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ARTICLE

Effects of short-term high-fat overfeeding on genome-wide DNA methylation in the skeletal muscle of healthy young men

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Abstract
Aims/hypothesis Energy-dense diets that are high in fat are associated with a risk of metabolic diseases. The underlying molecular mechanisms could involve epigenetics, as recent data show altered DNA methylation of putative type 2 diabetes candidate genes in response to high-fat diets. We examined the effect of a short-term high-fat overfeeding (HFO) diet on genome-wide DNA methylation patterns in human skeletal muscle.
Methods Skeletal muscle biopsies were obtained from 21 healthy young men after ingestion of a short-term HFO diet and a control diet, in a randomised crossover setting. DNA methylation was measured in 27,578 CpG sites/14,475 genes using Illumina's Infinium Bead Array. Candidate gene expression was determined by quantitative real-time PCR.
Results HFO introduced widespread DNA methylation changes affecting 6,508 genes (45%), with a maximum methylation change of 13.0 percentage points. The HFO-induced methylation changes were only partly and non-significantly reversed after 6–8 weeks. Alterations in DNA methylation levels primarily affected genes involved in inflammation, the reproductive system and cancer. Few gene expression changes were observed and these had poor correlation to DNA methylation.
Conclusions/interpretation The genome-wide DNA methylation changes induced by the short-term HFO diet could have implications for our understanding of transient epigenetic regulation in humans and its contribution to the development of metabolic diseases. The slow reversibility suggests a methylation build-up with HFO, which over time may influence gene expression levels.

Keywords DNA methylation · Epigenetic · Gene expression · Genome-wide · High-fat overfeeding · Human · Illumina Infinium 27k Bead Array · Reversibility · Skeletal muscle · Type 2 diabetes

Abbreviations
HFO High-fat overfeeding
IR Insulin resistance
SOM Self-organising map

Introduction

DNA methylation at CpG sites is an important and potentially heritable epigenetic modification of the mammalian genome [1]. DNA methylation can affect gene expression and

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chromosome stability, potentially influencing phenotypic outcomes in health and disease [2, 3]. Examples of such modifications are known from some types of cancer, where hypomethylation of oncogenes and hypermethylation of tumour suppressor genes are supposed components in cancer development [2, 4, 5]. Epigenetic profiles are influenced by genetics [6–8], but ageing and environmental factors including diet, chemicals and smoking may take centre-stage in the control of DNA methylation [9–12]. Studies have indicated that DNA methylation may play a role in metabolic diseases such as diabetes. Differential methylation has been identified in the promoter region of the key metabolic regulator peroxisome proliferator-activated receptor gamma coactivator 1 alpha (*PPARGC1A*) gene and in 1.8% of 14,475 genes examined in recent methylation profiling in pancreatic islets from type 2 diabetic patients [13, 14]. Furthermore, increased methylation of *PPARGC1A* has been found in the skeletal muscle of patients with glucose intolerance and type 2 diabetes [15], as well as in muscle from low-birthweight individuals with an increased risk of type 2 diabetes [16].

Diet is a highly influential factor in the origin of metabolic disease, and both specific dietary components as well as shifts in overall dietary regimens can affect DNA methylation levels. It is well established that diets rich in genistein [17] and methyl donors [12] are able to modulate DNA methylation patterns in the rodent offspring of mothers consuming such diets, influencing the offspring's incidence of obesity, diabetes and cancer in a potentially transgenerational manner [1, 12, 17]. Diets high in fat have been shown to increase DNA methylation of the leptin promoter [18] and to prolong the presence of general DNA methylation induced by treatment with carcinogenic agents in rats [19, 20]. Limited data are available in humans. However, we recently found that a high-fat overfeeding (HFO) diet increased DNA methylation of the *PPARGC1A* promoter in the skeletal muscle of young healthy men in a reversible manner [16]. This finding is supported by studies of human muscle cells, where exposure to fatty acids in vitro likewise increased methylation of *PPARGC1A* [15]. These data suggest that DNA methylation changes could be associated with the development of skeletal muscle insulin resistance (IR) in type 2 diabetes and in people at risk of this disease [15, 16]. Based on these recent findings, we hypothesised that a short-term HFO diet may introduce additional and potentially widespread DNA methylation changes in human skeletal muscle, affecting genes belonging to distinct pathways influencing the risk of metabolic disease.

Methods

Participants A total of 26 men aged 24.6±1.1 (mean ± SD) years were recruited according to a previous description [21]. The participants were born at term with birthweights in the 50–90th percentile. None of the participants had a family history of diabetes, BMI above 30 kg/m^2 or high physical activity levels (>10 h/week). The protocol conformed to the Helsinki Declaration, and was approved by the Ethics Committee for Copenhagen County and the Danish Data Protection Agency. All the participants gave informed consent before study participation.

Dietary intervention In a randomised crossover setting, all 21 participants completed both the HFO intervention and the control period separated by a washout period of 6–8 weeks (Fig. 1). The study design has previously been thoroughly described [21]. In brief, the participants were standardised with respect to physical activity, alcohol consumption and diet 5 days prior to both examinations and were asked to remain weight stable between the examination periods. The HFO diet was delivered to the participants for 5 days, starting 5 days prior to excursion of skeletal muscle biopsies. The HFO intervention diet contained 50% extra calories distributed as 60% fat, 32.5% carbohydrate and 7.5% protein. While the standardisation of the control period also included 5 days in total, compliance was optimised even further during the last 3 out of the 5 days by providing identical meals to all participants. The control diet contained 35% fat, 50% carbohydrate and 15% protein.

Clinical examinations Detailed clinical and metabolic examinations were performed in the participants and have been previously published [21]. Blood samples were drawn in the fasting state and throughout the examination period to measure, among others, glucose, insulin, NEFA, triacylglycerol and cholesterol levels. The *M* value was calculated as: glucose infusion (μmol/min)/fat-free mass in kg. Hepatic IR index was calculated as: endogenous (hepatic) glucose production [(μmol/min)/fat-free mass in kg] × fasting serum insulin (pmol/l). Biopsies were excised from musculus vastus lateralis

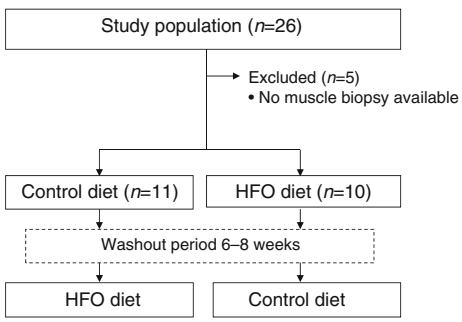

Fig. 1 Design overview. A total of 21 participants were randomised into one of two dietary groups. The control diet first group (n=11) consumed a control diet followed by an HFO diet, while the HFO first group (n=10) received an HFO diet initially followed by a control diet. The two dietary periods were separated by a washout period of 6–8 weeks

using a Bergström needle, immediately frozen in liquid nitrogen and stored at −80°C.

DNA methylation profiling: Illumina's 27k Bead Array Methylation was assessed at 27,578 CpG sites associated with 14,475 genes using Illumina's 12-sample Infinium Bead Array Methylation Chip (Illumina, San Diego, CA, USA). Genomic DNA was extracted using the DNeasy kit (Qiagen, Valencia, CA, USA). A total of 600 ng DNA was bisulfite-treated with the EZ DNA Methylation kit (Zymo Research, Orange, CA, USA). Each sample was whole-genome amplified and enzymatically fragmented. The whole-genome amplified–DNA samples were purified and hybridised to two different bead spots, methylated-Cy5 or unmethylated-Cy3, followed by single-base extension using DNP- and biotin-labelled dideoxynucleoside triphosphates. The methylation status at each CpG site was averaged over 30 replicate measurements and determined by the ratio of the fluorescent signal from Cy5 relative to the combined intensity, and recorded as β values between 0 and 1 corresponding to 0–100% methylation using BeadStudio Methylation Module v.3.2 (Illumina).

Validation of results from the bead array Validation of selected array results was performed using Sequenom's MassARRAY EpiTYPER (Sequenom, San Diego, CA, USA) or Qiagen's Pyrosequencing (Qiagen, Valencia, CA, USA). Initially, 200–500 ng DNA was bisulfite-treated with the EZ Gold DNA Methylation kit (Zymo Research).

MassARRAY EpiTYPER PCR was performed with bisulfite-specific primers (EpiDesigner; Sequenom). Transcription and cleavage was completed using the MassCleave kit (Sequenom, San Diego, CA, USA) and mass spectra acquired using the MassARRAY mass spectrometer (Sequenom, San Diego, CA, USA). DNA methylation levels were calculated by comparing mass signal intensity between methylated and non-methylated template DNA. The spectra were analysed using the EpiTYPER software v.1.0.1.

Pyrosequencing PCR was performed by the PyroMark PCR kit (Qiagen, Valencia, CA, USA) with primers designed using the PyroMark Assay Design Software 2.0. The samples were prepared using the PyroMark Q96 Vacuum Workstation and sequenced on the PyroMark Q96 ID instrument with bisulfite conversion controls. Data were analysed using the Pyrogram software v.2.5.7.

Quantitative real-time PCR Gene expression was determined for 13 selected candidate genes of type 2 diabetes, methyltransferase enzymes and biologically relevant genes showing large or significant DNA methylation changes following HFO. Total RNA was extracted from the muscle biopsies using TRI Reagent (Sigma-Aldrich, St Louis, MO, USA). cDNA was synthesised using the QuantiTect Reverse Transcription Kit (Qiagen). mRNA expression was detected with the ABI 7900 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using gene-specific primer/probe pairs for *ACAT2* (Hs00255067_m1), *ADAMTS9* (Hs00172025_m1), *AKT2* (Hs01086102_m1), *DNM2* (Hs00974698_m1), *DNMT1* (Hs00154749_m1), *DNMT3A* (Hs01027166_m1), *DNMT3B* (Hs00171876_m1), *ESRRG* (Hs00155006_m1), *LMNA* (Hs00153462_m1), *MGMT* (Hs01037698_m1), *MRC1* (Hs00267207_m1), *MYST4* (Hs00202463_m1) and *PPARGC1A* (Hs00173304_m1) (Applied Biosystems). All samples were run in duplicate and the standard curve approach was used for quantification. The transcript quantity was normalised to mRNA levels of *PPIA* (4326316E, Applied Biosystems), which did not vary with experimental conditions.

Statistical methods The bisulfite-treated DNA samples were distributed on nine different 12-sample arrays with an even division of control and HFO samples to eliminate any technical effects. The raw β values were normalised with the BeadStudio software, as recommended by Illumina. Illumina's internal controls supported successful array preparation and poor sample performance was eliminated. According to the detection p value (>0.05), 0.2% of the data points were eliminated alongside three CpG sites with a mean detection p value of >0.05. Statistical analyses were performed using the programming language R version 2.9.0 (www.r-project.org). Normality was evaluated by Kolmogorov–Smirnov's normality test and normality plots. Data were analysed with parametric tests, and data are presented as mean ± SD and differences between two methylation percentages are given as percentage points. p values ≤0.05 were considered statistical significant and p values ≤0.10 in the validation analyses were defined as borderline statistically significant. Benjamini–Hochberg's false discovery rate was applied to correct for multiple testing (α=0.10). χ^2 tests were used to evaluate proportions of change.

Pathway analyses were performed using Ingenuity Canonical Pathway Analysis v.7.5 (Ingenuity Systems, Redwood City, CA, USA), applying the complete Illumina Infinium Array as the reference set. The unsupervised clustering method self-organising maps (SOM) was employed using Pearson's correlation (α=0.01) with two endpoint classes (MeV v.4.8). Data inclusion criteria for both Ingenuity's Pathway Analysis and SOM analyses were: CpG sites significant after correction for multiple testing with a methylation change larger than the average methylation change of ±3.5 percentage points. In the validation procedure, the selected CpG site from the bead array was compared only with the same CpG site analysed by Pyrosequencing or MassARRAY EpiTYPER. Statistical testing was performed using a one-sided paired t test, based on the a priori hypothesis of replicating findings from the bead array. The dataset is available at the NCBI Gene

Expression Omnibus (www.ncbi.nlm.nih.gov/geo, accessed 8 August 2012) under accession number GSE36166.

Results

DNA methylation profiling in human skeletal muscle We employed Illumina's Infinium Bead Array to compare DNA methylation of 27,578 CpG sites in close proximity to 14,475 gene transcription start sites in human skeletal muscle tissue after control and HFO diets. The CpG sites were located up to 1,499 base pairs from transcription start, of which 53% were located within 300 base pairs of transcription start and 73% in CpG islands. We found that 66% of the CpG sites were hypomethylated (methylated <25%), whereas 14% were hypermethylated (methylated >75%) during the control diet (n=21) (electronic supplementary material [ESM] Fig. 1).

According to our a priori hypothesis, the randomised cross-over design would minimise periodic, sequence and carry-over effects, which is why we initially analysed all 21 participants collectively. A total of 4,857 CpG sites (18%) distributed over 4,316 genes (30%) changed significantly after the HFO diet, although this was non-significant when corrected for multiple testing. However, by employing an unsupervised cluster analysis with SOM including all 21 participants, we detected an effect of the sequence in which participants received the control vs HFO diet. SOM allocated the participants into two groups based on similarities in their DNA methylation response following the dietary treatment, resulting in two clusters with 11 and ten participants, respectively. Nine out of the 11 participants grouped in the first cluster had received the control diet first, followed by the HFO diet (Fig. 2). Eight out of the ten participants who shifted from the initial HFO diet to the control diet were grouped in the second cluster. Following this observation, we analysed the participants according to the sequence by which they had received each dietary treatment, enabling us to document methylation changes induced by HFO and the extent to which these were reversible (Fig. 1). The group that received the control diet followed by HFO is referred to as the 'control diet first' group (n=11) and the group that received the HFO diet followed by the control diet is referred to as the 'HFO first' group (n=10).

Clinical and metabolic changes All participants remained weight stable and maintained their WHR between the intervention periods (Table 1). In the control diet first group, HFO led to significantly higher plasma glucose levels and a higher hepatic IR index, whereas NEFA and triacylglycerol levels decreased. Fasting insulin, total cholesterol and M value remained unchanged. A shift from the HFO diet back to the control diet in the HFO first group led to an increase in fasting NEFA and total cholesterol and a decrease in the hepatic IR index. No changes were observed for fasting glucose, insulin, triacylglycerol and M value (Table 1).

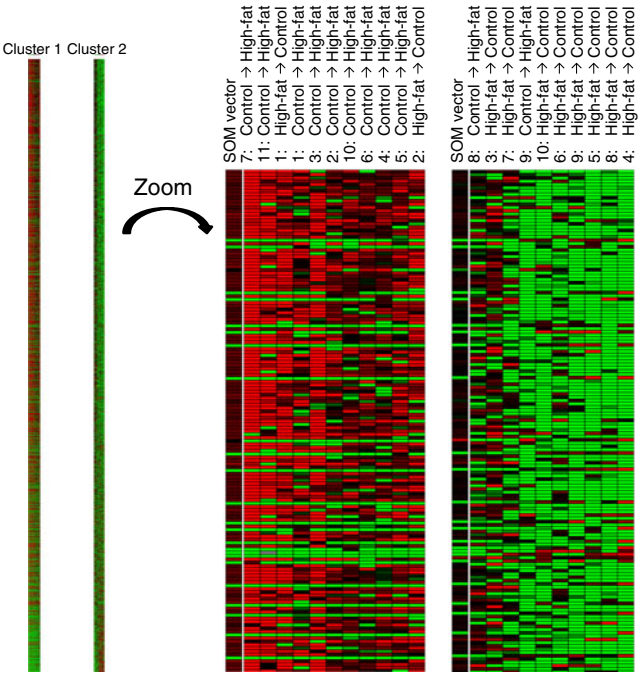

Fig. 2 Unsupervised cluster analysis with SOMs. All 21 samples were objectively divided into two clusters based on similarities in DNA methylation changes following the HFO diet. Each vertical column represents a participant and each horizontal line the methylation change for one CpG site. The participants were each labelled with a number and the order of dietary treatment. Individuals in the control diet first group are labelled 'Control→High-fat' and individuals in the HFO first group are labelled 'High-fat→Control'. Increased methylation is indicated by green and decreased methylation by red

HFO induces DNA methylation changes (control diet first group, n=11) In the control diet first group and after correction for multiple testing, 7,909 CpG sites (29%) corresponding to 6,508 genes (45%) changed significantly in response to HFO. Thus, the proportion of genes with altered CpG methylation far exceeded the 724 genes (5%) that would be expected to change by chance (χ^2 p<0.0001). The average absolute methylation change was 3.5±2.0 percentage points, with a maximum methylation change of 13.0 percentage points (Fig. 3 a, b). HFO led to an increase in DNA methylation in 83% of the affected CpG sites, among which 98% were classified as hypomethylated (Fig. 3a). The top 20 most significant genes included *DNM2*, *MGMT*, *SLC2A3/GLUT3*, *MRC1* and *ACAT2* (all p<0.0001) (ESM Table 1). Furthermore, the top 20 genes showing the largest percentage points increase or decrease in methylation following HFO included *APOH*, *DCC*, *ESRRG*, *FOLH1*, *GTF2I*, *MC4R* and *MYST4* (all methylation changes >10 percentage points) (ESM Table 2). We selected 12 CpG sites/genes showing the quantitatively largest significant methylation changes following the HFO diet in the control diet first group for further validation

Table 1 Clinical and metabolic characteristics of study participants

Variable	Control diet first group (n=11)		HFO first group (n=10)	
	Control	HFO	HFO	Control
Weight (kg)	78.5±8.3	77.5±7.0	76.7±11.3	76.5±10.1
BMI (kg/m^2)	23.0±1.9	23.0±1.8	23.5±3.2	23.5±2.9
WHR	0.86±0.05	0.87±0.05	0.90±0.06	0.88±0.05
Fasting plasma glucose (mmol/l)	4.51±0.49	5.02±0.40*	5.08±0.45	4.71±0.48
Fasting serum insulin (pmol/l)	30.2±13.9	35.0±17.4	50.2±36.1	33.2±16.8
Fasting NEFA (μmol/l)	359±131	201±87*	201±69	335±137†
Fasting triacylglycerols (mmol/l)	0.86±0.29	0.62±0.20***	0.74±0.45	0.88±0.39
Fasting total cholesterol (mmol/l)	3.90±0.76	3.89±0.63	4.10±0.75	4.37±0.60†††
M value (μmol [kg FFM]$^{-1}$min^{-1})	72.22±11.94	73.17±11.56	76.00±26.72	80.22±15.56
Hepatic IR index (μmol [kg FFM]$^{-1}$min^{-1} [pmol/l]$^{-1}$	371.1±218.9	577.8±360.0***	700.0±321.1	395.0±153.3††

Data are means ± SD

Control diet first group: control vs HFO: $^{*}p<0.05$, $^{***}p<0.001$

HFO diet first group: control vs HFO: $^{\dagger}p<0.05$, $^{\dagger\dagger}p<0.01$, $^{\dagger\dagger\dagger}p<0.001$

FFM, fat-free mass

with the two independent methods, Pyrosequencing and MassARRAY EpiTYPER, respectively. The validation analyses confirmed a response in the same direction for all 12 CpG sites/genes, with statistical significance for five and borderline statistical significance for one of the genes (ESM Table 3). Importantly, we also observed a good correlation between the bead array expression data and each of the two validation methods for nine out of the 12 CpG sites/genes, reaching statistical significance for seven and borderline statistical significance for two genes (ESM Table 3).

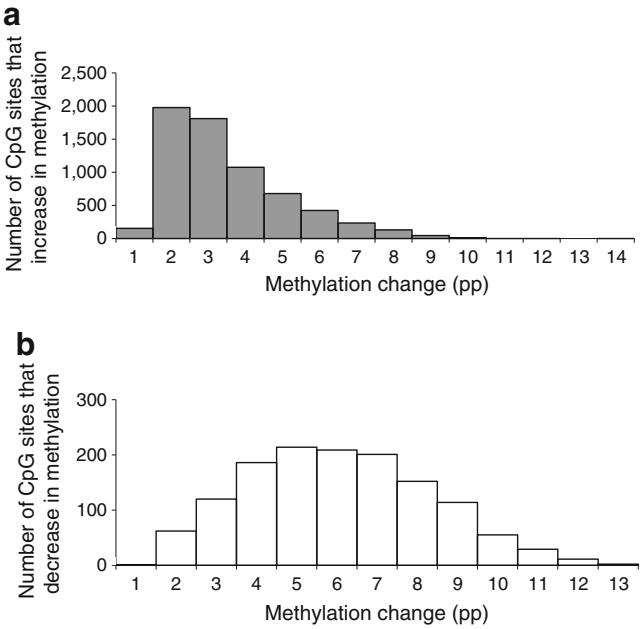

Fig. 3 DNA methylation changes induced by the HFO diet in the control diet first group (n=11). DNA methylation changes are given as percentage points (pp). (**a**) The number of CpG sites that showed an increase in methylation following the HFO diet and the magnitude of the absolute methylation changes. 0–1%: 154, 1–2%: 1,979, 2–3%: 1,811, 3–4%: 1,075, 4–5%: 679, 5–6%: 423, 6–7%: 235, 7–8%: 131, 8–9%: 46, 9–10%: 12, 10–11%: 5, 11–12%: 2, 12–13%: 0, 13–14%: 1. (**b**) The number of CpG sites that showed a decrease in methylation following the HFO diet and the magnitude of the absolute methylation changes. 0–1%: 1, 1–2%: 62, 2–3%: 120, 3–4%: 186, 4–5%: 214, 5–6%: 209, 6–7%: 201, 7–8%: 152, 8–9%: 114, 9–10%: 55, 10–11%: 29, 11–12%: 11, 12–13%: 2

Pathways and candidate genes affected by HFO (control diet first group, n=11) Three consecutive pathway analyses were performed to identify pathways with altered methylation following HFO. The individual analysis included either hypomethylated, hypermethylated or both hypo- and hypermethylated CpG sites collectively, which were objectively and stringently selected as described in the statistics section. Significant pathways for each group are presented in ESM Table 4. When summarising significant pathways into common diseases and disorders by Ingenuity for each analysis, pathways relevant to common and potentially lifestyle-related diseases including cancer (p=0.005–0.05), the reproductive system (p=0.006–0.05) and inflammatory systems (p=0.0003–0.04) were predominantly represented (Table 2).

We used a candidate gene approach to search for associations between HFO and type 2 diabetes by focusing on 43 type 2 diabetes susceptibility genes. Significant changes were found in 24 genes following HFO, for which methylation changes were present in more than half of the CpG sites on the array for *AKT2*, *PDX1/IPF1*, *SLC30A8*, *CDKN2A*, *CDKN2B* and *PPARG* (ESM Table 5).

DNA methylation changes induced by HFO are slowly reversed (HFO first group, n=10) As noted previously, we

Table 2 Ingenuity Canonical Pathway Analysis, showing common diseases and disorders with the largest significant methylation differences following the HFO diet in the control diet first group (n=11)

Pathway analysis	CpG sites/genes in analysis	Molecules	Common diseases/disorders	p value
Hypomethylated CpG sites	1,989/1,853	375	Cancer	0.005–0.05
		151	Reproductive system disease	0.006–0.05
		115	Gastrointestinal disease	0.02–0.04
Hypermethylated CpG sites	862/812	60	Inflammatory disease	0.0003–0.04
		95	Inflammatory response	0.0004–0.04
		35	Ophthalmic disease	0.0004–0.03
Both hypo- and hypermethylated CpG sites	3,162/2,914	99	Cancer	0.007–0.05
		83	Reproductive system disease	0.02–0.05
		4	Infection mechanism	0.02

The top three common diseases and disorders are presented according to hypomethylated, hypermethylated and both hypo- and hypermethylated CpG sites/genes

observed an obvious difference in methylation response based on the sequence by which the participants received the control and HFO diets using SOM (Fig. 2). Only 341 genes (2%) changed in the HFO first group, which shifted from initial HFO back to the control diet, of which none were significant after correction for multiple testing (mean change 2.9±1.7 percentage points, maximum change 13.1 percentage points). The proportion of genes that changed in the control diet first group (45%) thus far exceeded the changes seen in the HFO first group (2%) (χ^2 p<0.0001). Despite the limited changes observed in the HFO first group, we were able to establish a general trend towards reversibility of the DNA methylations, as indicated by the reversed colour pattern between the control diet first and HFO first group on the heatmap depicting the entire array and by the SOM analysis (Fig. 2, ESM Fig. 2). To further address the concept of reversibility, we examined the top 10% of genes (650 genes) that were most differentially methylated after correction for multiple testing in the control diet first group, to determine whether these showed signs of reversibility in the HFO first group. We observed that 66% of the genes that changed with the HFO diet had a methylation change in the opposite direction when switched back to the control diet; however, this was significant for only 5%. This tendency was supported by an increase in mean methylation of all 6,508 genes from 33.4% to 34.5% in the control diet first group, and a decrease in mean methylation from 34.1% to 33.7% in the HFO first group.

DNA methylation and gene expression To address the potential transcriptional effects of the observed methylation changes, we investigated the mRNA expression of methyltransferase enzymes and selected candidate genes of type 2 diabetes, as well as biologically relevant genes showing large or significant DNA methylation changes following HFO. Few gene expression changes were observed in either the control diet first or HFO first groups (ESM Table 6). A borderline increase was observed for *DNMT3A* (p=0.08) and *DNMT1* (p=0.10) during the HFO diet. The number of CpG sites present on the array for each gene investigated varied between one and 26, and correlations were performed individually for each site (ESM Table 7). Significant correlations between DNA methylation and gene expression were observed for a minor proportion of the CpG sites and with inconsistent direction.

Discussion

In this study, we extended our previous finding of increased promoter methylation by short-term HFO of the metabolic regulator *PPARGC1A* [16] to include almost half (45%) of the CpG sites present on the bead array, documenting a generalised regulatory epigenetic phenomenon introduced by HFO. Although modest in absolute magnitude, the widespread DNA methylation changes induced by HFO in this study are quantitatively similar to those previously reported to be influenced by HFO in selected candidate genes implicated in growth and metabolic disease, including type 2 diabetes [14, 16, 18, 22, 23]. However, the extent to which general epigenetic changes play a role in the short-term regulation of metabolic functions in muscle by HFO, including IR, remains to be established.

The functional read-out of altered DNA methylation has traditionally been thought to involve altered mRNA expression, and significant correlations between DNA promoter methylation and distinct gene expression has previously been reported in some [13, 15] but not all human studies, including the Human Epigenome Project, supporting the notion that the relationship between DNA methylation and gene expression is not always straightforward [14, 16, 24]. Few significant correlations were observed between DNA

methylation and gene expression levels for a number of candidate genes in the present study. Although this may question the immediate functional relevance of the widespread methylation changes, it remains possible that the methylation changes may influence the expression levels of some genes if HFO is maintained over longer periods of time, as indicated by the slow reversibility of the HFO-induced methylation changes. The methylation changes may also only influence gene expression during specific metabolic challenges or demands, such as in response to acute exercise [25, 26], or perhaps in subgroups predisposed to metabolic disease, such as individuals born with low birthweight [16]. To this end, the possibility that the methylation changes induced by HFO actually prevented potentially detrimental effects of HFO on gene expression, representing a homeostatic mechanism, should be mentioned. It has even been suggested that changes in methylation could actually represent a consequence—and not a cause—of altered transcriptional activity [27]. In addition, the bead array screens a minor portion of the CpG sites in the genome, and we cannot exclude that other regions, such as enhancer regions, are regulatory important for gene expression [28]. All together, the extent to which the observed changes in DNA methylation in this study affect gene expression over time, in a subset of individuals at risk or during certain specific metabolic challenges remain to be determined.

The induction of DNA methylation changes after 5 days of HFO supports the growing awareness of DNA methylation as a dynamic signal that is possibly relevant to short-term day-to-day metabolic adaptations, including acute exercise [26, 29, 30]. However, our finding of a slow reversibility rate indicates the demethylation process may be somewhat impeded compared with the induction of methylation changes by diet, which could have implications for the preservation or build-up of CpG methylation over time. Diverging DNA methylation levels between elderly, but not young, genetically identical twins indicate that environmental exposures throughout life may permanently influence DNA methylation, suggesting some preservation of de novo DNA methylation in adults [9]. A slow reversibility of DNA methylation induced by carcinogenic agents has likewise been observed due to ingestion of high-fat diets in rodents [19, 20]. As skeletal muscle is a terminally differentiated tissue, methylations induced by HFO could be introduced by de novo or maintenance DNA methylation enzymes, including *DNMT3A*, *DNMT3B* and *DNMT1* [31, 32]. We observed no significant methylation changes in these genes. However, we did observe borderline significant increased expression levels of *DNMT3A* and of *DNMT1* during HFO, similar to observations in transgenic mice following a high-fat diet [33]. More data are needed to determine the role of specific DNA methyltransferases in the regulation of DNA methylation induced by high-fat diets.

Applying Ingenuity's pathway analyses, we found increased methylation of CpG sites associated with genes involved in the reproductive system and cancer; conversely, we found decreased methylation of genes encoding inflammatory pathways. It is well known that obesity is associated with decreased fertility and certain types of cancers [34, 35]. Likewise, a high level of NEFAs is associated with IR and low-grade inflammation and may potentially be involved in the development of type 2 diabetes [36–38]. The decreased plasma NEFA levels following the HFO diet indicate decreased lipolyses, but could also reflect increased NEFA uptake and storage in the muscle [38, 39]. We did not find any disproportionately large or distinct methylation changes in 43 selected type 2 diabetes genes, emphasising the general nature of the response. Future studies are required to elucidate the role of altered methylation in specific genes or pathways and its potential impact on disease development.

As in all biological research, positive findings may have occurred by chance or random variation of data. However, we do not believe that this is the case with the methylation changes induced by HFO in the present study. First and most importantly, the SOM analysis classified the samples into two groups based on the order of the dietary treatments with great accuracy, providing strong intrinsic validity of the array results. In other words, the SOM analysis showed relatively smaller variation between arrays per se, as opposed to the effects of the HFO intervention. Second, the control and HFO samples were evenly distributed on different arrays and run on the same day, which eliminates the consideration that variation from one or two arrays could be responsible for the HFO response. Third, validation studies using two independent DNA methylation assessment methods supported the findings from the bead array analyses showing DNA methylations changing in the same direction for all 12 out of 12 CpG sites/genes tested and with good concordance between the methods. Given a variation in methylation of 5% between repeated measurements for both validation methods and the relatively small methylation differences detected with the bead arrays in response to HFO, we do not find it surprising or against our array findings that not all methylation changes were of the exact same magnitude, and that some failed to show statistical significance in the validation analyses. In addition, the lack of statistical significance for some DNA methylation changes in the validation process is likely to be due to the fact that limited muscle biopsy material was available, reducing the sample size to eight for some genes, which of course also reduced the statistical power compared with the array analyses. Finally, we did observe that the Pyrosequencing technique tended to be less sensitive in detecting low degrees of DNA methylations, and since *SNX7* and

STC1 showed the lowest degrees of methylation, we ascribe the limited DNA methylation changes to insensitivities of the Pyrosequencing method at low methylation levels. Regardless, the data of course need to be replicated in other studies.

In summary, we report for the first time that short-term HFO introduces DNA methylation changes on a genome-wide scale in human skeletal muscle. These changes were only partly reversed after 6–8 weeks. These broad changes, while modest in magnitude, highlight the plasticity and reversibility of DNA methylation levels in response to dietary interventions in humans. The relevance of these findings in causing or buffering human disease, including type 2 diabetes, remains to be determined.

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Contribution statement SCJ acquired data, analysed and interpreted data and drafted the manuscript. CB designed the study, analysed and interpreted data and critically revised the manuscript. AFF, BY, EL, EH and VC acquired data and critically revised the manuscript. JB, SM, RR, MFF, EN, SWJ, CL and PP analysed and interpreted data and critically revised the manuscript. AV designed the study and critically revised the manuscript. All authors approved the final version of the manuscript.

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