

# HIF3A association with adiposity: the story begins before birth

**Aim:** Determine if the association of *HIF3A* DNA methylation with weight and adiposity is detectable early in life. **Material & methods:** We determined *HIF3A* genotype and DNA methylation patterns (on hybridization arrays) in DNA extracted from umbilical cords of 991 infants. Methylation levels at three CpGs in the *HIF3A* first intron were related to neonatal and infant anthropometry and to genotype at nearby polymorphic sites. **Results & conclusion:** Higher methylation levels at three previously described *HIF3A* CpGs were associated with greater infant weight and adiposity. The effect sizes were slightly smaller than those reported for adult BMI. There was also an interaction within *cis*-genotype. The association between higher DNA methylation at *HIF3A* and increased adiposity is present in neonates. In this study, no particular prenatal factor strongly influenced *HIF3A* hypermethylation. Our data nonetheless suggest shared prenatal influences on *HIF3A* methylation and adiposity.

**Keywords:** birth weight • DNA methylation • embryonic and fetal development • epigenomics • *HIF3A* protein • human • obesity

DNA methylation states at particular loci have been associated with a range of disease states and environmental exposures. It is desirable to know whether particular DNA methylation changes occur before disease symptoms or after as a consequence of the disease, as epigenetic biomarkers have been suggested as a way of tracking developmental trajectories to disease [1].

A large epigenome-wide association study (EWAS) with replication in two independent cohorts reported that methylation levels at three CpGs in the first intron of the hypoxia inducible factor 3A (*HIF3A*) locus were positively associated with BMI in adult Caucasian whole blood and adipose tissue [2]. The finding has since been independently replicated [3]. The authors postulated that *HIF3A* has a role in acquired obesity, perhaps in regulating adipocyte differentiation.

There is considerable evidence of developmental pathways to obesity [4–7], beginning before birth and at least partly mediated by epigenetics [8–10]. Factors acting prenatally

may affect methylation levels at particular loci, which in turn affect transcription and phenotype later in life [1]. Birth weight is often used as a surrogate for the quality of the *in utero* environment and both low and high birth weights are associated with an increased risk of obesity and metabolic syndrome [11,12]. An association of birth weight or neonatal adiposity with *HIF3A* methylation at birth would be compatible with the developmental origins of obesity hypothesis. The effects of the prenatal environment on the epigenome could be genotype dependent [13] as we have previously found that umbilical cord methylation levels can associate with various *in utero* environmental factors in interaction with infant genotype [14]. *HIF3A* methylation was shown to associate with *cis*-genotype but the genotype was not associated with adult BMI [2].

The present study has three objectives. First, we tested if *HIF3A* gene methylation levels in umbilical cords were associated with birth size (weight and length) and adiposity. Second, due to the strong association between

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*cis*-SNPs and methylation within the *HIF3A* locus, we studied if the association of methylation with birth weight is independent of *cis*-genotype. Third, we sought to identify prenatal environmental variables associated with both birth weight and *HIF3A* methylation.

## Materials & methods

### Study population

Mother–offspring pairs in our study were drawn from the Growing Up in Singapore towards Healthy Outcomes (GUSTO) birth cohort [15]. Women aged at

**Table 1. Characteristics of participants in the GUSTO cohort studied in the analysis.**

Characteristic	Time point	n (%)	Mean (standard deviation)
Ethnicity:	Delivery		
Chinese		568 (57)	
Malay		251(25)	
Indian		172 (17)	
Child sex:			
Male		530 (53)	
Female		461 (47)	
Gestational age (weeks)			38.9 (1.0)
Weight (g)	Delivery	961	3150 (376)
	6 months	872	7744 (919)
	12 months	848	9405 (1073)
	18 months	808	10,770 (1304)
	24 months	820	11,996 (1542)
Length (cm)	Delivery	961	49 (2)
	6 months	876	67 (3)
	12 months	850	75 (3)
	18 months	695	82 (3)
	24 months	720	88 (4)
BMI (g/cm <sup>2</sup> )	Delivery	961	1.3 (0.1)
	6 months	872	1.7 (0.2)
	12 months	847	1.6 (0.1)
	18 months	693	1.6 (0.1)
	24 months	720	1.6 (0.1)
Subscapular skinfold (mm)	Delivery	962	5.0 (1.2)
	18 months	673	6.4 (1.4)
	24 months	760	6.4 (1.6)
Triceps skinfold (mm)	Delivery	963	5.5 (1.2)
	18 months	714	8.6 (1.7)
	24 months	734	8.8 (1.8)
Subscapular: triceps	Delivery	962	0.93 (0.17)
	18 months	648	0.76 (0.14)
	24 months	723	0.73 (0.14)
Methylation of cg27146050	Delivery	991	15% (4%)
Methylation of cg16672562			19% (5%)
Methylation of cg22891070			31% (6%)
rs8102595 minor allele frequency (G)			0.22
rs3826795 minor allele frequency (T)			0.41

**Table 2. Association between umbilical cord methylation at three sites in *HIF3A* and six neonatal anthropometric outcomes (weight, length, BMI, subscapular skinfold, triceps skinfold and ratio of subscapular to triceps skinfold) at birth.**

	cg27146050			cg16672562			cg22891070		
	Est.	95% CI	p-value	Est.	95% CI	p-value	Est.	95% CI	p-value
Birth weight (g)	3.61	(0.68–6.63)	<b>0.015</b>	3.34	(1.4–5.32)	<b>0.00068</b>	2.05	(0.32–3.82)	<b>0.020</b>
Birth length (cm)	0.60	(-0.29–1.5)	0.19	0.46	(-0.13–1.05)	0.13	0.35	(-0.18–0.88)	0.20
BMI at birth (g/cm <sup>2</sup> )	2.38	(0.23–4.58)	<b>0.030</b>	2.4	(0.97–3.84)	<b>0.00096</b>	1.35	(0.07–2.64)	<b>0.039</b>
Subscapular skinfold at birth (mm)	-0.77	(-5.97–4.72)	0.78	5.44	(1.76–9.25)	<b>0.0035</b>	3.27	(0–6.64)	<b>0.050</b>
Triceps skinfold at birth (mm)	-1.28	(-6.42–4.14)	0.64	0.75	(-2.75–4.38)	0.68	0.25	(-2.90–3.51)	0.88
Subscapular: triceps at birth	0.50	(-3.85–5.04)	0.83	4.67	(1.66–7.77)	<b>0.0022</b>	3.02	(0.34–5.78)	<b>0.027</b>

Regression coefficients (Est.) and 95% CI are reported as percentage change in outcome for 10% increase in methylation level. p-values are two-sided and p-values less than 0.05 are shown in bold. Analysis was done by linear regression of log-transformed outcome against methylation at each CpG site, adjusting for child sex, ethnicity, cell type proportions and interactions between ethnicity and cell type proportions.

least 18 years were prospectively recruited from the KK Women's and Children's Hospital (KKH) and the National University Hospital (NUH) in Singapore during their first trimester of pregnancy. Written informed consent was obtained. The GUSTO study was approved by the ethics boards of both KKH and NUH. To be eligible, women had to hold Singapore citizenship or permanent residency, intended to reside in Singapore for the next 5 years, were of Chinese, Malay or Indian ethnicity, had homogeneous parental ethnic background and had the intention of delivering at either NUH or KKH. Women who were on chemotherapy, psychotropic drugs or had diabetes mellitus were ineligible. Interviewer-administered questionnaires were used to obtain demographic, medical and obstetric data to assess eligibility. Multiple pregnancies, preterm and intrauterine growth restriction births were excluded from this analysis. There were 1052 live singleton births after excluding 124 preterm and intrauterine growth restriction births. This analysis used 991 offspring with complete information available on *HIF3A* methylation at three sites (cg27146050, cg16672562, cg22891070), genotype at two *cis*-SNPs (rs8102595 and rs3826795) and at least one anthropometric outcome.

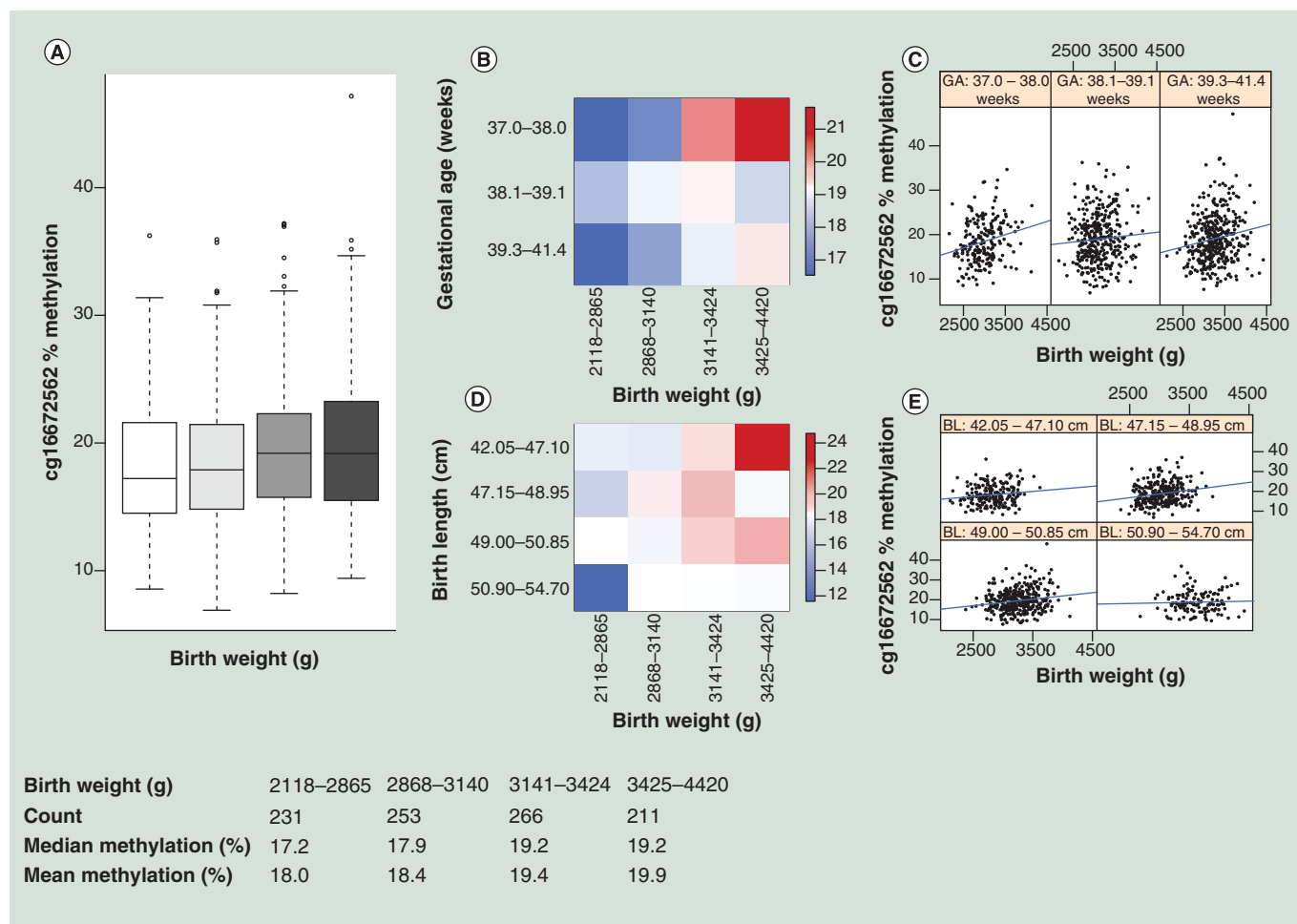
### Offspring anthropometry

Offspring weight and recumbent length were measured at birth, 6, 12, 18 and 24 months of age. Weight was measured using a calibrated infant scale (SECA 334 Weighing Scale, SECA Corp) and recorded to the nearest gram. Length was measured using a SECA infant mat (SECA 210 Mobile Measuring Mat, SECA Corp) and recorded to the nearest 0.1 cm. Measurements were

taken in duplicates for reliability. BMI was derived as weight (g) divided by length<sup>2</sup> (cm<sup>2</sup>) at all time points. Subscapular and triceps skinfolds were measured at birth, 18 and 24 months and taken in triplicates using Holtain skinfold calipers (Holtain Ltd, Crymych, UK) on the right side of the body, recorded to the nearest 0.2 mm. Subscapular to triceps skinfold ratio was derived by dividing subscapular skinfold (mm) by triceps skinfold (mm).

### Prenatal environmental exposures

An interviewer-administered questionnaire was conducted at 26–28 weeks of gestation to obtain information on occupational activity during pregnancy, exercise during pregnancy, alcohol usage before and during pregnancy and smoking patterns before and during pregnancy (Supplementary File 1; for supplementary information please see online at: [www.futuremedicine.com/doi/full/10.2217/EPI.15.45](http://www.futuremedicine.com/doi/full/10.2217/EPI.15.45)). Maternal height and weight were measured during the same time period. Prepregnancy weight was self-reported during study recruitment in the first trimester of pregnancy. Pregnancy weight gain was calculated as the difference between prepregnancy and 26–28-week weights. Maternal prepregnancy BMI was derived as prepregnancy weight divided by height squared. Maternal glucose levels, indicative of gestational diabetes, (2 h postglucose and fasting) were ascertained at 26–28 weeks using an oral glucose tolerance test (OGTT) of 75 g after overnight fasting (8–10 h). Information on maternal glucose levels (2 h postglucose and fasting) were available for 922 subjects. Using the 1999 WHO standard criteria ( $\geq 7.8$  mmol/l for 2-h postglucose and/or  $\geq 7.0$  mmol/l for fasting glucose), 162 out of 922 subjects would be diagnosed as having ges-



**Figure 1. Association between percentage methylation at cg16672562 and birth weight.** (A) Box plot of birth weight (horizontal axis) binned into four bins of equal numbers of unique values, against % methylation at cg16672562 (vertical axis). Number of individual data points, median and mean % methylation of each bin is displayed in the table below the horizontal axis. (B) Heatmap displaying % methylation at cg16672562, maximum, medium and minimum methylation are shown in red, white and blue, respectively. Subjects are split by binned (four bins of equal number of unique values) birth weight (horizontal axis) and binned (four bins of equal number of unique values, last two bins are combined as there are few observations in the last bin) gestational age (vertical axis). The progression from blue to red (low to high methylation) as birth weight increases (left to right) is visible in each gestational age bin (top to bottom). (C) Scatter plots of birth weight (horizontal axis) against % methylation at cg16672562 (vertical axis), each panel displays data for binned gestational ages (four bins of equal number of unique values, last two bins are combined as there are few observations in the last bin), ranges for each bin are displayed in panel headers. (D) Heatmap displaying % methylation at cg16672562, maximum, medium and minimum methylation are shown in red, white and blue, respectively. Subjects are split by binned (four bins of equal number of unique values) birth weight (horizontal axis) and binned (four bins of equal number of unique values) birth length (vertical axis). The progression from blue to red (low to high methylation) as birth weight increases (left to right) is visible in each birth length bin (top to bottom). (E) Scatter plots of birth weight (horizontal axis) against % methylation at cg16672562 (vertical axis), each panel displays data for binned (four bins of equal number of unique values) birth length, ranges for each bin are displayed in panel headers.

For color images please see online at: [www.futuremedicine.com/doi/full/10.2217/EPI.15.45](http://www.futuremedicine.com/doi/full/10.2217/EPI.15.45)

tational diabetes in this group. Maternal plasma micro-nutrient levels, including vitamin D, vitamin B12, copper, iron, zinc and folate, were tested using serum drawn at 26–28 weeks of gestation. Birth order and mode of delivery were abstracted from hospitals’ medical records.

### Methylation & genotyping data

Bisulfite-converted gDNA from umbilical cords was interrogated on Infinium® Human Methylation 450

Bead Chip arrays [16] as per manufacturer’s instructions and as described previously [14]. Data were processed as previously described [17]. Briefly, signal extraction was performed in GenomeStudio™ Methylation Module and raw values were extracted. Probes with data from two beads or fewer for any sample, or with signal detection p-values (calculated from signal vs background of the individual bead intensities) greater than 0.01, for any sample, were discarded for all samples. The green

**Table 3. Association of two *cis*-single nucleotide polymorphisms (rs8102595, rs3826795) with methylation at three sites in *HIF3A*.**

Model	cg27146050			cg16672562			cg22891070		
	Est.	95% CI	p-value	Est.	95% CI	p-value	Est.	95% CI	p-value
CpG ~ rs8102595	1.2	(0.96–1.5)	<b>5.7E-19</b>	2.9	(2.5–3.3)	<b>1.6E-45</b>	3.2	(2.8–3.7)	<b>5.7E-47</b>
CpG ~ rs3826795	0.64	(0.40–0.87)	<b>1.1E-07</b>	1.6	(1.2–1.9)	<b>3.2E-18</b>	1.5	(1.1–1.9)	<b>3.2E-14</b>

Above are results from six different linear regression models. In each linear regression model, methylation at each CpG site is regressed against each SNP. Regression coefficients (Est.) and 95% CI reflect changes in % methylation per copy of the effect allele, adjusting for child sex, ethnicity, cell type proportions and interactions between ethnicity and cell type proportions. p-values are two-sided and p-values less than 0.05 are shown in bold. The effect allele of rs8102595 is G and the effect allele of rs3826795 is C.

and red channel signals were normalized and the background signal from the negative probe control values was removed. Probe  $\beta$ -values or % methylation values were derived from the data.  $\beta$ -values are the ratio of the methylated probe intensity and the overall intensity,  $\beta$ -value for an  $i$ th interrogated CpG site:

$$\beta_i = \frac{\max(Y_{i, \text{methy}}, 0)}{\max(Y_{i, \text{unmethy}}, 0) + \max(Y_{i, \text{methy}}, 0) + a}$$

**Equation 1**

where  $y_{i, \text{methy}}$  and  $y_{i, \text{unmethy}}$  are the intensities measured by the  $i$ th methylated and unmethylated probes, respectively, averaged over the replicate beads;  $a$  is a constant offset, which is by default 100. Therefore,  $\beta$ -values range between 0 and 1 with 0 representing no methylation and 1 representing 100% methylation.  $\beta$ -values were further processed to scale the % methylation range of the type 2 probes to the type 1 probes using the procedure suggested by [18]. All data from the sex chromosomes were removed and the remaining data were subjected to quantile normalization. As part of the experimental design, subjects were randomized such that key variables (birth weight, child sex, ethnicity, gestational age) were randomly distributed across batch/chip/position. Batch effects were observed between different runs in the processed data and removed by a commonly used empirical Bayes method known as COMBAT [19]. Even though chip/position were associated with the three *HIF3A* methylation sites in question (Supplementary Table 1), chip/position were not associated with any of the six neonatal outcomes studied in this report (Supplementary Table 2)

due to experimental design. As chip/position were associated only with the independent variable (methylation) but not the outcome, this minimizes confounding bias due to chip/position.

The same umbilical cord DNA samples were genotyped using the Illumina Omniexpress + exome arrays. Genotyping was performed by the service provider, Expression Analysis Inc. Data were processed in Genome Studio Genotyping Module™. Genotyping calls were made by the Gen Call software, which incorporates a clustering algorithm (GenTrain) and a calling algorithm (Bayesianmodel). Gen Call score of each single nucleotide polymorphism (SNP) probe and call rate of each sample are generated. Genotypes with a Gen Call score less than 0.15 were not considered.

**Accounting for cellular heterogeneity from methylation data**

Umbilical cord tissue consists of a mixture of cell types with differing epigenetic profiles. To control for cellular heterogeneity in our samples, we applied three methods: one requiring a reference panel of methylation levels in discrete cell types [20] and two reference-free methodologies [21,22]. For the former, we estimated the proportion of fibroblasts, B cells and T cells using a reference panel’s cell-specific methylation profiles [20]. The methylation dataset obtained from EMBL-EBI European Genome-Phenome Archive [23] under accession number EGAD00010000460 was used as our reference panel [24]. The estimated cell fractions were then adjusted as covariates in the regression models. Interestingly, the associations between the estimated cellular

**Table 4. Association between two *cis*-single nucleotide polymorphisms at the *HIF3A* locus (rs8102595, rs3826795) and birth weight.**

Single nucleotide polymorphisms	Est.	95% CI	p-value
rs8102595	0.39	(-0.89–1.7)	0.55
rs3826795	0.059	(-1.0–1.2)	0.92

Regression coefficients (Est.) and 95% CI are reported as percentage change in birth weight per copy of the effect allele. p-values are two-sided. Analysis was done by linear regression of log-transformed birth weight against each SNP (additive genetic model), adjusting for ethnicity and child sex. The effect allele of rs8102595 is G and the effect allele of rs3826795 is C.



**Table 5.** Association between umbilical cord methylation at three sites in *HIF3A* and six neonatal anthropometric outcomes (weight, length, BMI, subscapular skinfold, triceps skinfold and ratio of subscapular to triceps skinfold) at birth, adjusting for rs8102595 and rs3826795.

Outcome	cg27146050			cg16672562			cg22891070		
	Est.	95% CI	p-value	Est.	95% CI	p-value	Est.	95% CI	p-value
Birth weight (g)	3.67	(0.62–6.81)	<b>0.018</b>	3.87	(1.7–6.09)	<b>0.00044</b>	2.28	(0.34–4.26)	<b>0.021</b>
Birth length (cm)	0.63	(-0.3–1.57)	0.18	0.55	(-0.11–1.21)	0.10	0.4	(-0.19–1)	0.18
BMI at birth (g/cm <sup>2</sup> )	2.37	(0.13–4.67)	<b>0.038</b>	2.74	(1.14–4.36)	<b>0.00075</b>	1.46	(0.03–2.91)	<b>0.046</b>
Subscapular skinfold at birth (mm)	-0.62	(-6.05–5.11)	0.83	7.08	(2.92–11.41)	<b>0.00073</b>	4.31	(0.64–8.11)	<b>0.021</b>
Triceps skinfold at birth (mm)	-0.06	(-5.46–5.65)	0.98	2.64	(-1.33–6.77)	0.19	1.7	(-1.85–5.39)	0.35
Subscapular: triceps at birth	-0.6	(-5.06–4.08)	0.8	4.34	(1–7.79)	<b>0.011</b>	2.57	(-0.4–5.63)	0.091

Analysis without adjusting for rs8102595 and rs3826795 are shown in **Table 2**, results of both are similar. Regression coefficients (Est.) and 95% CI are reported as percentage change in outcome for 10% increase in methylation level. p-values are two-sided and p-values less than 0.05 are shown in bold. Analysis was done by linear regression of log-transformed outcome against methylation at each CpG site, adjusting for rs8102595, rs3826795, child sex, ethnicity, cell type proportions and interactions between ethnicity and cell type proportions.

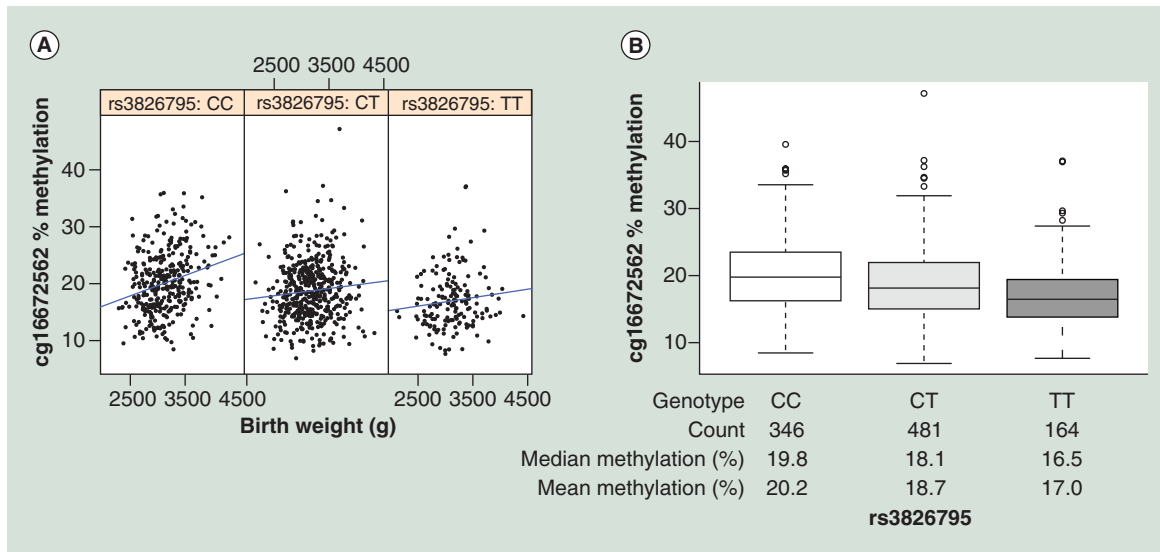
proportions and child anthropometry were generally ethnic-dependent (**Supplementary Tables 3 & 4**). Therefore, interaction terms between cellular proportions and ethnicity were included in all regression models. As a sensitivity analysis, we also applied two reference-free methods [21,22] to investigate the association between methylation levels at *HIF3A* and log-transformed child anthropometric measures, adjusting for infant sex and ethnicity. Leek *et al.* [22] estimates surrogate variables which capture cellular heterogeneity, the surrogate variables are then adjusted as covariates in regression analysis. We note that this sensitivity analysis using Leek *et al.* [22] would also act to adjust for residual unknown confounding effects, including chip/position effects since the estimated surrogate variables can potentially capture both cellular heterogeneity and residual batch/chip/position effects [21,25–27]. The method proposed by Houseman *et al.* [21] is similar to the surrogate variable analysis proposed by Leek *et al.* [22] with an additional biological mixing assumption. Houseman *et al.* [21] correct the cellular heterogeneity without explicitly estimating the relative cell proportions or returning the surrogate variables.

### Statistical analysis

Linear regression models were used to examine the association of child anthropometric measures with *HIF3A* methylation  $\beta$ -values, with adjustment for child sex, ethnicity, cellular composition and interactions between ethnicity and cellular proportions. Child anthropometric measures were log-transformed to satisfy statistical assumptions of normality. Due to the log-transformation, effect sizes are reported as percentage increase in child outcome for 10% increase in methylation  $\beta$ -values. As a sensitivity analysis, we also report results additionally adjusted for gestational age. To examine if the association between child anthropometry with *HIF3A* methylation were independent of *cis*-genotypes, we additionally adjusted for both *cis*-genotypes. To identify prenatal environmental exposures associated with both birth weight and *HIF3A* methylation, we regressed birth weight on various prenatal exposures, and adjusted for child sex and ethnicity. We further regressed *HIF3A* methylation on various prenatal exposures (and their interactions with genotype), adjusting for child sex, ethnicity, cellular composition and interactions between ethnicity and cellular proportions.

**Table 6.** Two-sided p-values from testing for interaction term between log-transformed birth weight and single nucleotide polymorphism (rs8102595, rs3826795), with methylation as outcome, adjusting for main effect of log-transformed birth weight, main effect of single nucleotide polymorphism, child sex, ethnicity, cell type proportions and interactions between ethnicity and cell type proportions.

Model	cg27146050	cg16672562	cg22891070
	p-value	p-value	p-value
log(BW)* rs8102595	0.32	0.077	0.30
log(BW)* rs3826795	0.34	<b>0.019</b>	<b>0.033</b>



**Figure 2. Association between % methylation at cg16672562 and birth weight, stratified by rs3826795 genotype.** (A) Scatter plots of birth weight (horizontal axis) against % methylation at cg16672562 (vertical axis), each panel displays data for each genotype. (B) Box plot of rs3826795 genotype (horizontal axis) against % methylation at cg16672562 (vertical axis). Number of individual data points, median and mean % methylation of each bin is displayed in the table below the horizontal axis.

## Results

### *HIF3A* methylation in umbilical cords was positively associated with birth weight & adiposity

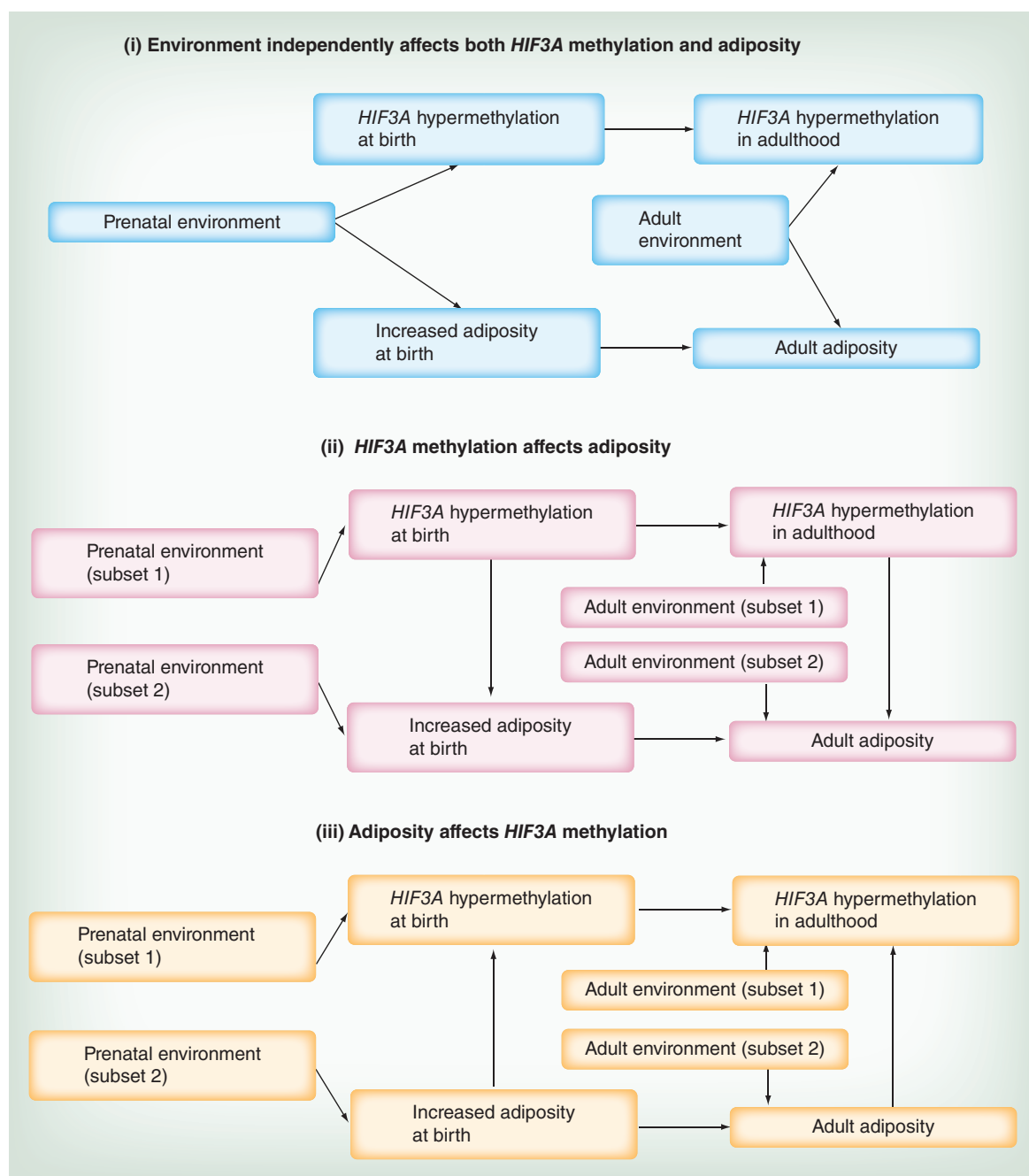
Table 1 gives the characteristics of the 991 infants investigated in the current analysis. Higher umbilical cord *HIF3A* methylation level at all three CpGs was associated with greater infant birth weight (Table 2 & Figure 1A), after adjusting for infant sex, ethnicity, estimated cellular proportions and interactions between ethnicity and cellular proportions [20]. There were also significant associations with adiposity at birth measured by BMI and, for two of the three CpGs, by subscapular skinfold and the ratio of subscapular to triceps skinfold (thought to reflect fat deposition on the trunk rather than limbs [28]) (Table 2). Similar results were obtained at two of the CpGs when we used reference-free methodologies [21,22] to account for cellular heterogeneity (Supplementary Tables 5 & 6). Despite the strong association between gestational age and birth weight in our dataset ( $r = 0.32$ ;  $p < 0.0005$ ), the associations of *HIF3A* methylation at all three CpGs with birth weight remained significant, after adjusting for gestational age (Supplementary Table 7). The association between methylation and birth weight was consistent across gestational age categories and was perhaps strongest in neonates with the earliest and latest gestational ages (Figure 1B & C & Supplementary Table 8). Similarly, despite the strong correlation between birth weight and length in the dataset ( $r = 0.67$ ;  $p < 0.0005$ ), *HIF3A* methylation was not significantly associated with birth

length and the association between methylation and birth weight was consistent across birth length categories (Figure 1D & E), suggesting a link between *HIF3A* methylation and adiposity at birth rather than birth size in general.

For every 10% increase in methylation of cg27146050, cg16672562 and cg22891070, birth weight was 3.61% (95% CI: 0.68–6.63), 3.34% (1.4–5.32) and 2.05% (0.32–3.82) higher, respectively (Table 2). These effect sizes are slightly smaller than the corresponding effect estimates on adult BMI of 7.8% (5.1–10.4), 3.2% (2.0–4.4) and 3.6% (2.4–4.9), as previously reported [2].

### The association of *HIF3A* methylation & birth weight remained significant after adjusting for *cis*-acting SNPs; but birth weight & *HIF3A* methylation was more strongly correlated in one genotypic group

Using the same 991 GUSTO umbilical cord samples, we genotyped the SNPs identified as *cis*-influencing *HIF3A* methylation in the Dick *et al.*'s study [2]. We found that rs8102595 and rs3826795 were strongly associated with methylation levels at all three CpGs sites (Table 3). However, neither SNP was associated with birth weight (Table 4) and the associations of birth weight with methylation at all three sites were similar when adjusted for genotype at both SNPs (Table 5). This mirrors the findings of [2]. We also tested if *HIF3A* methylation levels were explained by an interaction of *HIF3A* genotype and infant birth weight (adjusting for



**Figure 3. Three of the simplest possible scenarios to explain the relationship between *HIF3A* methylation and adiposity.**

main effects of both genotype and infant birth weight), as we have previously found that neonatal methylation patterns are often a product of the interaction of the *in utero* environment and genotype. The interaction term was significant at  $p < 0.05$  for two of the CpGs and rs3826795 (Table 6). In the CC genotype group, birth weight was more strongly correlated with *HIF3A* methylation (Figure 2 & Supplementary Table 9). The CC genotype was also associated with higher methylation values at the *HIF3A* locus (Table 3 & Figure 2). This

suggests the CC genotypic group could be more plastic to environmental exposures *in utero*.

**The prenatal environmental variables examined were not consistently associated with *HIF3A* methylation & birth weight**

As we hypothesized that *HIF3A* methylation and birth weight were related to the prenatal environment, we examined for significant associations with both *HIF3A* methylation and birth weight for a range of factors

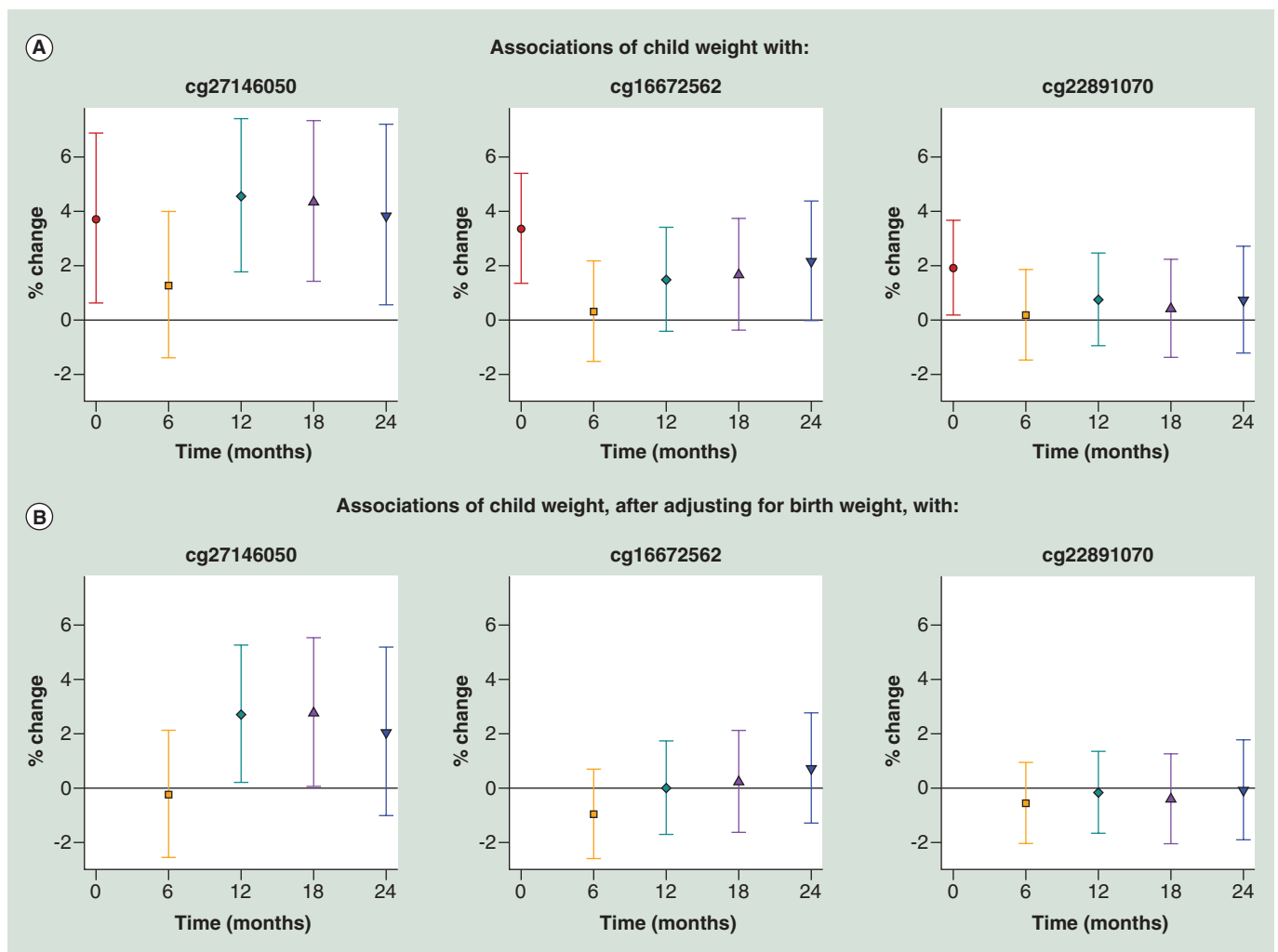


related to the prenatal environment, both as main effects and in interaction with *HIF3A* genotype. Factors investigated included antenatal lifestyle factors such as activity level and smoking, maternal prepregnancy BMI and pregnancy weight gain, maternal glucose tolerance during pregnancy indicative of gestational diabetes, maternal micronutrient levels, maternal age and parity (Supplementary File 1). There were some suggestive associations; for example, maternal glucose tolerance (indicative of gestational diabetes) was significantly associated with both *HIF3A* methylation at one of the CpGs and birth weight. Furthermore, maternal weight gain during pregnancy was significantly associated both with infant birth weight and *HIF3A* methylation (again

at only one CpG) in interaction with genotype. However, we did not find any prenatal environmental variables that showed consistently significant association with all three *HIF3A* sites and birth weight and none yielded substantial effect sizes.

**Discussion**

We have extended the finding that *HIF3A* DNA methylation in adults associates with BMI [2], to show that the link between *HIF3A* DNA methylation with weight and adiposity can be detected at birth. Interestingly, the association was limited to measures of adiposity (i.e., weight, BMI and skinfolds) and not other determinants of birth size or putative proxies for gestational



**Figure 4. Association between umbilical cord methylation at three sites in *HIF3A* and child weight measured at 0, 6, 12, 18 and 24 months. (A) Without adjusting for birth weight. (B) Adjusted for log-transformed birth weight. Regression coefficients (Est.) and 95% CI are reported as percentage change in child weight for 10% increase in methylation level. P-values are two-sided. Using linear regression models estimated using generalized estimating equations to account for repeated measures, we regressed log-transformed weight against methylation at each CpG site, adjusting for fixed effects of time, child sex, ethnicity, cell type proportions and interactions between ethnicity and proportions. Time was coded using a binary variable for each distinct time point and interaction terms of time with all variables (methylation, child sex, ethnicity, cell type proportions and interactions between ethnicity and proportions) were included.**

quality such as gestational age and birth length. As such, the relationship of increased methylation at *HIF3A* CpGs with birth outcomes is reminiscent of the effect of maternal diseases, like Type 1 diabetes, on the offspring, whereby weight is increased, but length is not [29].

Although pertaining to the same three CpGs within the *HIF3A* gene that were previously reported, our data were derived from a different tissue (umbilical cord vs blood and adipose), in a different population (Asian vs Caucasian), in a population-based cohort (normative range vs study population for metabolic disorders and controls) and most importantly at a different stage in the life-course (neonates vs adults). The effect size reported here are slightly smaller than those reported by Dick *et al.* [2]. Reasons the effect sizes are smaller could be statistical, population dependent (our population are healthy babies, Dick *et al.* studied a disease cohort with a high level of obesity) or developmental (the effect is smaller at birth and increases during the lifecourse as adiposity increases).

Replicated positive associations such as the one described here can guide future hypothesis-free EWAS. Lack of statistical power is a big challenge in EWAS, especially in cohorts of modest size. The integration of effects of cellular heterogeneity, *cis* and *trans* genotype and the potential influence of pregnancy conditions is also challenging. Therefore, a detailed case study of a proposed association (*HIF3A* with adiposity replicated across populations and ages) is important. It will help set the standards for statistical replication and delineate the issues to consider when evaluating hits from more open-ended screening.

Dick *et al.* [2] considered three possibilities that could explain the association between *HIF3A* methylation and adiposity in adults: (i) a confounding factor (e.g., environment) independently affects both *HIF3A* methylation and adiposity, (ii) increased *HIF3A* methylation causes increased adiposity and (iii) increased adiposity causes increased *HIF3A* methylation. To put our findings in neonates in context with the findings in adults, the same three simplified possible scenarios are schematically depicted in Figure 3. We note that more complicated scenarios or combinations of these simplified situations are possible and likely.

Using a Mendelian randomization reasoning [30] from the observation that *HIF3A* genotype was associated with *HIF3A* methylation but not adult BMI, Dick *et al.* [2] suggested that scenario (iii) (adiposity results in *HIF3A* hypermethylation) is more likely than scenario (ii) (*HIF3A* hypermethylation leads to increased adiposity). However, in this context, Mendelian randomization assumes the genotype can affect the phenotype only through DNA methylation and not through other biological pathways. The applicability of this assumption is unknown for *HIF3A* genotype. We cannot confer cau-

sality in our dataset but following the same arguments, increased DNA methylation at the *HIF3A* locus could result from increased birth weight/adiposity and not cause it, but the aforementioned limitation also applies in our case.

Dick *et al.* considered scenario (i) less likely as they did not find an association between *HIF3A* methylation and BMI-related characteristics like diabetes. However, the absence of an association between *HIF3A* methylation and diabetes could also be due to the same confounding factor having a direct effect on glucose tolerance that is independent of increased adiposity. We postulate that scenario (i) could involve a prenatal environmental factor that independently affected both birth weight and *HIF3A* methylation. We also find that, as may be expected from other studies [14,31], interindividual variation in *HIF3A* methylation can be explained by an interaction of genotype and birth weight, suggesting there may be a genotype group (CC) particularly sensitive to gestational environment (Table 6 & Figure 2). However, in this study, we were unable to definitively identify a factor acting prenatally that is consistently associated with both *HIF3A* methylation and birth weight, despite the extensive dataset on pregnancy environments collected from the GUSTO cohort and examined in this study. Nevertheless, *HIF3A* methylation at birth after much further study, may prove to be a useful surrogate for likely metabolic trajectory determined by gestational environment [32], and could be more useful than birth weight as it would be independent of the inherited genetic and environmental determinants of birth weight.

Transgenerational transmission of obesity and the importance of the very early environment in determining metabolic trajectories have been much studied [33,34]. If metabolic trajectory is programmed during gestation, a biomarker of likely trajectory would aid intervention [1]. Birth weight has been shown to predict later weight [35] and is strongly associated with later weight in the GUSTO cohort. Interestingly, *HIF3A* methylation levels at birth were also associated with weights at later ages, albeit possibly with slightly lower effect sizes (Figure 4A & Supplementary Table 10). Only a few of the associations survive at borderline significance when adjusted for birth weight (Figure 4B & Supplementary Table 11), thus the association of *HIF3A* methylation with later weights may reflect the growth trajectory set at birth. The 'dip' in the effect size at 6 months could be due to the effects of weaning or the decreased influence of genetic factors [36,37], although it is interesting to note that birth weight is more correlated with weight at 6 months than with weight at any of the other time points (Supplementary Tables 12 & 13).

Future studies should track both *HIF3A* methylation and offspring size and adiposity longitudinally during gestation, at birth, and at subsequent ages to determine if the association of *HIF3A* methylation with adiposity is consistent. As blood and adipose are invasive tissues to sample in infants, *HIF3A* methylation levels should be investigated in other tissue types such as saliva, buccal swabs or circulating fetal cells obtained prenatally. Longitudinal studies can help determine, if *HIF3A* is a biomarker for obesity risk and assess the utility of using *HIF3A* methylation to evaluate the efficacy of interventions such as lifestyle modification. Future studies should also examine the impact of *HIF3A* methylation changes on gene expression in relevant tissues. Dick *et al.* [2] found a negative association between *HIF3A* methylation levels and *HIF3A* gene expression in adipose tissue. It is a limitation of our study that we

cannot examine gene expression in a relevant tissue due to the unfeasibility of sampling adipose tissue in neonates or infants.

## Conclusion

The association between increased DNA methylation at *HIF3A* and increased adiposity is present in neonates. This suggests *HIF3A* methylation is a potential biomarker for metabolic trajectory and/or implicated in metabolic syndrome and further study is warranted.

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## Executive summary

### Introduction

- Variation in DNA methylation between individuals is a product of influences from the environment and individual genetic backgrounds.
- DNA methylation levels at particular loci may closely map trajectories to diseases such as obesity, which have environmental and genetic components.
- A recent study linked methylation within the *HIF3A* gene to BMI and obesity in adults.
- Individual susceptibility to obesity is influenced by early environmental exposures.
- This study investigates whether the association of *HIF3A* methylation with adiposity can be detected at birth.

### Material & methods

- The study was conducted in the 991 subjects from the GUSTO birth cohort.
- We determined *HIF3A* genotype and DNA methylation patterns in DNA extracted from umbilical cords of the 991 infants using Illumina® Infinium HumanMethylation and Omniexpress arrays.
- Associations of methylation levels and birth outcomes were adjusted for sex, ethnicity, cellular composition of umbilical cords and interactions between ethnicity and cellular composition.

### Results

- Higher DNA methylation within *HIF3A* measured in umbilical cord tissue was associated with greater infant birth weight and adiposity.
- The association of *HIF3A* methylation and birth weight remained significant after adjusting for *cis*-acting single nucleotide polymorphisms; but the association was stronger in one genotypic group.
- None of the prenatal environmental variables examined were consistently associated with *HIF3A* methylation and birth weight.

### Discussion

- We have extended the previous finding that *HIF3A* DNA methylation in adults associates with BMI to show that the link between *HIF3A* DNA methylation with weight and adiposity can be detected at birth.
- Although pertaining to the same three CpGs within the *HIF3A* gene that were previously reported, our data were derived from a different tissue (umbilical cord vs blood and adipose), in a different population (Asian vs Caucasian), in a population-based cohort (normative range vs study population for metabolic disorders and controls) and most importantly at a different stage in the lifecourse (neonates vs adults).
- This suggests that prenatal factors may influence *HIF3A* methylation but despite the extensive data collected in the GUSTO, we were unable to definitively identify a responsible prenatal factor.
- We were unable to imply causality in our data and do not know whether adiposity causes *HIF3A* hypermethylation, *HIF3A* hypermethylation causes adiposity or if some independent factor influences both.
- In their study of *HIF3A* methylation and adult BMI, Dick *et al.* used Mendelian randomization to suggest that *HIF3A* hypermethylation is a consequence of increased adiposity. We could draw the same conclusion from our data but note that some of the assumptions of Mendelian randomization might not be satisfied.
- We suggest that as the association between *HIF3A* methylation and adiposity is detectable so early in life, *HIF3A* may be a potential biomarker of metabolic trajectory.

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### Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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