A tale of two [adipose] tissues: DNA methylation markers associated with weight-loss and tissue specificity


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Introduction

Epigenetics provides mechanisms whereby environmental factors can influence complex diseases such as obesity and type-two diabetes[1,2]. The nutritional environment in early life can affect the epigenome, leading to changes in gene expression and increased risk of obesity and other complex diseases. Adult epigenome variation is also observed, and significance is further underscored by the potential for heritability.

Gastric bypass provides a model to investigate obesity and weight-loss in humans; it is used to treat obesity and can result not only in significant weight-loss but also substantially reduced type-2 diabetes[3,4,5,6].

Adipose tissue plays a key role in obesity related metabolic dysfunction, and different adipose depots in the body have differing structure, biochemistry and effects on metabolic risk[7,8].

Therefore, one epigenetic mechanism, DNA methylation, was assayed in matched intra-abdominal & subcutaneous abdominal adipose tissue before surgery and after subsequent weight-loss (>27% initial kg) using the Illumina 450K platform.

RESULTS

DNA methylation was investigated independently in intra-abdominal and subcutaneous abdominal adipose tissue in 15 females (clinical and anthropometric data presented in Table 1).

Global patterns of methylation

Global DNA methylation was significantly different before and after weight-loss. Plotting this at the level of genomic gene annotation revealed clear differences in overall methylation between different regions, a pattern consistent between the two adipose tissue types and other tissues (independent publically available data), Figure 1.

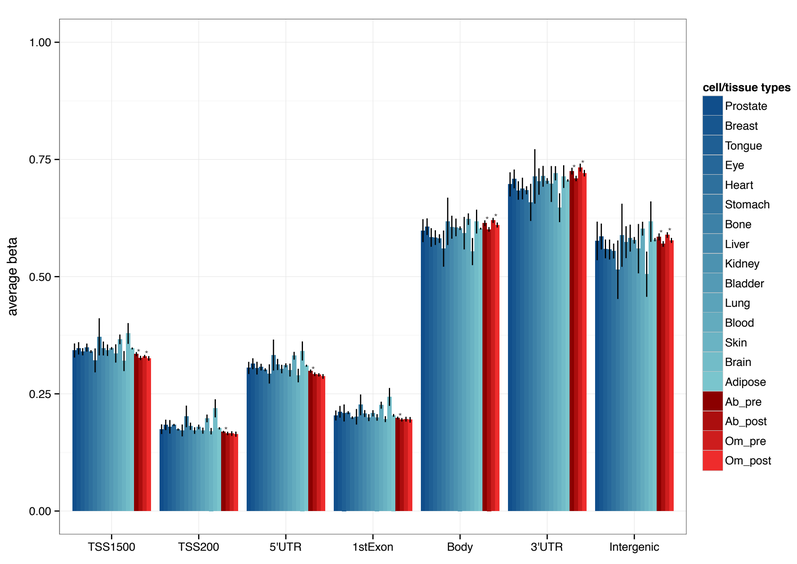

Figure 1: Global methylation profile of subcutaneous abdominal and intra-abdominal adipose tissue and 15 different healthy tissues.

*Mean methylation was calculated across all probes within each gene region (TSS200, TSS1500, 5'UTR, 1st exon, gene body, 3'UTR, intergenic) annotated as the Illumina 450K manifest. Standard deviation is represented by a bar. For subcutaneous abdominal (Ab) and intra-abdominal (Om) adipose a significant difference between tissue taken before (pre) and after (post) weight-loss is shown by *(adj p<0.05). Publically available data for healthy tissues was obtained through catalogues provided by the R package Marmal-aid[9].*

DIFFERENTIAL METHYLATION BEFORE AND AFTER WEIGHT-LOSS

Paired analyses before surgery & after subsequent weight-loss revealed significant differential methylation (Bonferroni $p<1x10^{-7}$) in intra-abdominal & subcutaneous abdominal adipose at 15 & 3601 CpGs respectively; these mapped within 11 and 1889 unique genes, with a greater proportion of CpG sites showing hypermethylation before weight-loss than afterwards (Figure 2). Table 2 provides a summary.

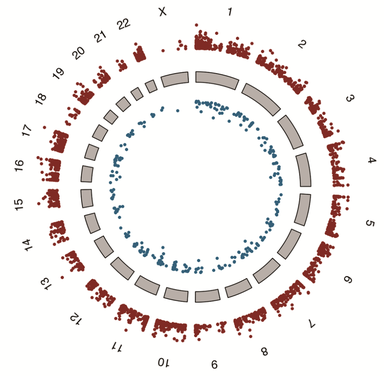

Figure 2: Genomewide differential methylation in subcutaneous adipose tissue before and after gastric bypass and weight-loss.

Difference in methylation (Δbeta) for all CpG sites passing Bonferroni correction is plotted. Δbeta is weighted by t-statistic such that distance from central core (grey) indicates increasing levels of statistical significance. Red dots represent CpG sites hypermethylated and blue dots CpG sites hypomethylated respectively before weight-loss. Chromosomes are shown clockwise from 1 through X.

Table 2: Summary of differentially methylated CpG sites passing Bonferroni correction.

Tissue	Number of CpG sites	CpG sites mapping to known genes (unique)	CpG sites mapping to intergenic regions	More methylated before weight-loss	More methylated after weight-loss	Absolute range Δbeta	Number of CpG sites with Δbeta > 10% (20%)
Intra-abdominal adipose	15	11 (11)	4	14	1	0.034-0.112	1 (0)
Subcutaneous adipose	3601	2458 (1889)	1143	3281	320	0.020-0.273	2401 (145)

Subcutaneous abdominal adipose

In subcutaneous adipose 9/57 and 10/59 obesity and type-two diabetes loci[10] represented on the Illumina 450K platform showed differential methylation at at least one CpG site. Obesity: *LEPR, STAB1, ZNF608, HMGA1, MSRA, TUB, NRXN3, FTO, MC4R;* type-two diabetes: *PROX1, BCL11A, RBMS1, IRS1, TCF7L2, KCNQ1, FTO, LMNB2, INSR, AKT2*. Differential methylation was also observed in genes with roles in epigenome regulation, and development: *DNMT3A, DNMT3L, HDAC7, HDAC4, HDAC7, HDAC10* and *HOX* gene clusters *A, B* and *D*. Pathways analyses of the 1889 unique genes showing robust differential methylation revealed enrichment for inflammation, tissue remodelling, TGFβ, insulin and leptin signalling, and adipogenesis.

Intra-abdominal adipose

4/11 genes showing differential methylation at at least one CpG loci had known or potential roles in obesity, *MYO1C, PLIN4, PARD3* and *PDE7B*. Pathways analysis of the 920 unique genes to which 1347 CpG loci passing a relaxed multiple testing threshold ($p<1x10^{-5}$) mapped revealed enrichment for tissue remodelling, phospholipid binding, and insulin signalling.

Validation

Pyrosequencing of a number of CpG loci showed strong correlations with the Illumina 450K data. Differential mRNA gene expression levels were also investigated for a number of loci for which changes in DNA methylation were observed. Changes in mRNA level before and after weight-loss were seen for *PLIN4* (in intra-abdominal adipose) and *ACACA, CETP, CTGF, S100A8* and *S100A9* (subcutaneous abdominal adipose).

BIOMARKER DETECTION

We applied a combination of statistical tools and graph theoretical algorithms in a machine learning environment to identify putative biomarkers indicative of biological response to gastric bypass surgery.

Differentiation of tissue taken before and after surgery

Initial analysis of subcutaneous adipose revealed 20013 sites with positive merit values. After filtering this number reduced to 6593 sites, which were compelling. However, further analysis revealed that one sample pair was contributing significant noise. When this sample pair was removed the discriminatory power of the methylation signature improved markedly, with the top 100 sites providing almost ideal separation (Figure 3A), and a single site also showing excellent discriminatory power (Figure 3B).

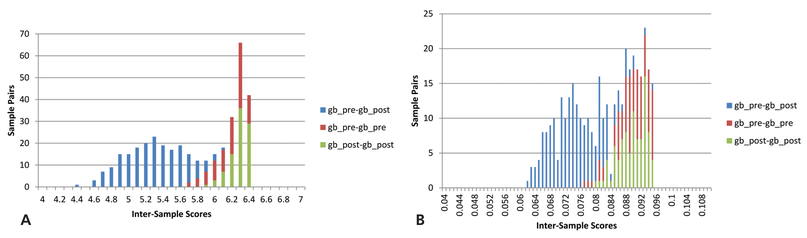

Figure 3. A. Plots showing discriminatory power of A: top 100 CpG sites and B: 1 CpG site for subcutaneous adipose tissue before (gb_pre) and after (gb_post) surgery and weight-loss

When this was repeated for the intra-abdominal tissue 4424 sites passed initial analysis, and 1758 subsequent filtering steps. While the separation obtained was not as clean as for the subcutaneous adipose, intra-abdominal adipose tissues did cluster by sampling time point and once again the top 100 sites performed as well as the initial filter set.

Differentiation of adipose tissue type

Next we used our algorithms to investigate whether the two types of adipose tissue could be distinguished at each time point. This analysis resulted in near perfect discrimination between tissue types both before and after surgery and weight-loss (Figure 4). Interestingly 8624 sites passed filtering after weight-loss, whereas 4900 sites passed in the before surgery comparison.

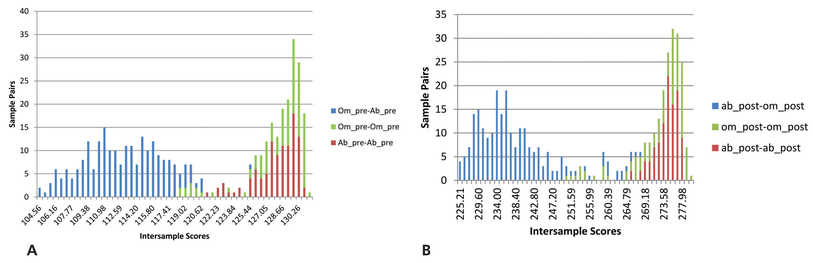

Figure 4. Plots illustrating the discriminatory power of 4900 and 8624 CpG sites to distinguish between subcutaneous abdominal adipose (Ab) and intra-abdominal adipose (Om) before weight-loss (A) and after-weight-loss (B) respectively.

Differentiation of individuals based on metabolic outcome after gastric bypass

Finally, we tested the ability of our approach to distinguish between patient outcomes after gastric bypass and weight-loss. We examined differences in fasting glucose at each time point and categorised individuals as follows: "good response" – decrease observed in fasting glucose or "poor response" – increase, or no difference observed in fasting glucose after gastric bypass and weight-loss. Appropriate data was available for 13 individuals (excluding the previously identified outlier) of which 8 were classified as "good" and 5 as "poor". When we applied our graph theoretical methods to the data for subcutaneous adipose tissue taken before gastric bypass we were able to identify an initial 4689 sites, which on filtering reduced to 2314. This provided a compelling separation (Figure 5A), which showed gradual, but tolerable loss of discriminatory power as the number of sites was reduced to 100 and then to 10 (Figures 5B and 5C respectively).

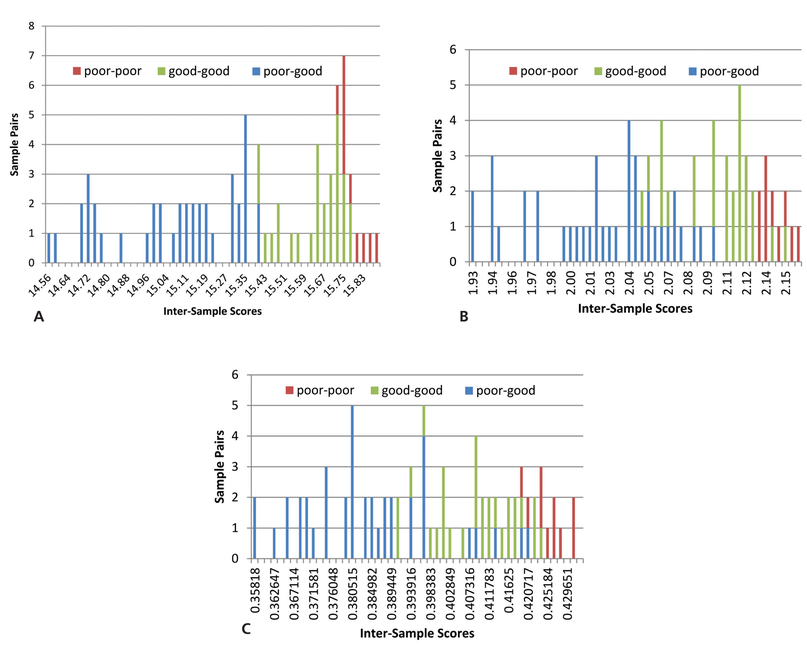

Figure 5: Plots illustrating the discriminatory power of 2314 (A), 100 (B) and 10 (C) CpG sites in subcutaneous adipose before gastric bypass to predict fasting glucose outcome after weight-loss.

DISCUSSION/MAIN POINTS

This study extends analysis of global DNA methylation after gastric bypass to adipose tissue, with one previous report for skeletal muscle[11].

Significant differential DNA methylation was observed in both subcutaneous adipose and intra-abdominal adipose, before and after gastric bypass and weight-loss, with the majority of CpG sites hypermethylated before weight-loss.

The potential discriminatory power of DNA methylation loci as biomarkers is compelling.

Differences between the two adipose tissues likely reflect their distinct biological functions. Subcutaneous adipose acts as an energy storage organ, expanding to "protect" other metabolic tissues from lipid build-up. Weight-loss will have resulted in a sizeable reduction of adipose mass and inflammation, as well as potential tissue remodelling, which is consistent with the pathways enrichment analyses. Perturbation of intra-abdominal adipose is associated with increased risk of obesity related co-morbidities[7,8]. Thus, the different role of this organ and potentially limited tolerance for disruption may be reflected in the observation of significantly less differential methylation than in subcutaneous adipose.

This study provides a foundation for future work focused on the role of epigenetics, & adipose tissue DNA methylation in particular, in obesity, highlighting key loci for functional analyses.

IN CONCLUSION

The potential significance of this work to human health is manifest:

- key loci with potential roles in obesity which are regulated by DNA methylation have been identified – these warrant further investigation.
- methylation biomarkers associated with obesity that are modifiable by gastric bypass & weight-loss have been identified – these may be of clinical utility and potentially receptive to alternative therapeutic inventions such as nutrition or specific drugs.

MATERIALS AND METHODS

Study Individuals: All subjects provided written informed consent and The Central Regional Ethic Committee, Wellington, NZ approved the study. The subjects included 15 obese females undergoing gastric bypass by a single surgeon at Wakefield Hospital, Wellington, NZ who returned for a second operation. Clinical and anthropometric data are summarised in Table 1.

Samples: Intra-abdominal and subcutaneous adipose tissue were biopsied at surgery and immediately snap frozen. Genomic DNA was extracted using a QIAamp DNeasy Tissue Kit (Qiagen).

Methylation: 500ng of genomic DNA was used to profile 485,577 individual cytosines using the Illumina 450K platform, in Eric Hoffman's lab at George Washington University School of Medicine and Health Sciences, Washington DC, USA. Pyrosequence analysis was performed by EpigenDX, USA.

Analysis: Initial analyses were performed using R version 3.03 (http://www.r-project.org), Bioconductor packages and custom bash scripts. Background correction and control normalisation was implemented in minfi, and probes with low intensity, multiple mappings to the human genome (hg19, using BOWTIE2), and located on the Y chromosome were removed. Methylation is expressed as a beta value ranging from 1 "fully" methylated to 0 "fully" unmethylated.

Differential methylation was assessed using a paired samples t test. Corrections for multiple testing were made using Bonferroni ($p<1x10^{-7}$).

The approach we used for discriminatory DNA methylation biomarker detection was based on the case-control differential analysis toolchain described in Langston *et al* 2005[12]. Statistical methods were employed to assign merit values to methylation sites, based on apparent utility in separating pre- and post-operative samples. With clique and dominating set graph algorithms, the site list was winnowed and refined in order to identify those sites that best cover others. A Wilcoxon signed rank test was set to apply a *p<0.001* threshold. Parameterized algorithms were utilised to achieve asymptotic efficiency. Additional technical details[13, 14].

Table 1. Clinical and anthropometric data for individuals in the study.

	Before weight-loss	After weight-loss
Age	44 (+/-10)	
BMI kg/m2	47.6 (+/- 11.3)	29.3 (+/-5.5)
Glucose mmol/L	5.5 (+/- 1.2)	4.6 (+/- 0.6)
Insulin pmol/L	176.2 (+/- 168.1)	33.4 (+/- 19.3)
Triglycerides mmol/L	1.6 (+/- 0.7)	1.0 (+/- 0.3)
Total Cholesterol mmol/L	5.3 (+/- 0.8)	4.6 (+/- 0.6)
HDL mmol/L	1.4 (+/- 0.4)	1.8 (+/- 0.9)
LDL mmol/L	3.2 (+/- 1.1)	2.6 (+/-0.6)
Systolic blood pressure	134 (+/-19)	115 (+/-9)
Diastolic blood pressure	77 (+/- 14)	72 (+/-9)

Means and standard deviations are shown. All blood measures are overnight fasted.

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