DNA methylation and body-mass index: a genome-wide analysis

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Summary

Background **Obesity is a major health problem that is determined by interactions between lifestyle and environmental and genetic factors. Although associations between several genetic variants and body-mass index (BMI) have been identified, little is known about epigenetic changes related to BMI. We undertook a genome-wide analysis of methylation at CpG sites in relation to BMI.**

Methods **479 individuals of European origin recruited by the Cardiogenics Consortium formed our discovery cohort. We typed their whole-blood DNA with the Infinium HumanMethylation450 array. After quality control, methylation levels were tested for association with BMI. Methylation sites showing an association with BMI at a false discovery rate q value of 0·05 or less were taken forward for replication in a cohort of 339 unrelated white patients of northern European origin from the MARTHA cohort. Sites that remained significant in this primary replication cohort were tested in a second replication cohort of 1789 white patients of European origin from the KORA cohort. We examined whether methylation levels at identified sites also showed an association with BMI in DNA from adipose tissue (n=635) and skin (n=395) obtained from white female individuals participating in the MuTHER study. Finally, we examined the association of methylation at BMI-associated sites with genetic variants and with gene expression.**

Findings **20 individuals from the discovery cohort were excluded from analyses after quality-control checks, leaving 459 participants. After adjustment for covariates, we identified an association (q value ≤0·05) between methylation at five probes across three different genes and BMI. The associations with three of these probes—cg22891070, cg27146050, and cg16672562, all of which are in intron 1 of *HIF3A*—were confirmed in both the primary and second replication cohorts. For every 0·1 increase in methylation β value at cg22891070, BMI was 3·6% (95% CI 2·4–4·9) higher in the discovery cohort, 2·7% (1·2–4·2) higher in the primary replication cohort, and 0·8% (0·2–1·4) higher in the second replication cohort. For the MuTHER cohort, methylation at cg22891070 was associated with BMI in adipose tissue ($p=1·72\times10^{-5}$) but not in skin (p=0·882). We observed a significant inverse correlation (p=0·005) between methylation at cg22891070 and expression of one *HIF3A* gene-expression probe in adipose tissue. Two single nucleotide polymorphisms—rs8102595 and rs3826795—had independent associations with methylation at cg22891070 in all cohorts. However, these single nucleotide polymorphisms were not significantly associated with BMI.**

Interpretation **Increased BMI in adults of European origin is associated with increased methylation at the *HIF3A* locus in blood cells and in adipose tissue. Our findings suggest that perturbation of hypoxia inducible transcription factor pathways could have an important role in the response to increased weight in people.**

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Introduction

Obesity and its associated comorbidities constitute a major and growing health problem worldwide.[1] Therefore, understanding the mechanisms that affect body-mass index (BMI)—the most widely used measure of obesity—and any downstream effects is an important health priority. BMI is a complex phenotype determined by lifestyle (eg, physical activity), environmental factors (food availability and intake), and genetic factors.[2] In the past few years, a major effort to identify genetic determinants of BMI through genome-wide association studies has shown that more than 30 single nucleotide polymorphisms (SNPs) are associated with BMI, which together explain about 1·5% of interindividual variation in BMI.[3]

DNA methylation is the reversible and heritable attachment of a methyl group to a nucleotide. The most common form of DNA methylation occurs at the 5′ carbon of cytosine in CpG dinucleotides, creating 5-methylcytosine.[4] CpG dinucleotides are often located in CpG islands (clusters of CpG sites) within the promoter region or first exon of genes, or upstream from genes within CpG island shores (DNA regions within 2 Kb of CpG islands) or shelves (within 2 Kb of shores).[4] DNA

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See **Online** for appendix

For the **InfiniumHD Methylation SNP list** see http://support.illumina.com/downloads/infinium_hd_methylation_snp_list.ilmn

For the **R Package minfi** see http://www.bioconductor.org/packages/release/bioc/html/minfi.html

methylation plays a part in transcriptional regulation of genes and miRNAs,[5] control of alternative promoter usage,[6,7] and alternative splicing.[6]

Both genetic and environmental factors can affect the extent of DNA methylation.[8,9] In view of the range of potential downstream functional outcomes of this epigenetic change, an effect on DNA methylation could integrate the impact of both genetic and environmental factors on a phenotype.[10] Alternatively, epigenetic changes caused by a phenotype can mediate its downstream effects by changing gene expression.[10]

Unlike genome-wide association studies of genetic variants, progress in systematic analysis of DNA methylation has hitherto been hampered by an absence of analogous platforms to study epigenetic phenomena. However, the newly developed Infinium HumanMethylation450 array (Illumina, San Diego, CA, USA) assays about 485 000 methylation sites spanning 99% of genes in the Reference Sequence database, with an average of 17 CpG sites per gene region. The array has been validated and consistently detects CpG methylation changes.[11] We used this array for a large-scale analysis of methylation patterns in whole-blood DNA in relation to BMI.

Methods

Participants

479 white individuals who had been recruited by the Cardiogenics Consortium[12] formed our discovery cohort. They either had a history of myocardial infarction (n=241; recruited from four centres: Leicester, UK; Lübeck, Germany; Regensburg, Germany; and Paris, France) or were healthy blood donors (n=238; recruited in Cambridge, UK). Genome-wide SNP genotypes had been previously obtained for all participants with the Human Quad Custom 670 array (Illumina, San Diego, CA, USA) and genome-wide gene expression data obtained for monocytes and derived macrophages with the HumanRef-8 v3 Beadchip array (Illumina, San Diego, CA, USA).[13]

For our primary replication cohort, we used data for 339 unrelated white patients of French origin who had venous thrombosis recruited into the MARseille THrombosis Association (MARTHA) cohort.[14] These patients had been genotyped with the Human 610/660W-Quad arrays (Illumina, San Diego, CA, USA).[14]

We analysed methylation sites that showed a significant association in the primary replication cohort in a second replication cohort of 1789 white participants from Germany who had been recruited for the KORA (Cooperative Health Research in the Region of Augsburg) F4 survey.[15] Genome-wide genotyping was done for KORA F4 with the Affymetrix 6.0 GeneChip array (Santa Clara, CA, USA).

To investigate whether the association between methylation at *HIF3A* sites and BMI that we observed in blood DNA would also be seen in other tissues, we analysed data for white female individuals from the UK obtained as part of the Multiple Tissue Human Expression Resource (MuTHER) study.[16] HumanMethylation450 arrays had been done for 635 subcutaneous adipose tissue biopsies and for 395 skin biopsies. The adipose tissue samples came from 249 twin pairs (93 monozygotic and 156 dizygotic twins) and 137 singletons. Skin samples came from 108 of the 249 twin pairs (44 monozygotic and 64 dizygotic) and 179 singletons. The collection and processing of the biopsy samples in the MuTHER study have been described previously.[17] In addition to the methylation arrays, genome-wide genotype data (obtained with a combination of HumanHap300, HumanHap610, and 1M-Duo and 1·2M-Duo Illumina arrays; Illumina, San Diego, CA, USA) and genome-wide expression profiles in adipose tissue (obtained with the IlluminaHT-12 v3 array; San Diego, CA, USA) were available for the MuTHER participants.[17] All individuals provided written informed consent to participate in the primary studies and to allow DNA analysis of their samples.

Procedures

Details of the methylation assay done for the discovery cohort and the quality checks that were undertaken are given in the appendix (p 2). Methylation is described as a β value, which is a continuous variable ranging between 0 (no methylation) and 1 (full methylation). In any one sample, a probe with a detection p value (a measure of an individual probe's performance) of more than 0·05 was assigned missing status. If a probe was missing in more than 5% of samples, we excluded it from all samples. We excluded 830 probes on this basis. To avoid spurious associations, we also excluded probes containing genomic sites where variation is already known according to the HumanMethlyation450 annotation files or the InfiniumHD Methylation SNP list that had a minor allele frequency of more than 1%, leaving 351699 probes. Before analysis, methylation values were corrected for background values and then normalised with SWAN[18] in the R Package minfi. We used the array annotations provided by Illumina (version 1.1) to assign probes to their corresponding genes.

We used the same Illumina HumanMethylation450 array in the replication cohorts and in the MuTHER samples, following similar experimental procedures. We did post-array processing in a similar way for all studies and normalised methylation values before analysis with SWAN[18] for the two blood replication cohorts and by quantile normalisation[19] for the MuTHER study samples.

Statistical analysis

BMI was not normally distributed in the discovery cohort and therefore was transformed on the log scale. Regression analysis of log-transformed BMI with methylation level at each probe was adjusted for age, sex, smoking status, methylation array batch, and centre. Adjustment for centre also adjusted for whether patients

had had myocardial infarction. Chip assignment was not associated with BMI and was therefore not included in the model. For models in which the dependent variable (BMI in this case) has been log transformed, the β coefficients from the regression analysis can be interpreted as the change in the dependent variable by 100×(coefficient) for an increase in one unit in the independent variable. Therefore, we present β coefficients as percentage change. A correction for genomic control (λ=1·092) was applied (appendix p 11). We estimated q values for false discovery rates[20] and associations with a false discovery rate q value of 0·05 or less were taken forward for replication.

We did sequential replication for the MARTHA and KORA cohorts with linear regression analysis of log-transformed BMI adjusted for age, sex, smoking status, and array batch. We assessed significance after Bonferroni correction.

In the MuTHER cohort, to account for family structure, we fitted a linear mixed effects model for log-transformed BMI with the lme4 package in R. We adjusted the model for age, array batch, and smoking status (fixed effects), and for family identification number and zygosity (random effects). We used the likelihood ratio test statistic to assess significance and calculated the p value from the χ^2 distribution with one degree of freedom.

We assessed associations between methylation level for sites showing a correlation with BMI and genotypes at adjacent SNPs (within 1 Mb) in the discovery cohort, assuming an additive allele effect. We used a linear mixed effects model with age, sex, smoking status, centre, BMI, and methylation batch array as fixed effects, and methylation chip as a random effect. We applied Bonferroni correction for multiple testing to the results. We analysed significant and independent associations in a similar manner in the replication cohorts and in MuTHER samples (with the addition of family identification number and zygosity as random effects and exclusion of sex). We also used the same model to analyse the association between methylation level or BMI with individual blood cell counts in the discovery cohort. We did power calculations with powerreg in Stata (version 12.1).

Role of the funding source

The sponsors of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. PD and NJS had full access to data for the discovery cohort, D-AT to data for the MARTHA cohort, CG for the KORA cohort, and PD for the MuTHER cohort. NJS had the final responsibility for the decision to submit for publication.

Results

20 individuals from the discovery cohort (two who had had myocardial infarction, 18 healthy blood donors) were excluded from analyses after quality-control checks of the methylation array data (appendix p 2), leaving 459 participants (table 1). As reported by others[21] at a genomic level, methylation at CpG dinucleotides in our discovery cohort had a bimodal distribution, with the most frequent level of methylation occurring at a β value of 0–0·05 with a second, slightly lower peak at 0·90–0·95

For more on the **lme4 package** see http://cran.stat.sfu.ca/web/packages/lme4/lme4.pdf

	Discovery cohort (Cardiogenics)		Primary replication cohort (MARTHA; n=339)	Second replication cohort (KORA; n=1789)	MuTHER cohort	
	Individuals who had had myocardial infarction (n=239)	Healthy blood donors (n=220)			Adipose tissue samples (n=635)	Skin samples (n=395)*
Age (years)	55·2 (6·8)	55·2 (6·8)	43·8 (14·2)	60·9 (8·9)	58·8 (9·3)	58·8 (9·3)
Men	202 (85%)	125 (57%)	74 (22%)	871 (49%)	0	0
Body-mass index (kg/m^2)	28·3 (4%)	25·9 (3·6)	24·2 (4·4)	28·1 (4·8)	26·7 (4·9)	26·6 (4·7)
Ever smokers	185 (77%)	89 (40%)	145 (43%)	1003 (56%)	308 (49%)	187 (47%)
Height (cm)	174·5 (8·7)	172·5 (9·1)	166·6 (7·7)	167·8 (9·2)	161·5 (5·8)	161·5 (6·0)
Weight (kg)	86·5 (15·8)	77·2 (12·5)	67·5 (14·4)	79·4 (15·3)	69·8 (13·8)	69·5 (13·3)
Systolic blood pressure (mm Hg)	130·5 (19·1)	NA	NA	124·8 (18·7)	129·8 (16·2)	129·1 (16·0)
Diastolic blood pressure (mm Hg)	77·8 (10·9)	NA	NA	76·1 (9·9)	78·6 (9·4)	78·6 (9·5)
Diabetic	10 (4%)	NA	6 (2%)	163 (9%)	30 (5%)	16 (4%)
Methylation of cg22891070†	0·434 (0·110, 0·189–0·910)	0·453 (0·098, 0·211–0·740)	0·473 (0·118, 0·127–0·823)	0·515 (0·131, 0·154–0·906)	0·177 (0·045, 0·076–0·358)	0·272 (0·052, 0·165–0·536)
Methylation of cg27146050†	0·319 (0·051, (0·144–0·516)	0·328 (0·047, 0·191–0·495)	0·315 (0·042, 0·180–0·458)	0·380 (0·057, 0·179–0·622)	0·163 (0·037, 0·086–0·262)	0·232 (0·029, 0·161–0·368)
Methylation of cg16672562†	0·389 (0·116, 0·071–0·952)	0·409 (0·101, 0·157–0·745)	0·454 (0·125, 0·107–0·795)	0·438 (0·136, 0·091–0·900)	0·098 (0·039, 0·016–0·237)	0·174 (0·044, 0·064–0·422)

Data are mean (SD), n (%), or mean (SD, range). NA=not available. *From subset of participants who had also provided adipose tissue samples. †β values.

Table 1: **Characteristics of participants in the studied cohorts**

(appendix p 9). In a previous study (in which the Illumina HumanMethylation27 Bead Chip, the precursor of the HumanMethylation450 Bead Chip, was used),[22] a robust association between current smoking and methylation at the cg03636183 locus in *F2RL3* had been shown and replicated. As a form of overall validation of our discovery analysis, we examined the association of current or ever smoking with methylation at this site in our dataset. We recorded a similarly highly significant association ($p=3\cdot8\times10^{-33}$) between methylation at cg03636183 and smoking, with reduced methylation in smokers (appendix p 10).

The distribution of p values in the discovery cohort from regression of methylation level at each site and BMI is shown in figure 1. The quantile–quantile plot for expected versus observed χ^2 values is shown in the appendix (p 11). Five probes achieved a false discovery rate q value of 0·05 or less, including individual probes in *CLUH* on chromosome 15 and *KLF13* on chromosome 17 (appendix p 3), and three probes in *HIF3A* on chromosome 19 (table 2). We excluded the possibility that these probes showed cross-reactivity for several CpG sites.[23]

We took these five probes forward for analysis in our primary replication cohort (MARTHA). Although methylation level for the probes in *CLUH* and *KLF13* were not associated with BMI in this cohort (appendix p 3), all three *HIF3A* probes were significant after Bonferroni correction for multiple testing (table 2). We further tested the association of these three probes in our second replication cohort (KORA). All three probes were significantly associated with BMI, although the association was weaker than for the other cohorts (table 2).

The three identified *HIF3A* probes (cg22891070, cg27146050, and cg16672562) are neighbouring probes in intron 1 of the gene (figure 2). Methylation levels at cg22891070, cg27146050, and cg16672562 are all highly correlated with each other ($R^2=0\cdot89$–$0\cdot95$ in the discovery cohort). The three probes are flanked by others that had nominally significant associations with BMI in the discovery cohort (cg05286653: $p=2\cdot37\times10^{-4}$; cg12068280: $p=4\cdot89\times10^{-3}$) that did not meet our false discovery rate q value threshold of 0·05 or lower. Overall, there are probes for 25 CpG sites in *HIF3A* on the array, and the results for all the probes are shown in the appendix (p 4). Methylation at CpG sites in the other members of the

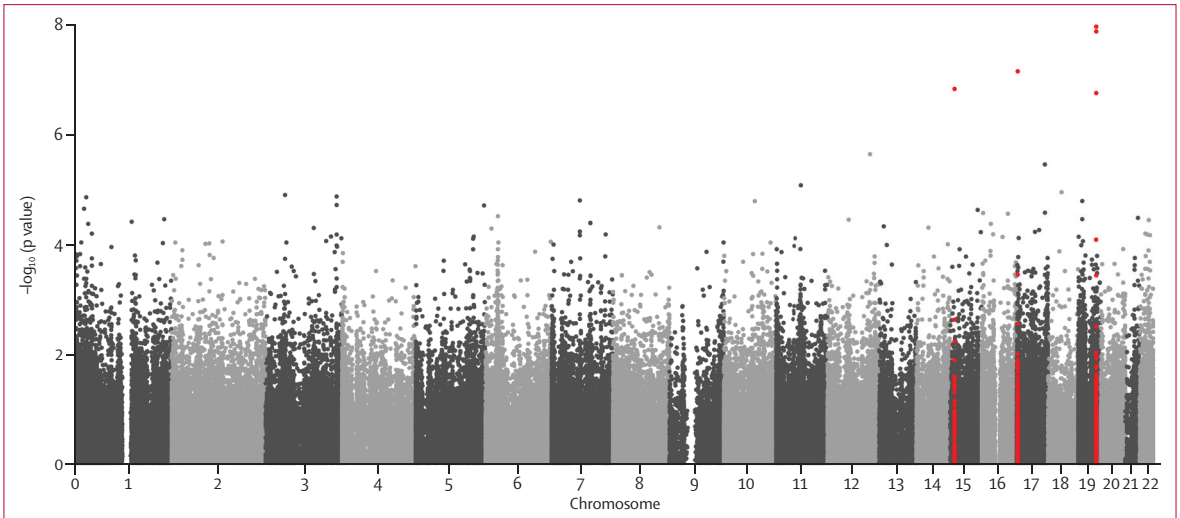

Figure 1: **Manhattan plot showing the distribution of p values of the association of methylation probes with body-mass index in the discovery cohort**
The red dots indicate probes that fall within *KLF13* (chromosome 15), *CLUH* (chromosome 17), and *HIF3A* (chromosome 19).

	Position	Discovery cohort (Cardiogenics)		Primary replication cohort (MARTHA)		Second replication cohort (KORA)	
		p value*	Percentage change in BMI (95% CI)†	p value	Percentage change in BMI†	p value	Percentage change in BMI†
cg22891070	46801642	$4\cdot00\times10^{-8}$	3·6% (2·4–4·9)	$3\cdot65\times10^{-4}$	2·7% (1·2–4·2)	$6\cdot69\times10^{-3}$	0·8% (0·2–1·4)
cg27146050	46801557	$4\cdot82\times10^{-8}$	7·8% (5·1–10·4)	$5\cdot09\times10^{-3}$	6·2% (1·8–10·4)	$2\cdot18\times10^{-3}$	2·1% (0·7–3·4)
cg16672562	46801672	$5\cdot36\times10^{-7}$	3·2% (2·0–4·4)	$3\cdot47\times10^{-3}$	2·1% (0·7–3·5)	0·011	0·7% (0·2–1·3)

The significance threshold after Bonferroni correction for multiple testing in the primary replication cohort is 0·01 and in the second replication cohort is 0·016. BMI=body-mass index. *λ corrected. †The β coefficients from the association analysis have been converted into percentage change in BMI for every 0·1 unit increase in methylation β value.

Table 2: **Association between methylation at sites in *HIF3A* on chromosome 19 in whole-blood DNA and BMI in the discovery and replication cohorts**

hypoxia inducible transcription factor family (*HIF1A* [13 probes], *EPAS1* [38 probes], and *ARNT* [17 probes]) was not associated with BMI (data not shown).

Because the DNA used in our methylation analysis is derived from a mixture of different white blood cell types, methylation in the *HIF3A* probes could vary between different white cell populations, and the correlation with BMI could simply be a result of varying proportions of these cell types in individuals with different BMIs. Therefore, using cg22891070 as an exemplar, we examined the association of methylation level of this probe with the number of each cell type in the discovery cohort using a linear mixed effects model. Additionally, we tested for an association between number of each cell type and BMI. We recorded a weak positive correlation (p=0·019) between methylation at cg22891070 and lymphocyte count that did not survive correction for multiple testing. We recorded no associations with other cell types (appendix p 5). Furthermore, adjustment for lymphocyte, monocyte, and neutrophil counts did not substantially attenuate the association between methylation at cg22891070 and BMI ($p=1·04\times10^{-7}$).

We also examined the association of DNA methylation at *HIF3A* with the two individual components of BMI—height and weight—in the discovery cohort. Methylation at cg22891070 was significantly associated with weight ($p=5·2\times10^{-7}$) but not with height (p=0·78). In exploratory analyses of the population-based KORA cohort, we did not find an association between methylation at cg22890170 and other characteristics associated with BMI, such as physical activity (p=0·955) or type 2 diabetes mellitus (p=0·680).

For the three significant sites in *HIF3A*, overall methylation β value in the discovery cohort ranged from 0·18 to 0·90 for cg22891070, from 0·14 to 0·52 for cg27146050, and from 0·07 to 0·95 for cg16672562 (appendix p 12). β values were similar in the replication cohorts (table 1). The correlation between methylation level at cg22891070 in blood DNA and BMI for the discovery cohort, and the change in methylation level at cg22891070 by quintile of BMI (and vice versa) are shown in the appendix (pp 13–14). Every 0·1 increase in methylation β value for cg22891070 was associated with a 3·6% higher BMI in the discovery cohort (table 2). For a person in the discovery cohort with the mean BMI (27 kg/m²), this 3·6% increase equates to a 0·98 kg/m² higher BMI on average. The increase in BMI was greater in participants who had had myocardial infarction (4·6%, 95% CI 2·9–6·3) than in the blood donors (2·3%, 0·4–4·1). The percentage changes in BMI in the replication cohorts for a 0·1 increase in methylation were smaller than in the discovery cohort (table 2), and in KORA was equivalent to a 0·22 kg/m² higher BMI on average.

In the MuTHER cohort, methylation level at the three *HIF3A* sites was strongly associated with BMI in adipose tissue but not in skin (table 3). The range of methylation β values was narrower in both tissues than in blood DNA (table 1). However, it was narrower in adipose tissue than in skin, which means that a reduced range cannot be a reason for why an association was not observed in skin. The direction of the association between methylation in *HIF3A* in adipose tissue and BMI was the same as that in blood, but the percentage change was greater.

We could analyse whether methylation at the *HIF3A* locus was correlated with *HIF3A* gene expression for the MuTHER adipose dataset, because genome-wide expression profiles were available. We recorded a weak (β value –0·025, SE 0·008) but significant (p=0·005) inverse correlation between methylation at cg22891070 and one (ILMN_1663015) of five *HIF3A* gene-expression probes on the array (appendix p 6). Although we had genome-wide expression data from monocytes and macrophages for the discovery cohort,[13] expression of *HIF3A* was below detectable levels in these cells so we could not directly examine whether variation in methylation level at cg22891070 is associated with expression of the gene in blood cells.

Because DNA sequence variation can be associated with methylation level, we looked for an association between SNPs within 1 Mb of cg22891070 and methylation at this probe, using the genome-wide SNP data available for the discovery cohort (appendix p 15). Two SNPs, rs8102595 and rs3826795, with an R^2 between them of 0·006 (D'=1), had independent associations with methylation at cg22891070 (table 4). rs8102595 had a stronger association than did rs3826795 (table 4).

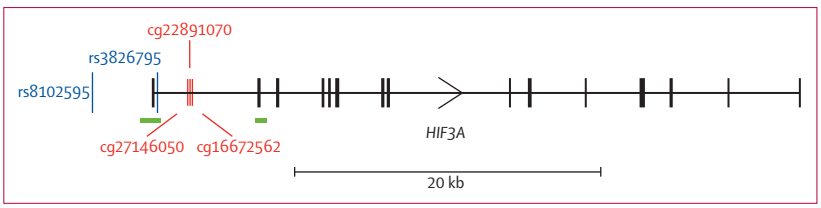

Figure 2: **Location of methylation probes associated with body-mass index and SNPs affecting methylation levels of these probes in the *HIF3A* locus**
Vertical black lines represent exons. The arrow indicates direction of transcription. The three methylation sites in intron 1 showing an association with body-mass index are shown in red. The two SNPs showing an association with methylation levels at these probes are shown in blue. The green blocks represent the position of CpG islands in this locus. SNP=single nucleotide polymorphism.

	Adipose tissue (n=635)		Skin (n=395)	
	p value	Percentage change in BMI*	p value	Percentage change in BMI*
cg22891070	$1·72\times10^{-5}$	6·2 (3·4 to 9·0)	0·882	–0·25 (–3·6 to 3·0)
cg27146050	$9·27\times10^{-7}$	11·9 (7·2 to 16·7)	0·011	–7·0 (–12·4 to –1·7)
cg16672562	$5·01\times10^{-6}$	7·9 (4·5 to 11·2)	0·862	–0·36 (–4·3 to 3·5)

Data in parentheses are 95% CIs. BMI=body-mass index. *The β coefficients from the association analysis have been converted into percentage change in BMI for every 0·1 unit increase in methylation β value.

Table 3: **Association between BMI and methylation at sites in *HIF3A* in adipose tissue and skin DNA in the MuTHER cohort**

	rs8102595			rs3826795		
	Frequency of effect allele*	β (95% CI)	p value	Frequency of effect allele†	β (95% CI)	p value
Discovery (Cardiogenics)	0·10	0·063 (0·042–0·083)	$6·29×10^{-9}$	0·81	0·039 (0·023–0·056)	$3·21×10^{-6}$
Primary replication cohort (MARTHA)	0·10	0·097 (0·062–0·121)	$1·41×10^{-9}$	0·79	0·051 (0·023–0·076)	$2·14×10^{-5}$
Second replication cohort (KORA)	0·09	0·073 (0·058–0·086)	$9·18×10^{-23}$	0·82	0·048 (0·037–0·059)	$2·26×10^{-18}$
MuTHER cohort: adipose tissue	0·10	0·041 (0·033–0·049)	$1·05×10^{-21}$	0·81	0·021 (0·014–0·028)	$3·61×10^{-9}$
MuTHER cohort: skin	0·10	0·062 (0·052–0·074)	$7·09×10^{-25}$	0·82	0·023 (0·013–0·034)	$1·77×10^{-5}$

The β values are from an additive model and are a unit change in methylation per copy of the effect allele. *G. †C.

Table 4: **Association between methylation level at cg22891070 and single nucleotide polymorphisms at the *HIF3A* locus**

rs8102595 is located 3·8 kb and rs3826795 1·2kb upstream of cg22891070 (figure 2). Associations between these SNPs and methylation at cg22891070 were also highly significant in the replication cohorts (table 4). Furthermore, the same associations were recorded in both adipose tissue and skin in the MuTHER cohort (table 4). Genetic variation in rs8102595 accounted for 6·4% of the variation in methylation at cg22891070 in the blood DNA in the discovery cohort, 9·9% in the MARTHA cohort, and 4·8% in the KORA cohort. This genetic variation also accounted for 14·3% of variation in methylation at cg22891070 in adipose tissue and 21·8% in skin in the MuTHER study.

In view of the association between the two SNPs and methylation at cg22891070, we next tested their association with BMI in the discovery and other cohorts, but observed no consistently significant association (appendix p 7). However, the power of these analyses was low (appendix p 7). Therefore, we also tested for associations between these SNPs and indices of body mass in the publicly available GIANT consortium datasets.[3] We found no significant association of either SNP with BMI (rs8102595: n=123 791, p=0·15; rs3826795: n=123 847, p=0·25; appendix p 8).

For the **datasets** see http://www.broadinstitute.org/collaboration/giant/index.php/GIANT_consortium_data_files

Discussion

We have identified and replicated a specific association between BMI and methylation of *HIF3A* in whole blood DNA. We recorded the same association in DNA from adipose tissue, which is of high relevance to bodyweight and obesity, implying that it is biologically relevant. Although some preliminary reports are available of whole-blood methylation profiles in relation to indices of body composition and obesity,[24–27] we are the first to have undertaken a large-scale analysis with replication of the principal finding (panel).

HIF3A is a component of the hypoxia inducible transcription factor (HIF), which regulates a wide variety of cellular and physiological responses to reduced oxygen concentrations by controlling expression of many target genes.[30] It is a heterodimer that is composed of a β subunit (ARNT) and one of three α subunits (HIF1A, EPAS1, and HIF3A). The binding of each α subunit to ARNT targets different sets of downstream genes in a cell-specific manner.[30] In the case of HIF3A, a further layer of complexity is added by the fact that the *HIF3A* locus is subject to much alternate splicing, leading to at least seven variants with differing targets.[31] The induction of target genes by HIF3A binding to ARNT is generally weaker than is that evoked by HIF1A and EPAS1 binding to ARNT.[30,31] Furthermore, especially in situations in which the amount of ARNT could be limiting, at least some isoforms of HIF3A seem to hinder the response to hypoxia by sequestering ARNT and restricting its binding to HIF1A and EPAS1.[32,33]

Although the main focus on HIF has been its role in cellular and vascular response to changes in oxygen tension during normal development or pathological processes (eg, cardiovascular disease and cancer[30]), compelling and increasing experimental data suggest that the HIF system also plays a key part in metabolism, energy expenditure, and obesity.[34–37] Specifically, targeted disruption of either HIF1A or ARNT in adipocytes in transgenic mice is associated with reduced fat formation and protection from obesity and insulin resistance induced by high-fat diets.[34] Similarly, systemic use of an antisense oligonucleotide to HIF1A for 8 weeks in mice with diet-induced obesity substantially suppresses *HIF1A* expression in liver and adipose tissue and is associated with increased energy expenditure and weight loss.[35] In the hypothalamus, HIF signalling (primarily via EPAS1) has a role in glucose sensing and regulation of energy balance and weight by affecting expression of pro-opiomelanocortin.[36]

Although HIF3A has not been investigated as thoroughly as the other α subunits in this context, it has been shown to have a role in the cellular response to glucose and insulin, and functions as an accelerator of adipocyte differentiation.[38,39] Furthermore, siRNA inhibition of *HIF3A* in Hep3B cells significantly downregulates mRNA expression of *ANGPTL4*,[31] which could have a role in acquired obesity.[40]

The cross-sectional nature of our analysis means that we cannot assign a cause–effect association directly from the association we observed between *HIF3A* methylation and BMI. Previous studies[41,42] have shown that DNA

sequence variation can affect levels of methylation at individual sites (methylation quantitative trait loci). To investigate directionality of the association between *HIF3A* methylation and BMI, we searched for genetic variants that associate with *HIF3A* methylation to establish whether these variants also associate with BMI in turn. We identified significant independent associations between genotypes at two SNPs—rs8102595 and rs3826795, upstream of *HIF3A*—and methylation at one of our identified *HIF3A* probes, cg22891070. However, we identified no association between these variants and BMI in our cohorts or in the large GIANT genome-wide association meta-analysis of BMI which included more than 123 000 individuals. Our analysis of GIANT data had more than 95% power to detect an association for both SNPs if one existed (appendix p 8). These findings suggest that the association between increased methylation and higher BMI is not causal. Furthermore, the finding that methylation in *HIF3A* in skin was not associated with BMI, despite a strong methylation quantitative trait locus for cg22890170 in this tissue, also indicates the absence of causal directionality. Therefore, our findings suggest that increased methylation at the *HIF3A* locus is a result of increased BMI.

An alternative possibility is that the association between methylation at *HIF3A* and BMI is due to a confounding factor which affects both variables. However, we did not observe the association between *HIF3A* methylation and BMI in skin. Furthermore, we did not observe any association with other characteristics associated with BMI, such as physical activity or diabetes.

The mechanism by which increased BMI could lead to rises in *HIF3A* methylation is unknown. Obesity predisposes individuals to obstructive sleep apnoea,[43] which is associated with intermittent hypoxia. In turn, hypoxia activates HIF signalling. Therefore, chronic upregulation of HIFs in response to obstructive sleep apnoea could result in secondary changes in methylation of the *HIF* genes. However, the association of methylation level at the *HIF3A* locus showed a linear correlation across the range of BMI levels, and increased methylation was not confined to obese individuals (appendix p 13). Furthermore, the association of BMI with variation in methylation was specific to *HIF3A* and was not noted for *HIF1A* and *EPAS1*.

We identified a significant inverse association between *HIF3A* methylation and *HIF3A* expression in adipose tissue. The association was only recorded with one of five *HIF3A* expression probes on the genome-wide expression array (appendix p 6), suggesting that the effect of methylation could be transcript-specific.[31] In this context, we note that all three CpG sites at the *HIF3A* locus that were associated with BMI are situated within regions of open chromatin as identified by formaldehyde-assisted isolation of regulatory elements (FAIRE) in H1-hESC cells and K562 cells, suggesting that these sites lie in a regulatory region.[44] However, two of the expression probes analysed (ILMN_1663015 and ILMN_1687481) are reported to tag the same *HIF3A* transcript (appendix p 6), and the reason for the discrepant findings for these two probes is unclear. Therefore, further work needs to be done to confirm the effect of methylation on expression and any transcript specificity. However, our finding supports the possibility that even if the association between increased methylation of *HIF3A* and BMI is secondary, an alteration in HIF signalling as a result of obesity-induced *HIF3A* methylation could still have an important role in some of the deleterious downstream effects of the disorder.

Although we recorded significant associations between increased *HIF3A* methylation in blood DNA and increased BMI in three different cohorts, the strength of the association varied substantially across the different cohorts. The gradient of the relation between methylation at *HIF3A* and BMI was four-times steeper in the discovery cohort than in the second population-based replication cohort (KORA), despite a similar distribution of methylation values. Whether this difference represents an element of winner's curse[45] or reflects other variation in the characteristics of the cohorts (including the presence of disease in some) is unclear. Even in the discovery cohort, we noted a difference in the level of association between the individuals who had had myocardial infarction and the healthy blood donors. The strength of the association in the blood donors was similar to that in the MARTHA cohort, which comprised patients with deep vein thrombosis, suggesting that the variation is not entirely related to disease status. Therefore, further

***Panel:* Research in context**

Systematic review

We searched Medline on Dec 1, 2013, with the terms "BMI & DNA methylation", "obesity & DNA methylation", "BMI & epigenetics" and "obesity & epigenetics". We identified hundreds of reports, many of which were reviews about the potential relevance of epigenetics in obesity. Of original research, some reports focused on methylation of specific genes already known to be associated with body-mass index (BMI) or obesity, such as *FTO* and *POMC*. In a few small genome-wide studies,[24-27] the association between methylation and BMI or other indices of obesity has been explored, without definitive findings. One study of overweight or obese adolescents[28] identified five regions that showed differential methylation levels between individuals who had a high and low response to a multidisciplinary weight-loss intervention. Another study showed significant changes in genome-wide methylation pattern in human adipose tissue after a 6-month exercise intervention.[29] Although further validation is necessary, these studies show that DNA methylation can be dynamic and could also affect whether weight changes in response to lifestyle and dietary measures.

Interpretation

Ours is the first large-scale genome-wide analysis of the association between adult BMI and DNA methylation. We have shown that BMI is associated with methylation of *HIF3A* in blood and adipose tissue. Our findings provide a strong foundation for further exploration of the part played by the epigenome in regulation of BMI and the downstream detrimental effects of increased bodyweight. Understanding of this role could identify novel therapeutic targets to tackle obesity.

studies are needed to identify factors that affect *HIF3A* methylation and modulate the association between BMI and *HIF3A* methylation in whole-blood DNA. Further work is also necessary to deduce the timing of the variation in methylation at the *HIF3A* locus in relation to BMI and whether it is dynamic or not.

Blood is readily accessible for DNA analyses. By contrast with genetic analyses, a challenge of epigenetic analyses is that circulating leucocytes—the source of DNA in blood—are composed of several different cell subtypes that could each show cell-type specific variation in DNA methylation patterns. To an extent, as we have shown, this variation can be assessed and statistical adjustment done. Perhaps a more fundamental issue for the epigenetics community is whether analysis of blood DNA methylation is worthwhile and can reflect changes in relevant tissues for a phenotype. In this regard, our finding of an association between BMI and specific *HIF3A* methylations sites in both blood and adipose tissue DNA supports the use of whole-blood DNA methylation profiling for identification of relevant epigenetic changes and provides a rationale for other studies of this type.

We used a strict sequential replication design to avoid the penalty of multiple testing for confirmation of the association of probes identified in the discovery cohort. We also started with a fairly small discovery cohort. Therefore, we recognise that we have probably missed associations between methylation of other genes and BMI. Meta-analyses of the datasets used in our study together with other datasets could yield additional insights into epigenetic changes associated with BMI.

In summary, we have reported a novel association of increased BMI in adults of European origin with increased methylation at the *HIF3A* locus in blood cells and in adipose tissue. The finding extends reports linking HIF and obesity in experimental models and provides direct evidence in people that perturbation of HIF signalling could have an important role in mediation of some of the downstream adverse responses to increased BMI.

Contributors

KJD, CPN, PD, and NJS conceived the study. JE, CH, FC, AHG, WHO, HS, and NJS were responsible for recruitment and phenotyping of the discovery (Cardiogenics) cohort. LT and EM generated methylation array data for the discovery cohort. KJD and CPN analysed data for the discovery cohort, supervised by JRT. DA, P-EM, FG, and D-AT provided data from the primary replication cohort (MARTHA) and did analyses. SW, HG, MW, AP, and CG provided data from the second replication cohort (KORA) and did analyses. JKS, TDS, and PD provided data from the MuTHER cohort and did analyses. KJD, CPN, and NJS wrote the report. All authors reviewed the report and provided comments.

Declaration of interests

We declare that we have no competing interests.

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