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Adipose tissue transcriptomics and epigenomics in low birthweight men and controls: role of high-fat overfeeding

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Abstract

Aims/hypothesis Individuals who had a low birthweight (LBW) are at an increased risk of insulin resistance and type 2 diabetes when exposed to high-fat overfeeding (HFO). We studied genome-wide mRNA expression and DNA methylation in subcutaneous adipose tissue (SAT) after 5 days of HFO and after a control diet in 40 young men, of whom 16 had LBW.

Methods mRNA expression was analysed using Affymetrix Human Gene 1.0 ST arrays and DNA methylation using Illumina 450K BeadChip arrays.

Results We found differential DNA methylation at 53 sites in SAT from LBW vs normal birthweight (NBW) men (false discovery rate <5%), including sites in the *FADS2* and *CPLX1* genes previously associated with type 2 diabetes. When we used reference-free cell mixture adjustments to potentially adjust for cell composition, 4,323 sites had differential methylation in LBW vs NBW men. However, no differences in SAT gene expression levels were identified between LBW and NBW men. In the combined group of all 40 participants, 3,276 genes (16.5%) were differentially expressed in SAT after HFO (false discovery rate <5%) and there was no difference between LBW men and controls. The most strongly upregulated genes were *ELOVL6*, *FADS2* and *NNAT*; in contrast, *INSR*, *IRS2* and the *SLC27A2* fatty acid transporter showed decreased expression after HFO. Interestingly, *SLC27A2* expression correlated negatively with diabetes- and obesity-related traits in a replication cohort of 142 individuals. DNA methylation at 652 CpG sites (including in *CDK5*, *IGFBP5* and *SLC2A4*) was altered in SAT after overfeeding in this and in another cohort.

Conclusions/interpretation Young men who had a LBW exhibit epigenetic alterations in their adipose tissue that potentially influence insulin resistance and risk of type 2 diabetes. Short-term overfeeding influences gene transcription and, to some extent, DNA methylation in adipose tissue; there was no major difference in this response between LBW and control participants.

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Abbreviations

CpG	Cytosine–phosphate–guanine
FDR	False discovery rate
HFO	High-fat overfeeding

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KEGG	Kyoto Encyclopedia of Genes and Genomes
LBW	Low birthweight
NBW	Normal birthweight
OXPHOS	Oxidative phosphorylation
qPCR	Quantitative real-time PCR
SAT	Subcutaneous adipose tissue

Introduction

Overfeeding is a risk factor for type 2 diabetes and may cause insulin resistance even before weight gain develops [1, 2]. Studies have consistently shown that individuals who had a low birthweight (LBW) due to a suboptimal fetal environment have higher risk of developing metabolic diseases including type 2 diabetes [3, 4]. Additionally, young, healthy LBW men develop more severe peripheral insulin resistance when exposed to 5 days of high-fat overfeeding (HFO) compared with men with a normal birthweight (NBW) [1].

Lifestyle-related factors may alter epigenetic and transcriptional patterns [5–12], and thereby affect the risk of metabolic diseases. Indeed, we observed widespread DNA methylation changes in skeletal muscle from healthy young men after 5 days of HFO [7]; in contrast, there were no significant epigenetic changes in matched LBW men after HFO [13]. We also observed extensive DNA methylation changes in subcutaneous adipose tissue (SAT) and skeletal muscle after regular exercise [10, 11]. However, genome-wide DNA methylation plasticity in response to overfeeding in human SAT has not been explored.

Several studies have suggested that insulin resistance is initiated in the adipose tissue and that this may induce insulin resistance in skeletal muscle and liver before the development of obesity and type 2 diabetes [14, 15]. Excess energy intake has unfavourable effects on whole-body insulin sensitivity independent of changes in body weight in both healthy lean [1, 16] and obese diabetic individuals [17]. Additionally, short- and long-term overfeeding may alter adipose tissue histology [18] and gene expression [18–21].

We hypothesised that transcriptional and epigenetic levels in SAT change with short-term HFO, and that the pattern and plasticity of these molecular modifications may be different in SAT from LBW vs NBW individuals, thereby potentially contributing to the metabolic differences seen in LBW individuals. Therefore, we examined the influence of 5 days of HFO on genome-wide mRNA expression and DNA methylation in SAT from 40 healthy young men, of whom 16 had a LBW and 24 were matched NBW controls. To replicate our epigenetic results in another cohort, we analysed DNA methylation in response to 7 weeks of overfeeding in SAT samples from another study.

Methods

Study participants and diets

HFO cohort This study included 40 healthy young men recruited from the Danish National Birth Registry [22]. Sixteen men had a LBW (≤10th percentile) and 24 were age- and BMI-matched controls who had a NBW (≥50th percentile and ≤90th percentile). These individuals form part of a dietary challenge study which includes 46 individuals; their detailed clinical characteristics have been described [1, 16]. Using a randomised crossover design, each participant received an individualised weight-maintaining 3-day control diet (35% of energy from fat) and a 5-day HFO diet (50% more energy, 60% of energy from fat) prior to clinical examination and SAT biopsy excision [16]. The diets were separated by a washout period of 6–8 weeks. The local ethics committee approved the study [22].

All individuals underwent a 3 h hyperinsulinaemic–euglycaemic clamp to estimate hepatic and peripheral insulin sensitivities [22] and the HOMA-IR was calculated [23]. Abdominal SAT biopsies were excised under local anaesthesia both during fasting and after insulin stimulation; these were frozen in liquid nitrogen within 30 s.

Twin cohort A total of 142 elderly monozygotic and dizygotic twins were included for replication of SAT gene expression analysis. Their clinical characterisation and SAT biopsies were performed as part of a larger study [24].

LIPOGAIN cohort Seventeen healthy young individuals from another diet intervention study were included for replication of overfeeding-induced DNA methylation changes in SAT [20]. Abdominal SAT biopsies were excised at baseline and after 7 weeks of overfeeding in which muffins high in saturated fatty acids were added to the habitual diet.

Genome-wide mRNA expression analysis

Total RNA was extracted from SAT using miRNeasy MiniKits and purified from small RNA molecules with RNeasy MiniElute Cleanup Kits (Qiagen, Valencia, CA, USA). RNA quality was assessed (RNA integrity number: mean, 8.3; range, 6.8–9.0) using a Bioanalyzer (Agilent Technologies, Basel, Switzerland). The expression of 28,829 transcripts representing 19,880 genes was analysed using GeneChip Human Gene 1.0 ST arrays (Affymetrix, Santa Clara, CA, USA) following the Affymetrix protocol. Data were background-corrected and quantile-normalised with a robust multiarray average

('RMA') approach using dedicated Bioconductor software [25].

Genome-wide DNA methylation analysis

Genomic DNA was isolated from SAT using QIAamp DNA Micro Kits (Qiagen; HFO cohort) and DNeasy Blood & Tissue Kits (Qiagen; LIPOGAIN cohort). DNA (500 ng) was bisulphite converted using EZ DNA Methylation Kits (Zymo Research, Irvine, CA, USA) and DNA methylation of 485,577 cytosines covering 21,231 genes was analysed with Infinium HumanMethylation450 BeadChip arrays (Illumina, San Diego, CA, USA) [26] according to the Infinium Protocol (User Guide part #15019519; HFO and LIPOGAIN cohorts). Paired samples (control and overfeeding) were placed on the same chip, and samples from LBW and NBW individuals were evenly distributed (see electronic supplementary material [ESM] Methods).

Technical validation by quantitative real-time PCR

Quantitative real-time PCR (qPCR) was used to analyse the mRNA expression of five genes in SAT from all men for whom cDNA was available (HFO cohort; see ESM Methods).

Statistical analysis

Overfeeding-induced expression and methylation changes were analysed using paired Wilcoxon tests (i.e. for HFO vs the control diet) based on the randomised crossover design [1, 16]. Similarly, paired Wilcoxon tests were used to analyse overfeeding-induced DNA methylation changes in the LIPOGAIN cohort. Comparisons between birthweight groups were made using two-sample Mann–Whitney tests. To account for multiple testing, we applied Benjamini–Hochberg false discovery rate (FDR) correction; a q value of <0.05 was considered significant. In addition to these tests, methylation data were adjusted for potential differences in cell mixture using the reference-free adjustment method recently developed by Houseman et al [27]. Here, we also adjusted for age, BMI and, for paired comparisons, birthweight. Random-effect linear models (PROC MIXED) in the twin cohort were adjusted for sex, age and, where appropriate, BMI using SAS Enterprise Guide (version 6.1, SAS Institute, Cary, NC, USA). This model adjusts for twin pair membership (a random-effects term) and zygosity (a fixed-effects term). According to a post hoc power calculation, we had 95% power to detect an absolute DNA methylation difference of 2% (SD 1.5%) in 16 LBW vs 18 NBW participants.

Results

Clinical characteristics

The men who had a LBW were shorter, had higher fasting glucose levels and were more insulin resistant based on HOMA-IR compared with NBW men (ESM Table 1). Peripheral insulin sensitivity and fasting levels of insulin, NEFA and triacylglycerols were not significantly different between the LBW and NBW groups during control diet feeding.

The clinical response of the 40 men to HFO is presented in Table 1. After 5 days of HFO, fasting levels of glucose and insulin were significantly increased, resulting in an elevated HOMA-IR. Hepatic insulin resistance increased and peripheral insulin sensitivity showed a tendency to decrease ($p = 0.06$). Fasting NEFA, triacylglycerol and VLDL-cholesterol levels were significantly lower and insulin-stimulated NEFA levels were higher after HFO than after the control diet (Table 1).

Impact of birthweight on gene expression and DNA methylation in SAT

After correction for multiple testing ($q < 0.05$), we found no significant differences in genome-wide SAT expression between LBW and NBW men during the control diet or after HFO. However, we found 13 cytosine–phosphate–guanine (CpG) sites during the control diet and 40 other sites after the HFO that had differential DNA methylation levels in 16 LBW compared with 18 NBW men ($q < 0.05$; Table 2), including sites in the *ACAT1*, *CPLX1*, *FADS2*, *GPRC5B*, *HCCA2* and *IGF2R* genes that have previously been associated with type 2 diabetes (ESM Table 2). The known or suspected general function and the potential diabetes-related function of all differentially methylated genes are presented in ESM Table 2. Five of the 13 sites with significant between-group differences under control diet conditions were also differentially methylated after HFO ($p < 0.05$). Likewise, nine of the 40 sites that were significantly different between birthweight groups during HFO were also differentially methylated during the control diet (Table 2). We investigated the DNA methylation response to HFO separately in LBW and NBW men and found no significant changes in either group.

Changes in SAT expression in response to HFO

Since there were no significant differences in mRNA expression between LBW and NBW men, the impact of 5 days of HFO on SAT gene expression was examined in both groups combined.

Interestingly, expression of 12.9% (3,713) of all analysed transcripts and 16.5% (3,276) of all analysed genes was significantly changed in SAT after the HFO compared with the control diet ($q < 0.05$; ESM Table 3): of these transcripts,

Table 1 Clinical and metabolic characteristics of the 40 young men in the HFO cohort

Variable	Control diet	High-fat overfeeding
Body composition		
Weight (kg)	78.3 ± 9.3	78.5 ± 9.8
BMI (kg/m^2)	24.0 ± 3.0	24.1 ± 3.1
Body fat (%)	19.1 ± 7.4	19.0 ± 7.3
WHR	0.88 ± 0.05	0.89 ± 0.05
Glucose and Insulin profile		
HbA$_{1c}$ (%)	5.2 ± 0.3	5.2 ± 0.2
HbA$_{1c}$ (mmol/mol)	33	33
Fasting plasma glucose (mmol/l)	4.8 ± 0.5	5.1 ± 0.4**
OGTT 2 h glucose (mmol/l)	4.9 ± 0.5	5.2 ± 0.5**
Fasting serum insulin (pmol/l)	36.0 ± 14.9	45.0 ± 26.7**
Insulin sensitivity		
HOMA-IR	1.1 ± 0.5	1.5 ± 0.9**
M value^a (mg [kg FFM]$^{-1}$ min^{-1})	13.8 ± 2.7	12.9 ± 3.5§
Hepatic insulin resistance index	83.3 ± 42.8	114.8 ± 59.1**
Lipid profile		
Fasting NEFA (μmol/l)	340.7 ± 197.7	197.7 ± 87.6**
Insulin stimulated NEFA (μmol/l)	9.3 ± 4.7	13.1 ± 6.9**
Fasting total cholesterol (mmol/l)	4.4 ± 0.8	4.2 ± 0.8
Fasting HDL-cholesterol (mmol/l)	1.3 ± 0.3	1.5 ± 0.3
Fasting LDL-cholesterol (mmol/l)	2.6 ± 0.8	2.4 ± 0.8
Fasting VLDL-cholesterol (mmol/l)	0.46 ± 0.17	0.33 ± 0.15**
Fasting triacylglycerols (mmol/l)	1.0 ± 0.4	0.7 ± 0.3**
Leptin (μg/ml)	4.6 ± 3.4	5.2 ± 3.7**
Adiponectin (μg/ml)	42.8 ± 24.2	51.3 ± 22.6**

Data are mean ± SD

Clinical data for the HFO population has already been published [1, 16]

^{a} Insulin-stimulated glucose disposal rate

Significant differences for paired comparisons between the control and HFO diets (**$p \leq 0.01$, §$p = 0.06$)

59% (2,207) were upregulated and 41% (1,506) were downregulated. Genes with the most pronounced increase (including *ELOVL6*, *FADS2*, *NNAT* and *DGAT2*) and decrease (including *SLC27A2* and *CIDEA*) in transcription are presented in Table 3. A total of 1,469 (40%) of the genes that showed significant differences in expression changed more than 10% (ESM Table 3).

We used Webgestalt (http://bioinfo.vanderbilt.edu/webgestalt) to test whether functionally related genes were systematically altered with HFO. Significantly upregulated transcripts could be mapped to 40 Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways that predominantly related to metabolism and energy metabolism (Fig. 1a, ESM Table 4). Oxidative phosphorylation (OXPHOS; Fig. 1b–f), citrate cycle and pyruvate metabolism were the most significant KEGG pathways among transcripts that were upregulated by HFO. Moreover, many genes involved in the fatty acid metabolism, glycolysis/gluconeogenesis and biosynthesis of unsaturated fatty acids pathways were upregulated by HFO (Fig. 2a–c).

TGF-beta signalling was the only significantly enriched KEGG pathway among transcripts that were downregulated by HFO (ESM Table 5). Notably, genes in the insulin signalling pathway were either upregulated (27 genes) or downregulated (16 genes; ESM Tables 3 and 4): downregulated genes included *INSR* and *IRS2*; and upregulated genes included *AKT1, AKT2, IRS1* and *SLC2A4* (also known as *GLUT4*; Fig. 2d). A literature search was done to investigate whether genes of potential importance in adipose tissue were differentially expressed with HFO. We found that several adipose-derived hormones (adipokines) [14] and adipogenesis-induced transcription factors [28] were up- or downregulated by HFO (Fig. 2e, f). Moreover, two white adipose tissue markers (*HOXC8*, *HOXC9*) that are downregulated in human brown adipose tissue [29] were upregulated by HFO (ESM Table 3).

Table 2 CpG sites with differential DNA methylation (%) in LBW ($n = 16$) vs NBW ($n = 18$) men under control and HFO conditions

Gene symbol	Target ID	Gene region	DNA methylation (%)		Diff (%)	p value	q value	Diff (%)[a]	p value
			LBW ($n = 16$), mean ± SD	NBW ($n = 18$), mean ± SD					
Control diet								High-fat overfeeding	
C17orf97	cg11946719[b, d]	Intergenic	17.0 ± 2.9	26.1 ± 4.5	−9.1	4×10^{-8}	0.014	−7.0	1×10^{-4}
	cg19590578	Intergenic	69.7 ± 1.9	74.5 ± 1.9	−4.8	9×10^{-8}	0.014		
	cg20698501[b, d]	TSS1500	14.0 ± 2.5	21.5 ± 4.0	−7.4	9×10^{-8}	0.014	−6.6	2×10^{-4}
KAZALD1	cg12060422[b, d]	1st exon; 5 UTR	11.5 ± 2.7	7.8 ± 0.9	3.8	2×10^{-7}	0.023	2.0	0.002
	cg23045594	Intergenic	3.9 ± 0.4	4.5 ± 0.5	−1.1	2×10^{-7}	0.023		
SORBS2	cg18625630	Body; 5 UTR	82.8 ± 1.5	79.2 ± 1.9	3.6	3×10^{-7}	0.027		
	cg08035479[b, d]	Intergenic	78.8 ± 5.7	86.2 ± 2.3	−7.4	6×10^{-7}	0.032	−5.8	0.001
DPP10	cg05868547	Body	70.9 ± 1.0	73.5 ± 1.4	−2.6	6×10^{-7}	0.032		
C11orf35	cg00716245	Body	4.7 ± 0.4	3.8 ± 0.4	0.9	8×10^{-7}	0.032		
	cg09158015[b, d]	Intergenic	31.3 ± 7.0	18.1 ± 5.4	13.2	8×10^{-7}	0.032	10.6	9×10^{-5}
DNAJC22	cg11303127	TSS1500	4.1 ± 0.3	4.8 ± 0.4	−0.7	8×10^{-7}	0.032		
	cg19949955	Intergenic	9.2 ± 1.2	7.4 ± 0.7	1.9	8×10^{-7}	0.032		
MRPL51	cg22379905	3 UTR	88.0 ± 0.8	86.2 ± 1.1	1.8	1×10^{-6}	0.039		
High-fat overfeeding								Control diet	
KIF26A	cg13995140[c, d]	Body	68.6 ± 3.0	60.7 ± 3.8	7.9	2×10^{-7}	0.040	6.8	2×10^{-5}
	cg20758882	Intergenic	4.6 ± 0.4	5.7 ± 0.6	−1.1	3×10^{-7}	0.040		
PRDM12	cg13726887	Body	15.8 ± 1.4	12.4 ± 1.4	3.3	5×10^{-7}	0.040		
CPLX1	cg02689072[c, d]	Body	72.5 ± 4.5	63.5 ± 3.8	9.1	6×10^{-7}	0.040	8.7	0.003
CACNA2D2	cg11799511	Body	95.8 ± 0.4	94.6 ± 0.8	1.1	6×10^{-7}	0.040		
	cg13937361	Intergenic	89.1 ± 0.7	87.5 ± 0.7	1.5	8×10^{-7}	0.040		
FIGNL1	cg03731078	TSS1500	14.3 ± 1.0	16.5 ± 1.2	−2.3	1×10^{-6}	0.040		
FADS2	cg07999042[c, d]	Body	75.8 ± 2.5	71.1 ± 2.2	4.8	1×10^{-6}	0.040	3.9	3×10^{-4}
LOC100271832	cg09504692	Body	88.5 ± 1.0	86.0 ± 1.5	2.5	1×10^{-6}	0.040		
CUGBP2	cg11002404	5 UTR; 1st exon	4.2 ± 0.3	5.0 ± 0.3	−0.8	1×10^{-6}	0.040		
TTYH3	cg19506025	TSS200	8.2 ± 0.8	10.0 ± 1.0	−1.8	1×10^{-6}	0.040		
SEC22A	cg06652349[c, d]	TSS1500	82.7 ± 1.3	80.1 ± 1.4	2.6	1×10^{-6}	0.040	1.7	0.036
HCCA2	cg16723180	Body	84.8 ± 0.9	82.5 ± 1.2	2.3	1×10^{-6}	0.040		
	cg24487639[c, d]	Intergenic	12.1 ± 3.7	7.0 ± 1.7	5.1	1×10^{-6}	0.040	5.0	1×10^{-5}
SLC29A4	cg02632809[c, d]	Body	23.8 ± 1.6	26.9 ± 1.3	−3.1	2×10^{-6}	0.040	−2.1	0.001
PTPRN2	cg06094238	Body	82.1 ± 1.4	78.9 ± 1.8	3.2	2×10^{-6}	0.040		
ACAT1	cg07364154	3 UTR	89.3 ± 1.3	86.7 ± 1.3	2.6	2×10^{-6}	0.040		
NCLN	cg10554989	TSS200	5.8 ± 0.4	6.6 ± 0.6	−0.9	2×10^{-6}	0.040		
C7orf50	cg16079645	Body	72.0 ± 1.2	69.5 ± 1.2	2.6	2×10^{-6}	0.040		
LMTK2	cg20071506	3 UTR	78.3 ± 1.3	75.5 ± 1.2	2.8	2×10^{-6}	0.040		
CASZ1	cg20462360	Body	8.2 ± 0.6	9.7 ± 0.9	−1.5	2×10^{-6}	0.040		
	cg25887076	Intergenic	94.8 ± 0.3	93.9 ± 0.5	0.9	2×10^{-6}	0.040		
TUBG2	cg10180165	TSS1500	86.3 ± 1.0	84.3 ± 0.8	2.0	2×10^{-6}	0.040		
MIER2	cg11273000	Body	73.1 ± 1.7	69.9 ± 1.5	3.2	2×10^{-6}	0.040		
IGF2R	cg25484127	Body	88.9 ± 0.7	87.3 ± 0.8	1.5	2×10^{-6}	0.040		
	cg00459845[c, d]	Intergenic	34.6 ± 3.0	29.2 ± 2.6	5.4	3×10^{-6}	0.040	2.9	0.012
	cg01995468	Intergenic	5.5 ± 0.7	4.5 ± 0.4	1.0	3×10^{-6}	0.040		
USF1	cg03430800	TSS200	9.6 ± 0.4	10.5 ± 0.6	−1.0	3×10^{-6}	0.040		
FAM83H	cg08975720[c]	TSS200	10.1 ± 0.5	11.2 ± 0.5	−1.1	3×10^{-6}	0.040	−0.7	0.046
GPRC5B	cg09141413	TSS200	16.0 ± 1.0	17.8 ± 0.9	−1.8	3×10^{-6}	0.040		
PDLIM4	cg11843238[c, d]	TSS200	9.3 ± 1.8	6.5 ± 0.8	2.8	3×10^{-6}	0.040	2.0	0.008

Table 2 (continued)

Gene symbol	Target ID	Gene region	DNA methylation (%)		Diff (%)	p value	q value	Diff (%)[a]	p value
			LBW ($n = 16$), mean ± SD	NBW ($n = 18$), mean ± SD					
PRR5;ARHGAP8	cg14699765	Body; 5 UTR	82.5 ± 1.4	79.6 ± 1.8	2.9	3×10^{-6}	0.040		
CREB3L2	cg15975598	Body	86.2 ± 1.0	84.0 ± 1.1	2.2	3×10^{-6}	0.040		
MFN2	cg16040838	TSS200	3.4 ± 0.2	3.9 ± 0.3	−0.5	3×10^{-6}	0.040		
ARHGAP23	cg19735097	Body	84.1 ± 1.4	81.0 ± 1.6	3.1	3×10^{-6}	0.040		
NSMAF	cg22754569	Body	90.0 ± 0.6	88.4 ± 1.1	1.6	3×10^{-6}	0.040		
LNPEP	cg01180046	TSS200	4.1 ± 0.2	4.9 ± 0.6	−0.8	4×10^{-6}	0.047		
ARID1B	cg03561416	Body	80.4 ± 1.3	77.7 ± 1.5	2.7	4×10^{-6}	0.047		
UPK3A	cg09536337	Body; body	81.9 ± 1.3	79.6 ± 0.9	2.3	4×10^{-6}	0.047		
TCTN3	cg12748703	TSS200	2.4 ± 0.1	2.7 ± 0.1	−0.3	4×10^{-6}	0.047		

Data are means ± SD

The q values are adjusted for multiple testing (Benjamini–Hochberg FDR correction)

[a] Methylation differences ($p < 0.05$) between LBW and NBW groups (mean % for LBW minus mean % for NBW) after the diet (HFO/control) that is not represented in the column to the left

[b, c] The CpG site is also differentially methylated in the same direction in LBW vs NBW men during [b] HFO and [c] control diet ($p < 0.05$)

[d] The CpG site is differentially methylated in the same direction in LBW vs NBW men during both HFO and control diet ($q < 0.05$) after adjustment for age, BMI and potential differences in cell composition (Houseman et al [27])

Diff, difference

Comparison with mRNA data from other overfeeding studies

We found a large overlap in the transcriptional response to overfeeding in SAT between our HFO study and two previous short-term (7–14 days) overfeeding studies (ESM Table 6) [18, 19].

Technical validation of gene expression microarray data

There were significant correlations between microarray and qPCR expression data for all tested genes (CIDEA, ELOVL6, FADS2, GYS2 and SLC27A2) and significant changes in HFO-induced mRNA expression (up- or downregulation) identified with the microarray were confirmed by qPCR (ESM Table 7).

Replication of SLC27A2 expression in SAT

SLC27A2 encodes FATP-2 which functions as both a fatty acid transporter and an acyl-CoA synthetase that drives fatty acid uptake by trapping activated fatty acids (acyl-CoA esters) inside the cell [30]. We used qPCR to analyse SLC27A2 expression in SAT obtained during fasting and after hyperinsulinaemic–euglycaemic clamps to gain insight into in vivo regulation of this gene. SLC27A2 expression was lower in SAT biopsies obtained after the HFO compared with the control diet under both fasting and insulin-stimulated conditions (Fig. 3a–c). Moreover, hyperinsulinaemia reduced

SLC27A2 expression during the control diet but not during HFO (ESM Fig. 1a, b).

To obtain further insight into the biological role of SLC27A2, we analysed expression of this gene in SAT biopsies from 142 elderly monozygotic and dizygotic twins with a broad range of BMIs (17–39 kg/m^2) and different glucose tolerance statuses (ESM Table 8). SLC27A2 expression did not differ between sexes ($p = 0.87$) or between diabetic and non-diabetic individuals ($p = 0.38$; ESM Fig. 2a, b). However, SLC27A2 mRNA expression correlated negatively with several diabetes- and obesity-related traits (i.e. fasting glucose, fasting insulin, insulin resistance, BMI, WHR and triacylglycerol levels; ESM Table 9). We subsequently measured Spearman's correlation coefficients between SLC27A2 expression and clinical characteristics in the HFO cohort. In this analysis, SLC27A2 expression also correlated negatively with obesity- and diabetes-related traits such as BMI, HOMA-IR and plasma leptin levels during both diets (ESM Fig. 3a–d, ESM Table 9).

Changes in SAT DNA methylation in response to HFO

To investigate the effect of 5 days of HFO on the SAT epigenome, DNA methylation at 461,368 cytosines was analysed in SAT from 18 NBW and 16 LBW men after each diet. In all, methylation was significantly changed at 21,881 sites with HFO ($p < 0.05$; ESM Table 10). However,

Table 3 Transcripts with the largest increase (top 0.5%, 11 transcripts) and decrease (top 0.5%, 8 transcripts) in mRNA expression in response to HFO in SAT from all men ($n = 40$)

Gene symbol	Gene name	Probe ID	Gene expression (AU)		Fold change	q value
			Control diet, mean ± SD	HFO diet, mean ± SD		
Genes with increased mRNA expression in response to HFO						
ELOVL6	ELOVL fatty acid elongase 6	8102342	195 ± 126	683 ± 501	3.51	4×10^{-6}
FADS2	Fatty acid desaturase 2	7940565	855 ± 453	1,602 ± 558	1.87	5×10^{-7}
NNAT	Neuronatin	8062395	2,882 ± 988	5,048 ± 1,523	1.75	2×10^{-7}
DGAT2	Diacylglycerol O-acyltransferase 2	7942613	2,350 ± 810	4,092 ± 1,022	1.74	7×10^{-7}
ACLY	ATP citrate lyase	8015460	832 ± 249	1,396 ± 640	1.68	2×10^{-6}
TTTY9A	Testis-specific transcript, Y-linked 9A (non-protein coding)	8176692, 8177195	85 ± 36	141 ± 64	1.66	1×10^{-6}
LRRN3	Leucine rich repeat neuronal 3	8135488	230 ± 85	375 ± 139	1.63	2×10^{-6}
GYS2	Glycogen synthase 2 (liver)	7961673	139 ± 81	225 ± 133	1.62	0.0005
CKMT2	Creatine kinase, mitochondrial 2 (sarcomeric)	8106689	376 ± 120	604 ± 224	1.61	2×10^{-7}
SCD	Stearoyl-CoA desaturase (Δ-9-desaturase)	7935776	437 ± 164	703 ± 178	1.61	6×10^{-7}
SLC2A5	Solute carrier family 2 (facilitated glucose/ fructose transporter), member 5	7912224	98 ± 20	156 ± 59	1.60	1×10^{-6}
Genes with decreased mRNA expression in response to HFO						
SLC27A2	Solute carrier family 27 (fatty acid transporter), member 2 (FATP2)	7983650	273 ± 207	110 ± 88	−2.48	2×10^{-7}
GSDMB	Gasdermin B	8014903	465 ± 155	308 ± 99	−1.51	2×10^{-7}
THBS1	Thrombospondin 1	7982597	1,794 ± 784	1,225 ± 662	−1.46	5×10^{-5}
UTS2D	Urotensin 2 domain containing	8092741	172 ± 81	118 ± 45	−1.46	3×10^{-6}
PLA2G2A	Phospholipase A2, group IIA (platelets, synovial fluid)	7913216	1,000 ± 541	690 ± 374	−1.45	0.0001
PTCHD3	Patched domain containing 3	7932727	170 ± 174	120 ± 110	−1.43	0.0006
CIDEA	Cell death-inducing DFFA-like effector a	8020211	1,395 ± 471	979 ± 333	−1.42	7×10^{-7}
AGPAT9	1-Acylglycerol-3-phosphate O-acyltransferase 9	8096116	232 ± 142	166 ± 86	−1.40	0.0008

Data are means ± SD

The q values are adjusted for multiple testing (Benjamini–Hochberg FDR correction)

no individual methylation site had a q value of <0.05 after FDR correction.

We then merged data on genes with significant alterations in both mRNA expression (3,276 genes) and DNA methylation ($p < 0.05$). Here, the methylation level at 3,102 sites covering 1,648 genes changed with HFO ($p < 0.05$; ESM Table 11). Interestingly, the most significantly enriched gene sets among genes with both altered methylation and mRNA expression after HFO were mapped to the oxidative phosphorylation (34 genes) and insulin signalling (36 genes) KEGG pathways (ESM Table 12).

Epigenetic modifications can regulate gene expression [31]; therefore, we measured Spearman's correlation coefficients (r values) between mRNA and DNA methylation levels for genes that changed both their mRNA expression ($q < 0.05$) and DNA methylation status ($p < 0.05$) in SAT with HFO. Although negative or positive correlations with a p value of <0.05 were identified for 213 CpG methylation–mRNA expression combinations, none had a q value of <0.05 after FDR correction (ESM Table 13).

Replication of DNA methylation changes

To biologically replicate our DNA methylation findings from the HFO cohort in another cohort, we analysed genome-wide DNA methylation in SAT samples from 17 young individuals from the LIPOGAIN study in whom 7 weeks of overfeeding caused increased weight, BMI and HDL levels (ESM Table 14). We found 541 sites with increased and 111 sites with decreased methylation after overfeeding that were common to both cohorts ($p < 0.05$; ESM Table 15). Of the genes with DNA methylation changes in both cohorts, 112 were also differentially expressed ($q < 0.05$). These included CDK5, CIDEA, IGFBP5 and SLC2A4, which were previously linked to adipose tissue metabolism and/or differentiation (Fig. 3d–g, ESM Table 16).

Impact of HFO on enzymes regulating DNA methylation

To further dissect the mechanism controlling how overfeeding may alter DNA methylation in SAT, we

Fig. 1 Gene sets and OXPHOS genes with significantly increased mRNA expression in SAT after 5 days of HFO in all men ($n = 40$). (**a**) Twelve of the most significantly enriched KEGG pathways representing the genes with increased expression after HFO. Black bars, observed number of genes; white bars, expected number of genes; number in parenthesis, total number of genes in each pathway. $q < 0.05$ vs the expected number of genes (after Benjamini–Hochberg FDR correction). (**b–f**) Genes in the oxidative phosphorylation pathway that are significantly upregulated with HFO (black bars) compared with the control diet (white bars). (**b–f**) Genes in OXPHOS complexes are shown separately: (**b**) I, (**c**) II, (**d**) III, (**e**) IV and (**f**) V. †$q < 0.05$ (after FDR correction) vs the control diet. AU, arbitrary unit; PPAR, peroxisome proliferator-activated receptor; TCA, tricarboxylic acid
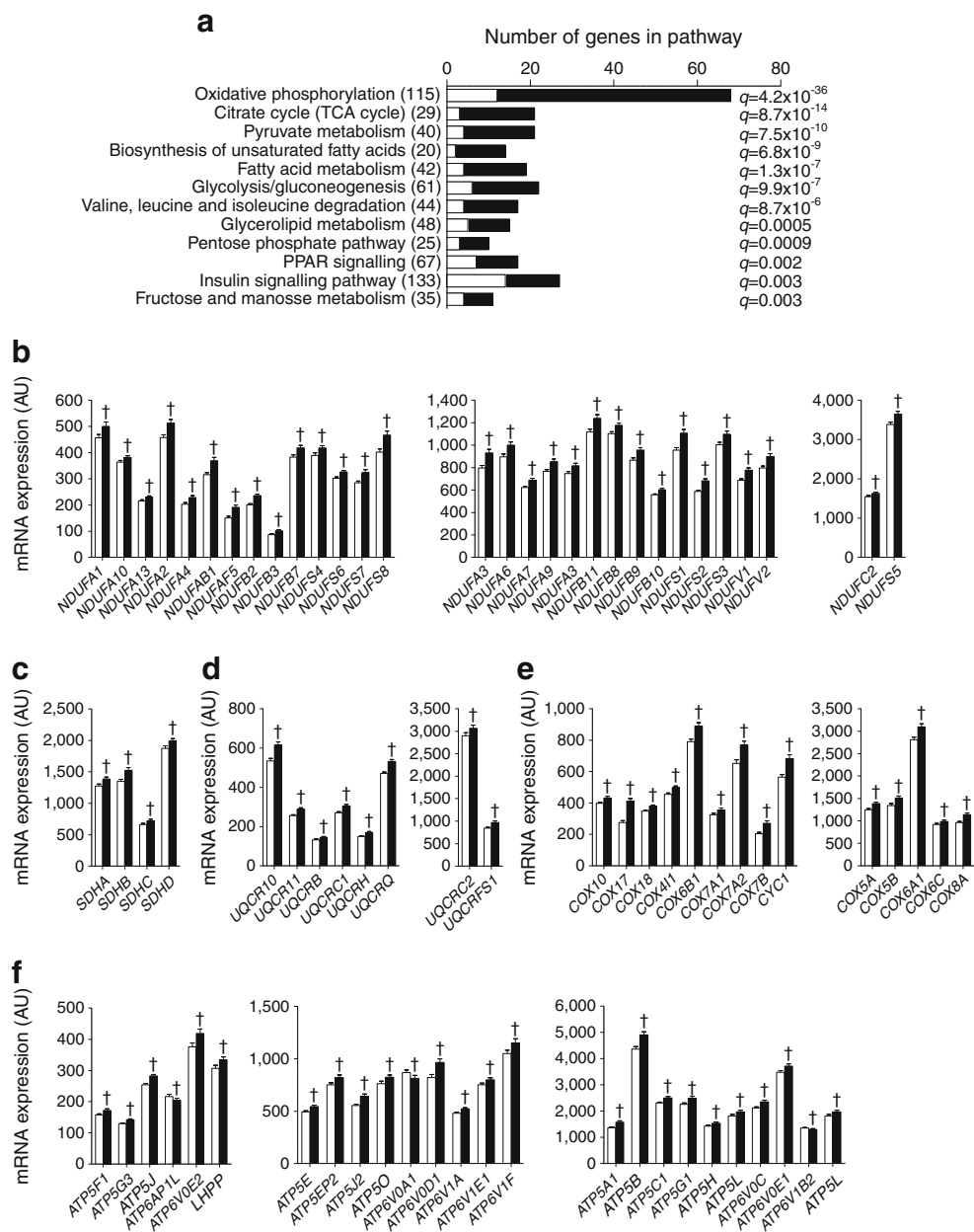
investigated the expression of genes encoding proteins that are important for regulating DNA methylation. *DNMT1* and genes encoding enzymes and modifiers involved in one-carbon metabolism and the generation of methyl donors were among the genes with altered expression after HFO (ESM Table 17).

Impact of birthweight and HFO on DNA methylation in SAT after reference-free cell mixture adjustment

Adipose tissue contains several different cell types, including adipocytes, fibroblasts, stem cells and immune cells. Therefore, differences in the cellular composition may affect the overall DNA methylation pattern in

adipose tissue. Using data on genome-wide DNA methylation patterns measured in purified cell types, one may perform cell mixture adjustments when studying DNA methylation in tissues and/or mixed cell populations. However, no such data currently exist for purified cells from adipose tissue. Instead, we used a reference-free cell mixture adjustment method recently developed by Houseman et al [27] to potentially adjust our methylation data for differences in cell mixture. Using this method, methylation at 27,999 sites during control diet and 31,866 sites during HFO was found to significantly differ in SAT from LBW vs NBW men ($q < 0.05$; ESM Table 18), including all 53 CpG sites that showed significant differences before cell
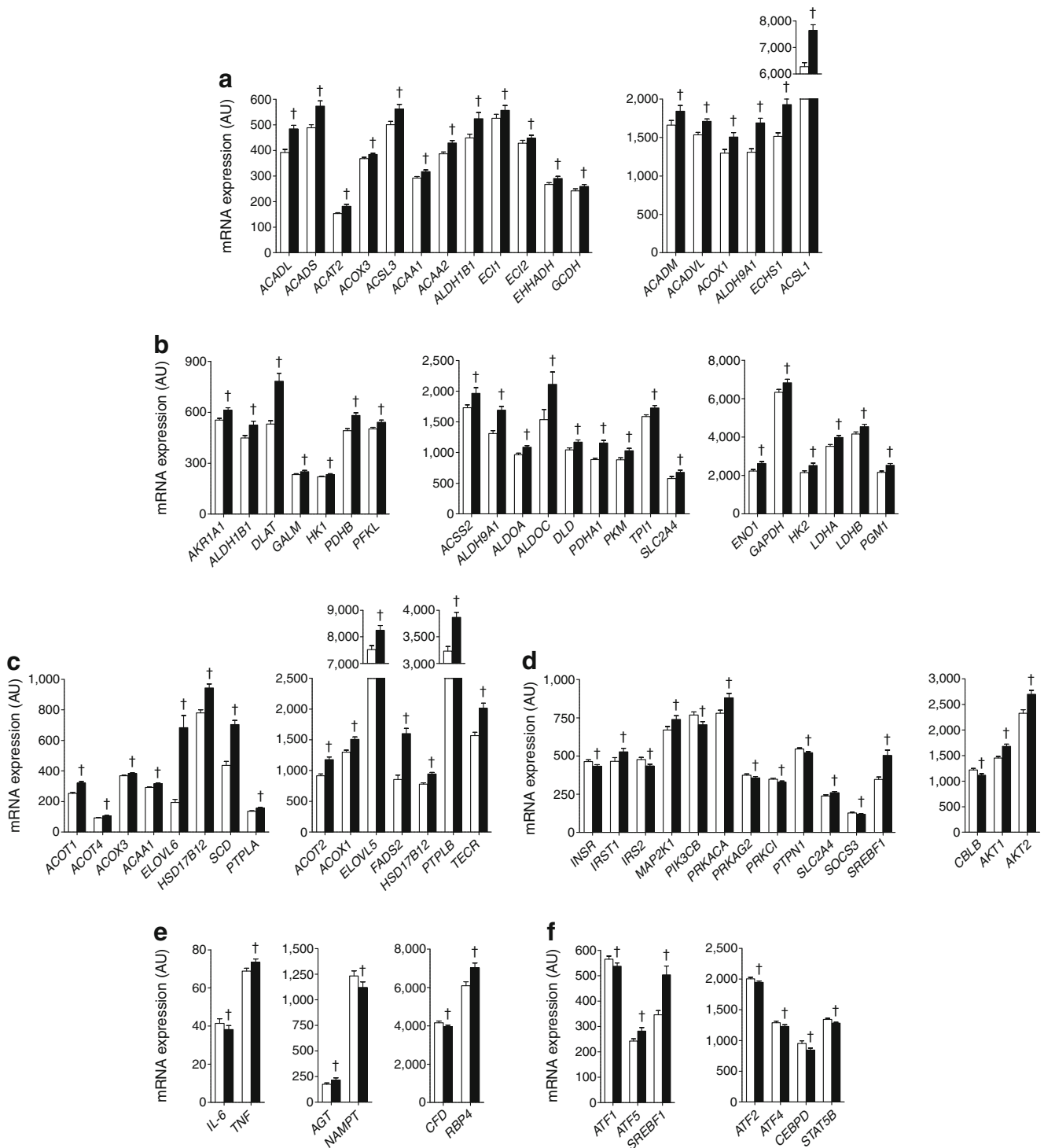
Fig. 2 Metabolically important genes with differential mRNA expression in SAT with 5 days of HFO (black bars) compared with the control diet (white bars) ($n = 40$). (**a–c**) Significantly upregulated genes in the (**a**) fatty acid metabolism, (**b**) glycolysis/gluconeogenesis and (**c**) biosynthesis of unsaturated fatty acids KEGG pathways. (**d–f**) Selected genes in the (**d**) insulin signalling KEGG pathway and (**e**) adipokines and (**f**) adipogenesis-specific transcription factors that were significantly up- or downregulated after 5 days of HFO. $^{†}q < 0.05$ (after FDR correction) vs the control diet

admixture adjustment (Table 2). Of these, 4,323 sites annotated to 2,448 genes differed significantly between LBW and NBW men under both control diet and HFO

conditions ($q < 0.05$; ESM Table 19). These included 13 out of the 14 CpG sites that had methylation differences during both diets before adjustment, including *CPLX1*

Fig. 3 Overfeeding-induced
mRNA expression and DNA
methylation changes in genes
important for metabolism and
insulin resistance, and the impact
of LBW on DNA methylation in
SAT. (a–c) Impact of 5 days of
HFO on *SLC27A2* mRNA
expression in SAT. (a) Fold
changes in *SLC27A2* mRNA
expression for each of the 40 men
(Affymetrix arrays). (b, c) Fold
changes in *SLC27A2* mRNA
expression as measured by qPCR
for (b) 14 fasting men and (c) 10
hyperinsulinaemic men.
***$p < 0.001$ vs the control diet.
(d–g) Selected genes previously
linked to adipose tissue
metabolism and/or differentiation
that showed differential DNA
methylation (HFO and
LIPOGAIN cohorts) and mRNA
expression (HFO cohort) levels
after overfeeding (black bars)
compared with the control diet
(white bars). *$p < 0.05$ and
†$q < 0.05$ (FDR correction) vs the
control diet. (h) CpG sites with
the largest differences (of >10%
during both control diet and HFO)
in SAT methylation between
NBW (white bars) and LBW
(grey bars) men after reference-
free cell mixture adjustment.
†$q < 0.05$ (FDR correction) vs
NBW. Ins stim, insulin stimulated
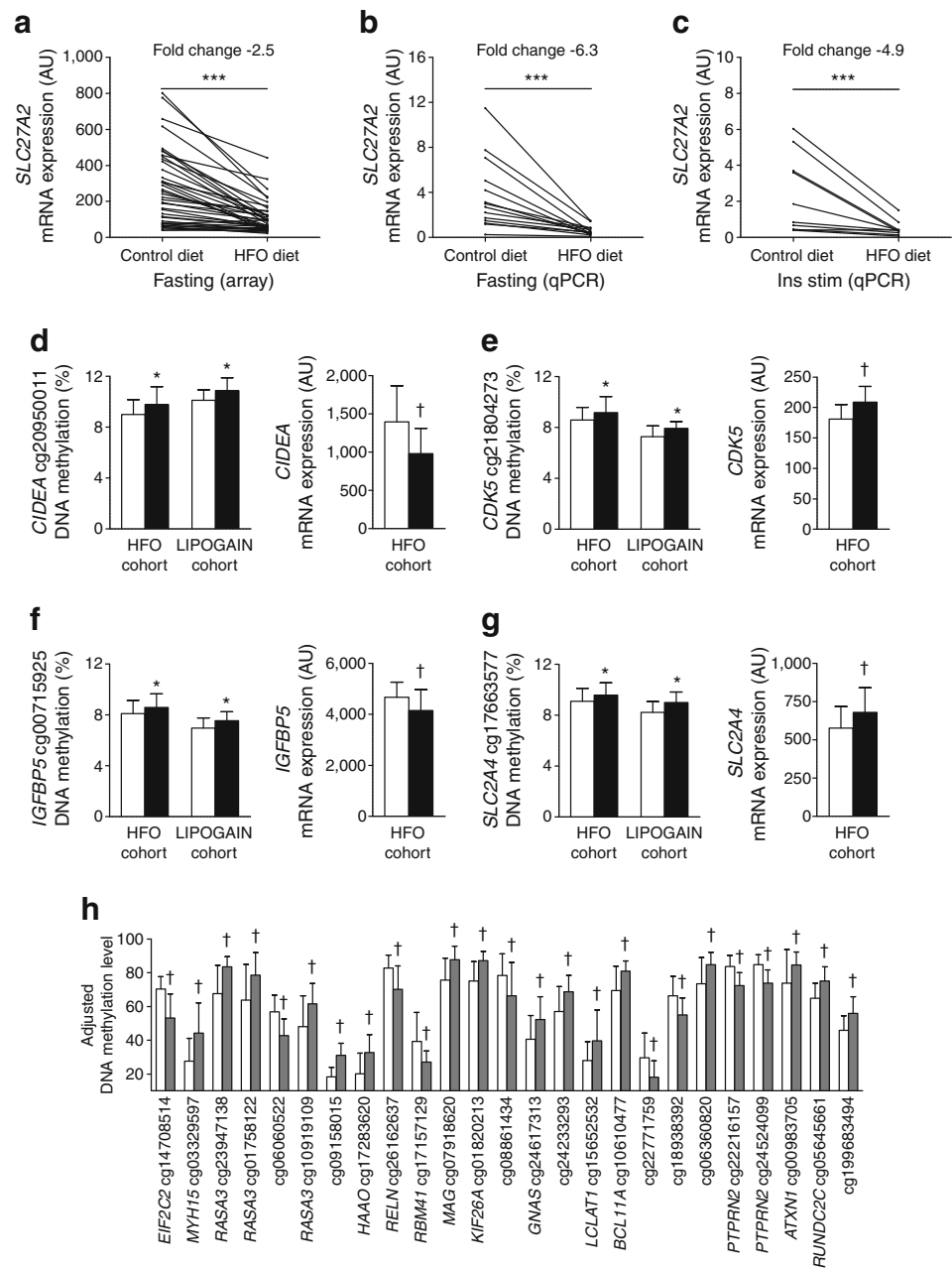
and *FADS2* (Table 2). The largest identified differences in SAT methylation between LBW and NBW men are presented in Fig. 3h. Moreover, we performed a pathway analysis on the 2,448 unique genes and found significant enrichment of genes in, for example, the protein digestion and absorption, endocytosis and calcium signalling pathways (ESM Table 20).

Finally, after reference-free cell mixture adjustment, SAT methylation at 1,009 sites annotated to 824 genes was significantly different under HFO vs control diet conditions ($q < 0.05$; ESM Table 21). These include 937 of the sites presented in ESM Table 10, including sites in *CIDEA*, *KLF14* and *PRDM16*.

Discussion

This study demonstrates that LBW individuals display multiple DNA methylation alterations in SAT in early adulthood. We also demonstrate that 5 days of a high-fat high-energy diet changes the expression of more than 3,000 genes in SAT from young men with or without a LBW. These changes may contribute to development of the insulin resistance seen in these men. In contrast to the transcriptome, only modest DNA methylation changes were observed in SAT in response to overfeeding.

A LBW affects key metabolic functions in multiple tissues, thereby increasing the risk of type 2 diabetes [4, 32]. This

might partly be due to an adverse intrauterine environment that causes epigenetic alterations during fetal development. Interestingly, we found significant differences in DNA methylation at 53 sites in SAT from LBW vs NBW men either during control diet or HFO. Of these, 14 sites were significantly changed during both diets, including sites in *FADS2* that has been genetically linked to type 2 diabetes [33] and in *CPLX1* which has been associated with glucose-stimulated insulin release [34]. Moreover, sites in *HCCA2* and *IGF2R* (which have also been genetically associated with type 2 diabetes) and in *ACAT1* and *GPRC5B* (for which adipose tissue expression has been associated with insulin resistance and diabetes; see ESM Table 2) were differentially methylated in LBW men. Although the DNA methylation differences observed between LBW and NBW individuals may appear small, ageing might induce bigger differences that may, by influencing mRNA expression, become functionally significant and thus represent key defects involved in the development of type 2 diabetes. Furthermore, when reference-free cell mixture adjustments were performed according to Houseman et al [27], many more epigenetic alterations were identified under both dietary conditions in LBW vs NBW men. Importantly, these include the majority of the sites that differed between groups during both diets when methylation data were analysed without reference-free cell admixture adjustment. These included sites in *CPLX1* and *FADS2*, as well as sites annotated to genes involved in, for example, protein digestion and absorption, endocytosis, and calcium signalling pathways. Nevertheless, it remains to be tested whether the adipose tissue cell composition differs between the LBW and NBW groups. In addition, the Houseman method uses mathematical modelling to adjust in silico for cell composition and other 'unknown' variables that might affect the data. To our knowledge, no other studies have applied this reference-free method in adipose tissue or investigated whether it generates consistent results in this tissue.

The altered transcriptional activity of SAT after 5 days of HFO included upregulation of numerous genes involved in energy metabolism. Previous short-term overfeeding studies mainly found SAT transcriptional changes in genes involved in lipid [18, 19] and carbohydrate metabolism [19]. Despite the excess lipid content of the HFO diet compared with the control diet [16], we found that genes upregulated by HFO were enriched in many (10 out of 15) of the KEGG pathways related to carbohydrate metabolism, but only to a few (4 out of 17) of the KEGG pathways related to lipid metabolism (www.genome.jp/kegg/pathway.html). Interestingly, several genes involved in de novo lipogenesis (e.g. *ACLY*, *ACACA*, *FASN*, *FADS1*, *FADS2* and *SCD*) were upregulated in SAT in both this and a previous overfeeding study [21]. An induction of adipose tissue lipogenesis (rather than hepatic lipogenesis) could mirror an increased efficiency of lipid storage during HFO, as reflected by the reduced fasting plasma NEFA and

triacylglycerol levels observed in the young men after HFO. Moreover, the finding that genes involved in glycogen synthesis (*GYS1* and *GYS2*) were among those most strongly upregulated by HFO is consistent with the suggestion that adipose glycogen turnover may help coordinate glucose and lipid metabolism in adipose tissue [35].

Six of the 12 most strongly upregulated transcripts are key genes in lipid and glycogen metabolism: *ACLY*, *DGAT2*, *ELOVL6*, *FADS2*, *GYS2* and *SCD*. Interestingly, we recently found that *ELOVL6*, *FADS1*, *FADS2* and *GYS2* are among the most strongly downregulated genes in SAT from monozygotic twins discordant for type 2 diabetes [9]. In addition, the gene sets that were most significantly upregulated by HFO in this study (oxidative phosphorylation, citrate cycle and pyruvate metabolism) were significantly downregulated in SAT from diabetic twins [9]. A disturbed adipocyte metabolism has been suggested to shift lipid storage into alternative tissues, such as skeletal muscle, the liver and the pancreas, resulting in insulin resistance [36]. Our data suggest that low mRNA levels or an inability to upregulate these genes during HFO may predispose individuals to type 2 diabetes.

Changes in the expression of several genes involved in insulin-signalling occurred in response to HFO: for example, *INSR*, *IRS2* and *PIK3CB* were downregulated; and *AKT1*, *AKT2*, *IRS1* and *SLC2A4* (encoding *GLUT4*) were upregulated. To our knowledge, the opposing effects of HFO-induced regulation on *IRS1* and *IRS2* (which encode important docking proteins) in SAT has not been previously observed.

Interestingly, expression of the most strongly downregulated gene, *SLC27A2*, showed inverse correlations with diabetes and obesity-related traits including insulin resistance and BMI. This indicates that transcriptional regulation of *SLC27A2* may be important in HFO-induced pathogenesis of insulin resistance and obesity. The encoded protein, very long-chain acyl-CoA synthetase (VLACS; also known as FATP-2) preferentially mediates the transport and conversion of long-chain and very-long-chain *n*-3 fatty acids into the corresponding *n*-3 acyl-CoAs, which are trafficked into pools destined for phosphatidylcholine and phosphatidylinositol incorporation [30, 37]. In line with our data, previous studies show *SLC27A2* downregulation in human SAT after 56 days of overfeeding [18] and after in vitro exposure to adipocytokines such as TNF-α and leptin [38]. A link between inflammation, *SLC27A2* regulation and insulin resistance has been proposed [38]. This is supported by another study in which *SLC27A2* expression was lower in SAT from insulin-resistant vs insulin-sensitive women [39]. Interestingly, we found both upregulation (*AGT*, *RBP4*, *TNF*) and downregulation (*CFD*, *IL6*, *NAMPT*) of genes encoding adipokines and cytokines, which may contribute to HFO-induced insulin resistance.

Furthermore, based on data from this and previous studies [30, 37], we speculate that diet-induced downregulation of *SLC27A2* may influence insulin resistance through reduced channelling of specific lipids (e.g. *n*-3 fatty acids) toward phosphatidylinositol synthesis, which could subsequently reduce phosphatidylinositol 3-kinase (PI3K) signalling and other insulin-signalling events downstream of AKT [40]. As *SLC27A2* expression was reduced by hyperinsulinaemia, the *SLC27A2* downregulation observed during HFO may be partly caused by increased insulin levels.

Some of the significant changes in gene expression in response to HFO were modest, and one may question whether such changes are of biological importance. However, small changes in the expression of numerous genes involved in key metabolic pathways may indeed have biological effects [41, 42]. Importantly, we technically validated our microarray data on all five analysed genes using qPCR. Additionally, the overlap in SAT gene expression data between our study and previous short-term overfeeding studies further supports the biological significance of our data [18, 19]. Nevertheless, small changes in gene expression should be interpreted cautiously. In addition, changes in cell type composition could be responsible for some of the changes in HFO-induced gene expression. However, when we investigated 24 cell-type-specific markers for adipocytes, pre-adipocytes, brown adipocytes, macrophages, cytokines and inflammation [12], only *LEP*, *TNF* (cytokines) and *CIDEA* (brown adipocyte marker) were differentially expressed after HFO. These data suggest that 5 days HFO does not alter the cellular composition of SAT.

Although 5 days of HFO altered the expression of more than 3,000 genes in SAT, the associated epigenetic changes were less extensive. We previously found HFO-induced DNA methylation changes in *PPARGC1A* (in both SAT and skeletal muscle) [5, 6] and extensive genome-wide DNA methylation changes in skeletal muscle from NBW but not LBW men [7, 13]. Thus, our data show that epigenetic changes in SAT are less pronounced than in skeletal muscle [7] in response to 5 days of HFO. However, our overfeeding-induced methylation changes were replicated at more than 600 sites in SAT from a different cohort [20]. Most of these sites showed increased methylation levels during overfeeding, consistent with increased expression of the *DNMT1* methyltransferase with HFO. The expression of several genes with altered DNA methylation in both overfeeding cohorts also changed with HFO including *CDK5* and *SLC2A4*, whose protein activity and gene expression in adipose tissue were previously associated with insulin resistance [43, 44]. Finally, the results generated after reference-free cell mixture adjustment support the hypothesis that epigenetic changes take place in response to overfeeding.

It should be noted that we previously identified extensive epigenetic alterations in human adipose tissue from other cohorts of a similar size to the one included in this study [9, 11]. These studies together with our post hoc power calculation suggest an appropriate statistical power in the present study. Importantly, however, the stringent correction for multiple testing of our genome-wide data together with the modest epigenetic changes may have increased the risk of false negatives. Moreover, because the 450K BeadChip only covers ~2% of genomic CpG sites, we cannot exclude the possibility that methylation changes occur at other genomic sites. Finally, a longer diet intervention may be required to induce more pronounced epigenetic changes in human SAT.

In conclusion, our study demonstrated that young healthy men with a LBW exhibit multiple DNA methylation alterations in adipose tissue which may contribute to their increased risk of type 2 diabetes. However, SAT gene expression was similar in LBW and NBW men. We also demonstrated that 5 days of HFO causes extensive gene expression and modest epigenetic changes in SAT, with no differences in the responses of LBW and NBW men. Overall, this study adds to our understanding of the molecular mechanisms in adipose tissue that are likely to contribute to the development of insulin resistance and type 2 diabetes in people with and without LBW when exposed to overfeeding.

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