The observed effects of short-term lifestyle changes on DNAm have been subtle and replication studies of these findings are still needed. Although notable physical activity intervention studies have observed differences in DNAm (Archer 2015), they lack consistency in the type/length of exercise program, study population characteristics (sex/age/ethnicity), and type of tissue examined. Weight-loss interventions, including caloric-restriction programs or gastric bypass, have also observed changes in DNAm but again results have been inconsistent (Campión et al. 2009, Milagro et al. 2011, Benton et al. 2015). Although this field is still in its infancy, the results
thus far of a relationship between DNAm and lifestyle changes related to physical activity and/or weight loss are encouraging.

Therefore, in the present study, we investigated DNAm patterns before and after a lifestyle intervention from a six-month pilot randomized control trial (Ashe et al. 2015) of previously inactive post-menopausal women. We focused on characterizing genome-wide DNAm changes associated with measures of physical activity, percent body weight change, and C-reactive protein (CRP).

Materials and Methods

Participants

This was a sub-study of a lifestyle intervention for community-dwelling older women from Vancouver, Canada (ClinicalTrials.gov identifier: NCT01842061). We provide a description of the methods and results, following CONSORT 2010 guidelines (Moher 2010), from the main study (1:1 parallel two-arm randomized pilot trial) in our previously published manuscript (Ashe et al. 2015). For this current study we included women aged 55-70 years that self-identified as not meeting physical activity of 150 minutes/week of moderate to vigorous physical activity (MVPA), but could walk 400 meters and climb a flight of stairs. Ethics were approved from the university institutional review board and all participants signed a written informed consent form prior to participation in the study.

Intervention

The six-month self-management intervention called “Everyday Activity Supports You” (EASY), was designed to reduce sitting time and promote physical activity through incremental increases
in activities of daily living and utilitarian walking (Ashe et al. 2015). The EASY model contains the following three key elements: group education, individualized sessions with an exercise professional, and the use of an activity monitor (Fitbit, USA). The intervention group was offered nine two-hour sessions (over six months) and concurrently the control group had six one-hour monthly sessions. The format for the EASY model was as follows: the first hour was an interactive educational session on a number of physical activity and health related topics. The second hour consisted of participatory group-based work to address action and coping plans to being active, and similar topics. In addition, all participants had an individual short session with an exercise professional. Participants’ programs were developed and progressed, based on feedback from the activity monitor, to reduce sitting time and increase their daily step count. The control group received six one hour sessions on health- (but not physical activity-) related topics. Descriptive information was collected and height, weight, and blood pressure [BpTRU BPM-200 BpTRU Medical Devices, Coquitlam, BC] were measured using standard procedures. Participants were also requested to wear a waist-mounted accelerometer, ActiGraph GTX3+ (LLC, Fort Walton Beach, FL, USA). Further details of the pilot study are described in detail elsewhere (Ashe et al. 2015).

Sample collection and data processing

We requested that participants provided a non-fasting blood draws at baseline and six months. Blood was drawn into Vacutainer Cell Preparation Tubes (Becton Dickinson) by antecubital venipuncture. A complete cell blood count was performed and no outliers were observed. Samples were inverted 10 times and then subjected to a density-gradient centrifugation at 1800 RCF for 20 minute at room temperature, within one hour of collection. 1ml of blood plasma was simultaneously collected and used for CRP measurement using an immunoassay (Meso Scale
Discovery, Maryland, USA). Approximately 3ml of buffy coat was transferred into a tube and topped up to 15ml with warm RPMI-1640 medium (Sigma-Aldrich), inverted five times, and centrifuged at 300 RCF for 10 minutes at room temperature, supernatant was discarded. Cell pellet was re-suspended with 10ml warm RPMI with a pipette, centrifuged at 300 RCF for 10 minutes at room temperature, and then the supernatant was discarded. Cell pellet was then re-suspended with 1ml RPMI, and thoroughly mixed by pipetting. 5ul of peripheral blood mononuclear cell (PBMC) suspension was aliquoted into 45ul of Trypan Blue Solution 0.4% (Sigma-Aldrich). 10ul of trypan blue/cell mixture was transferred into a Bright-Line™ Hemacytometer (Sigma-Aldrich) to count cells. Approximately six million cells were aliquoted per microfuge tube, and centrifuged at 13,000 RPM for 3 minutes, supernatant was discarded, and pellet was stored at -80°C until DNA extraction. One tube for each sample intended for DNAm quantification was lysed and homogenized in a QiaShredder Spin Column (Qiagen, Hilden, Germany). Genomic DNA was isolated using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). Quantity and quality of DNA was assessed using a NanoDrop™ 8000 Spectrophotometer (Thermo Scientific). Bisulfite conversion of 750ng of DNA was performed using the EZ-DNA Methylation™ Kit (Zymo Research, Irvine, CA, USA). We processed 47 samples (20 paired samples, three technical replicates, and four unpaired samples) using the Infinium HumanMethylation450 BeadChip per manufacturer’s instructions (Illumina, San Diego, CA, USA). For the purpose of our analyses we only included individuals who provided samples at both time points (n=20).

Data preprocessing included exporting colour corrected and background subtracted β-values from the Illumina GenomeStudio software (Illumina, San Diego, CA, USA). We subsequently imported and processed these data using R version 3.2.3. Probe filtering was standard and
included poor performing probes as well as probes known to potentially introduce bias (Price et al. 2013), resulting in 433,056 probes remaining for further analysis.

We accounted for known variation that had potential to confound or bias our results, such as probe design, batch effects, and cell type proportion. Specifically, we employed quantile normalization across samples and subset-quantile within array normalization across probes to adjust for technical bias (Maksimovic et al. 2012), ‘ComBat’ from the package ‘sva’ was used to adjust for microarray chip and position batch effects (Johnson et al. 2007), and we corrected for predicted blood cell type proportions using previously established methods (Houseman et al. 2012), (Jones et al. 2015). All analyses and reports of DNAm are in β-values ranging from 0-1 (0 = no methylation, 1 = fully methylated).

Statistics

To avoid examining sites that did not change across individuals, we performed a within sample variability filter to yield a reduced dataset of 39,238 CpGs that showed an inter-quantile range (80th – 20th percentile) above 5% DNAm (Lemire et al. 2015). We then regressed changes in methylation at each of these CpGs onto the following variables in separate linear models: intervention group status, daily percent change in step count, percent change in CRP, and percent change in body weight, while correcting for baseline BMI and age. We used a significance threshold of \( p \text{ value} = 5e-5 \), we also reported multiple test corrected values for statistical transparency.

As a second line of analysis, we investigated whether a potential epigenetic predictor using baseline DNAm was feasible for prediction of percent change in body weight (kg). Our approach
has two stages: 1) We estimated a sparse linear model using “lasso” (least absolute shrinkage and selection operator) a well-established machine learning method that considers the joint effect of multiple CpG sites and simultaneously selects only the informative sites (Friedman et al. 2010). Briefly, this method allows for the identification of the most informative CpGs that jointly are associated with a variable of interest. The penalty parameter was calibrated through 5-fold cross-validation. The statistical equation used for the five CpG-based prediction of percent weight change over the intervention period is as follows:

\[
\left(\frac{\Delta \text{weight}}{\text{weight}_{\text{Base}}}\right)_{\text{predicted}} = a + b_1 \beta_{\text{cg}17920653} + b_2 \beta_{\text{cg}25134701} + b_3 \beta_{\text{cg}24088639} + b_4 \beta_{\text{cg}22664307} + b_5 \beta_{\text{cg}08104023},
\]

where the intercept \(a\) and coefficients \(b_i\) for the methylation values \(\beta_{\text{cg}i}\) were estimated with the lasso regression.
Results

Cohort characteristics

Samples were obtained from participants of an exercise intervention program called Everyday Activity Supports You (EASY). A subsample of the EASY cohort was used and included 20 healthy middle-aged [mean (SD) 64.1 (4.6) years] women who self-reported as being physically inactive (not meeting physical activity guidelines) and resided in Metro Vancouver, British Columbia (Table 1). We previously reported significant differences between groups for some variables (step count, weight, and diastolic blood pressure) favoring the intervention group (Ashe et al. 2015).

DNA methylation across the intervention period

Initially, we selected CpG sites with a biologically meaningful DNAm change from the total dataset (433,056 CpGs), that being an absolute DNAm change of ≥ 5% across the intervention period. Using this method, we identified 39,268 variable CpGs (vCpGs) for downstream analyses. By applying a minimum threshold of DNAm change, this approach allowed us to assess DNAm changes that were less likely to be a result of technical noise and a more likely evidence of a biological difference. We investigated whether DNAm changed differently in the intervention group as compared to the control group by testing the change in DNAm at each of the 39,238 vCpG sites, while controlling for chronological age and baseline BMI. Two CpG sites passed a nominal $p$ value threshold ($p < 5e^{-5}$), cg09786593 ($p$ value = 2.89e-05, $q$ value = 0.737) and cg11630939 ($p$ value = 4.28e-05, $q$ value = 0.737), although these findings did not remain significant after applying a multiple test correction (Figure 1A, Supplementary table S1).
Since no statistically significant differences in DNAm were found between the intervention and control groups, we focused on the association between physiological variables measured at baseline and post intervention and the change in DNAm in all individuals. Using linear regression, we examined the association between DNAm changes at each vCpG and percent change in body weight (kg), and found 12 CpG sites that demonstrated a change in DNAm ($p$ value < 0.00005, $q$ value < 0.12) (Figure 1B, Table 2). DNAm at seven of these CpG sites were positively correlated with percent weight change, and five were negatively correlated with percent weight change. The majority of these sites were located within gene bodies (5/12), although some were located between genes (4/12) or within promoter regions (3/12); not entirely surprising, given these distributions are comparable to what is represented on the 450k array. The effect sizes of these identified CpGs ranged from 8.6% - 15.1%, with an average $\beta$-value range of 10.9% (Figure 2).

We did not find any association between the change in DNAm over time with either the percent daily step count or the percent change in serum CRP. We do however note that the change in CRP was considerably variable across individuals, ranging from -64 - 95% (Supplementary figure S1).

**A potential epigenetic predictor of weight loss**

To explore epigenetic predictors of weight loss, we first investigated previously reported DNAm loci that have been observed to predict weight loss (Nicoletti et al. 2016). We tested the association between the baseline DNAm at sites near or within *SERPINE-1* and changes in body weight in our study participants. Although we were restricted to sites represented on the 450k array, there were 12 sites that spanned a genomic region of 7bp – 12kbp upstream of the
SERPINE-1 transcription start site (TSS). We observed 1/12 sites near SERPINE-1 that reached nominal significance (cg17968347: p value = 0.0285, Supplementary figure S2).

To identify a new robust predictor of weight-loss, we used a penalized regression approach that incorporates relevant variable selection, and takes into account DNAm information at more than one CpG. We found that baseline DNAm at five CpG sites was able to predict the percent body weight change over the six-month period (cg17920653, cg25134701, cg24088639, cg22664307, and cg08104023), while controlling for baseline weight (Table 3). We found a correlation of \( r = 0.74 \) between the predicted weight loss and actual weight loss (Figure 3).

**Discussion**

In this study, our goals were to test whether a six-month lifestyle intervention was associated with epigenetic changes, and if it was possible to identify potential epigenetic predictors of the intervention. Although we did not find significant changes between DNAm with either the intervention or physical activity measures, we did observe a change in DNAm at 12 CpG sites that associated with weight loss. We also generated a potential epigenetic predictor of weight change, based on baseline DNAm at five CpG sites.

Weight loss was an unexpected significant outcome from the EASY model pilot study, with an average between group difference of approximately -4 kg, favouring the intervention (Ashe et al. 2015) and, interestingly, it has also previously been associated with DNAm (Martínez et al. 2014, Benton et al. 2015, Wahl et al. 2017). In the current study, one of the CpG sites (cg05004518) associated with weight change has also been reported previously to relate to changes in body weight. The site cg05004518 is located within an intron of nicotinamide
phosphoribosyltransferase (NAMPT). NAMPT is an enzyme involved in regulation of nicotinamide adenine dinucleotide (NAD), which is important in cellular redox reactions and decreases with age, as well as certain metabolic disorders (Garten et al. 2015). Furthermore, expression of NAMPT has been found to increase after weight loss in previously obese individuals (Rappou et al. 2016). We observed a decrease in DNAm at the NAMPT CpG was associated with greater weight loss at the intragenic NAMPT CpG. This relationship is generally associated with reduced gene expression; however, this relationship is not always linear (Jones 2012).

The other significant CpG (cg15014975) is located -777bp from the TSS of RUNX3, which encodes for a transcription factor that has a pivotal role in activating the immune response (Smeets et al. 2012). At this site, we found that a gain of methylation was associated with greater weight loss. While it is tempting to speculate that the observed associations between body weight change and DNAm within NAMPT and RUNX3 may have biological implications, we emphasize these findings are exploratory in nature and require further studies to confirm their significance.

The ten other genes identified with nearby body weight-related DNAm included ABR, SLCO4C, WNT7A, RASGRP3, CYP2E, CA13, KANK4, SMOC2, SLIT3, and GABRG3; although, to our knowledge these genes have not previously been implicated with weight loss. It is encouraging that the effect sizes at these sites, ranging from 8-15%, illustrated expected trends: participants who did not lose or gain weight during the intervention period had no change in DNAm (~0%) at the identified sites.
We also investigated whether a change in activity levels, objectively measured as MVPA, daily step count, and day spent in sedentary behavior, was associated with changes in DNAm over the intervention period. The EASY lifestyle intervention aimed to increase everyday activity, but did not focus on generating high intensity physical activity (e.g., strenuous exercise), and since the activity variables were highly correlated we decided to focus on step count as a representative variable for activity (Supplementary figure S3). However, we did not find associations between any change in DNAm and daily step count over the intervention period.

We also investigated inflammation (CRP) over time as this is a pro-inflammatory marker highly related to physiological processes, but did not see any association with the change in DNAm. We expect that the intensity level of physical activity may not have been vigorous enough to identify a uniform epigenetic signature across participants in this study, as other studies that observed DNAm changes concordant with exercise have included a more intense activity regime (Rönn et al. 2013). Additionally, the variability in CRP may have also originated from differential times of blood collection for each participant, as the non-fasting conditions of our study design could have actually introduced unwanted variation. For example, food consumption, specifically foods high in fat or carbohydrates, prior to blood sampling could have influenced CRP levels, as this has been shown to effect other blood inflammatory markers (Poppitt et al. 2008).

Finally, we explored a potentially novel epigenetic predictor of weight loss using a machine learning approach. The estimated predictor was based on five DNAm sites. Previous work has explored the use of epigenetic loci, specifically near the gene SERPINE-1, to estimate susceptibility to weight loss (Lopez-Legarrea et al. 2013), but we were unable to replicate this in our study sample. Therefore, we put forward a new tool to potentially predict weight loss. The
utility of a biological weight loss predictor has been discussed as having substantial clinical advantages (Moreno-Aliaga et al. 2005), such that specific programs can be tailored to an individual. This predictor, constructed in our small sample, needs to be tested in different cohorts to ensure its validity. Pending that, we expect the predictor may be potentially useful in identifying individuals more or less susceptible to specific weight loss intervention programs.

We note limitations in this study that should be considered when interpreting results. The use of PBMCs instead of a more relevant tissue, such as adipose, was based on feasibility, but does impose a limitation as concordance across these tissues has not been examined at the genomic sites identified. Another limitation stems from the small number of observations in our sample, as a much larger sample size is necessary to confidently establish associations in DNAm studies. In light of the limited power and the potential for over-fitting, our statistical results related to the potential DNAm predictor of weight loss should be interpreted with caution, pending further validation in additional cohorts.

This pilot study was designed to investigate whether changes at the epigenetic level occurred in response to a physical activity intervention, and although we did not find epigenomic effect of physical activity we do highlight a potential signature of body weight loss in the blood methylome. We believe these findings will provide a strong basis for a larger study of this nature.
Acknowledgments

This study is supported by Canadian Institutes of Health Research (CIHR) for operational funds for this project (funding reference number AAM-108607). The sponsors had no role in the study design; collection, analysis, and interpretation of data; writing the report; and the decision to submit the report for publication. All authors declare no competing interests. We gratefully acknowledge the generosity of our study participants and the support of the Centre for Hip Health and Mobility staff. We also acknowledge career award support for MCA from CIHR, Michael Smith Foundation for Health Research and the Canada Research Chair program. MSK is the Canada Research Chair in Social Epigenetics, Senior Fellow of the Canadian Institute for Advanced Research, and Sunny Hill BC Leadership Chair in Child Development. LM McEwen is supported by a CIHR Doctoral Research Award (F15-04283). Lastly, we would like to acknowledge Alexandre Lussier and Nicole Gladish for their editorial contributions.

Disclaimer

The authors report no conflicts of interest associated with this manuscript.
References


Tables

Table 1. Participant baseline characteristics*

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<th>Intervention (n= 12)</th>
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<td>5340 (1966)</td>
<td>6402 (2534)</td>
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<td><strong>MVPA (min/day)</strong></td>
<td>24.3 (32.7)</td>
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<td>67.8 (7.5)</td>
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<td><strong>Diastolic BP (mmHg)</strong></td>
<td>83.4 (9.3)</td>
<td>77.3 (6.9)</td>
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BP=Blood pressure; BMI=body mass index; MVPA=moderate to vigorous physical activity
*These data have been previously published in the original intervention study (Ashe et al. 2015)
### Table 2. Significant CpGs associated with percent weight change over six months

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Figure Captions

Figure 1. Volcano plots of the change in DNA methylation (DNAm) over six months. A) The change in DNAm over six months for 39,268 CpGs was modeled as the dependent variable and intervention group was the independent variable with BMI at baseline and chronological age as covariates. Red points = intervention group lost DNAm compared to controls, blue points = intervention group gained DNAm. B) The change in DNAm over six months modeled as the dependent variable and percent weight change in all samples was the independent variable with BMI at baseline and chronological age as covariates. Red data points = percent weight change was associated with a loss of DNAm compared to controls, blue data points = weight loss associated with a gain of DNAm over six months.

Figure 2. Scatterplot displaying 12 significant CpGs for percent change in weight against DNA methylation (DNAm) β-value change from linear regression analysis. Linear regression line is shown in black with 95% confident intervals displayed by grey shading. Dotted lines represent no change in DNAm and no change in weight.

Figure 3. Actual versus predicted weight percent change from a robust linear regression analysis. Linear regression line is shown in black with 95% confident intervals displayed by grey shading.
$R^2 = 0.84$