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Full title: *HIF3A* cord blood methylation and systolic blood pressure at 4 years – a population-based cohort study

Short title: Birth *HIF3A* methylation and 4-year blood pressure

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Keywords: DNA methylation, *HIF3A,* cardiovascular, paediatrics, cord blood

Abstract

Background: Methylation of the hypoxia-inducible factor 3α gene (*HIF3A*) in blood has been reproducibly linked to body-mass index (BMI) in adults. Despite emerging evidence implicating *HIF3A* in angiogenesis and metabolism, no studies have examined the link between *HIF3A* methylation in early life and cardiovascular health. Here, we investigated the relationship between *HIF3A* methylation in blood at birth and 12 months of age with cardiovascular measures at four years. We also examined influences of prenatal exposures, birth outcomes, and genetic variation on these relationships.

Methods: Methylation of two *HIF3A* promoter regions in cord blood was measured using Sequenom EpiTYPER mass-spectrometry. The first promoter region was also measured in 12-month blood. Four-year cardiovascular measures included blood pressure, pulse wave velocity, and aortic and carotid intima-media thickness. Strength of associations were tested using partial correlation tests and linear regression modelling.

Results: Methylation of the first *HIF3A* promoter in cord and 12-month blood was not associated with any four-year measures. There was modest evidence of a positive association between DNA methylation of the second *HIF3A* promoter in cord blood and four-year systolic blood pressure (n=353, r=0.12, p=0.03). In sex-stratified analysis, methylation of the second region was modestly associated with systolic (r=0.16, p=0.03) and diastolic blood pressure (r=0.16, p=0.03) in males only.

Conclusions: *HIF3A* methylation at birth shows some evidence of an association with later blood pressure in childhood. Further work should determine whether this relationship persists into later childhood, and should assess the potential functional link between *HIF3A* methylation and cardiovascular health more generally.

Key words: *HIF3A*, DNA methylation, epigenetics, developmental biology, infant, blood pressure.

Background

The trajectory towards adult metabolic and cardiovascular health begins very in early life, with evidence for a variety of environmental exposures in childhood impacting disease risk in adulthood [1]. Environmental exposures in early life can influence childhood cardiovascular health, which in turn has been linked to later risk of cardiovascular disease [2, 3]. Elevated blood pressure in childhood is associated with increased risk of hypertension, metabolic syndrome [4] and altered heart structure [5] in adulthood. Further, intima-media thicknesses of the aortic and carotid vessels in childhood have been used as measures of preclinical atherosclerosis [6, 7]. Evidence from animal models suggests these associations may be influenced by sex [8], but evidence from humans is less compelling [9]. Emerging data suggest that epigenetics plays a role in the 'biological embedding' of later life risk following early life exposures [10], and attention has recently turned to identifying genes where methylation levels in early life may predict later cardiovascular health [11].

Hypoxia-inducible factor 3α (HIF3A), encoded by the *HIF3A* gene, is part of a family of proteins that play a key role in angiogenesis, metabolism and obesity [12]. DNA methylation of one promoter of the *HIF3A* gene has been reproducibly linked to body-mass index (BMI) in adult blood [13], and more recently the link between *HIF3A* methylation at the same region and BMI has been investigated in childhood [14, 15]. However, most paediatric studies have focussed on early life associations rather than longitudinal associations between methylation and later phenotypes. An exception is a study that measured DNA methylation at this region at birth, age 7 and 17 years and examined associations with BMI at 7 and 17 years of age [15]. This study suggested that birth weight and BMI at 7 years was associated with later *HIF3A* methylation in blood at 7 and 17 years of age, respectively. The same study also reported evidence of a link between maternal pre-pregnancy BMI and *HIF3A* methylation levels at a second promoter region in cord blood. More recently, we found evidence that GDM, pre-eclampsia, infant sex, gestational age, and *HIF3A* genetic variation all

independently associated with different *HIF3A* methylation levels at this second promoter region in cord blood [16].

At present, there are no data on whether *HIF3A* methylation in early life is linked with cardiovascular phenotypes in children or adults. However, given the evidence for a link between pregnancy exposures we identified previously [16] and cardiovascular health in offspring, specifically exposure to pre-eclampsia with elevated systolic blood pressure [17] and risk of stroke [18], and exposure to gestational diabetes with risk of cardiovascular-related hospitalisations [19], we hypothesised that early life methylation of *HIF3A* is associated with later cardiovascular development in childhood.

Here, we investigated if *HIF3A* promoter methylation (two regions, *HIF3A.1* and *HIF3A.2*) in blood at birth and 12 months of age associated with BMI and/or measures of cardiovascular health at four years. We also considered whether specific prenatal exposures and birth outcomes, previously associated with *HIF3A* methylation and *HIF3A* genetic variation, might confound these relationships.

Methods

Study cohort – Barwon Infant Study

We used samples from the Barwon Infant Study (BIS), a population-based pre-birth cohort (n=1074), with maternal clinical data from pregnancy, infant outcomes at birth, and cardiovascular measures from four years of age. The BIS protocol was approved by the Barwon Health Human Research Ethics Committee (HREC 10/24), and mothers provided written informed consent. The details on eligibility, recruitment, and retention have been described previously [20].

Primary outcome – cardiovascular development at four years of age

Cardiovascular measures were taken during the participant's 4-year review, and included measurement of weight, height, blood pressure, heart rate, and pulse wave velocity, as well as measurement of aortic and carotid intima-media thicknesses (aIMT and cIMT, respectively) following ultrasound imaging using the GE Vivid-I (GE Healthcare), with an intra-reader intra-class correlation (ICC) of 0.92 and inter-reader ICC of 0.90, as previously described [21]. Measured weight and height-squared were used to calculate BMI. Brachial blood pressure, heart rate, and pulse wave velocity were averaged across three readings in a resting, supine position using SphygmoCor XCEL (AtCor Medical). The means for aIMT and cIMT were calculated from five images. Tests with mean aIMT were also adjusted for aortic diameter. Data availability for each measurement in this study is shown in **Table 1**. For analysis, tests including blood pressure measures were also adjusted for actual child age, height and sex.

Primary exposure – early life blood *HIF3A* methylation

DNA was extracted from cord and 12-month whole blood using the QIAamp DNA QIAcube HT Kit (QIAGEN, Hilden, Germany) according to manufacturer's instructions and stored at -80°C. Bisulphite conversion of DNA was performed with the MagPrep Lightning Conversion Kit (Zymo Research, Irvine, CA, USA). DNA methylation in two promoter regions of *HIF3A* was measured using the locus-specific Sequenom EpiTYPER mass-spectrometry platform (Agena Bioscience) as described previously [16]. Methylation at *HIF3A.1* (hg38:chr19:46,298,243-46,298,580), previously linked to BMI in adults [13], was measured in in a subset of cord blood (n=490) and 12-month whole blood samples (n=538). Methylation at *HIF3A.2* (hg38:chr19:46,303,864-46,304,196), associated with maternal pre-pregnancy BMI [15], was measured in cord blood for all available samples (n=938). The EpiTYPER platform utilises a process of reverse transcription and cleavage of the assayed region to create fragments (referred to here as 'CpG units'), each of which contains 1-4 CpG sites (the majority contain 1 CpG site). The resulting methylation level represents the average proportion of

methylation across all CpG sites on each CpG unit. The CpG units measured in each region are listed in **Supp. Table 1**.

As methylation at each CpG unit within each region was strongly correlated [16], the average methylation across each region was used as the main exposure measure , and individual CpG unit methylation was considered in sensitivity analysis. Participants with missing methylation data for any of the CpG units were excluded from the average methylation analysis.

To assess possible cellular heterogeneity in blood samples, flow cytometry (FACsCalibur, Becton Dickinson) was used to characterise the cellular composition of blood samples as described previously [22]. The proportions of monocytes, granulocytes and lymphocytes were considered in sensitivity analysis.

Other factors: pregnancy heath, child genetics and birth outcomes

Infant birth weight (z-score, adjusted for gestational age and sex [23]), sex and gestational age were considered as covariates. As there is evidence for maternal pre-pregnancy BMI, gestational diabetes and pre-eclampsia impacting both offspring *HIF3A* methylation [15, 16, 24] and offspring cardiovascular health [17, 19], these were considered as potential confounders . Pre-pregnancy BMI was calculated from self-reported weight, and gestational diabetes and pre-eclampsia were defined using standard clinical criteria [25, 26]. Socioeconomic status, measured using Socio-Economic Indexes For Areas (SEIFA) [27] and grouping mothers into tertiles, and maternal age were also considered as potential confounding factors.

Genome-wide genotyping and imputation was performed on all BIS infants as described previously [16]. After quality control, genotypes were available for 261 common SNPs (minor allele frequency >0.01) in and near the *HIF3A* gene (hg38: chr19:46,278,743-46,361,743). A total of 14 tag SNPs, identified with the HaploView software (Broad Institute), were used as proxies for clusters of

associated genetic variation ($r^2 > 0.1$) in analysis. There is previous evidence for several of these SNPs associating with *HIF3A* methylation levels, particularly *HIF3A.2* methylation [16].

Statistical analysis

A flowchart of participant inclusion in this analysis, and the number of participants with any cardiovascular phenotype for each of the methylation measures, is shown in **Figure 1**. The exact number of participants included in each test are shown in the corresponding results tables.

Pearson's correlation coefficients were calculated for the pairwise correlations of all methylation measures (both average methylation across each region and individual CpG unit methylation). Preliminary analysis used partial correlation tests to identify potential associations of interest between *HIF3A.1* and *HIF3A.2* methylation in infancy and four-year weight and cardiovascular outcomes. All tests were adjusted for actual age in years at the four-year time point and EpiTYPER batch, as well as the actual age in months at the 12-month time point for 12-month methylation associations. To consider potential sex-specific associations, analyses were additionally stratified by sex. The associations of interest from in the initial analysis were then investigated further in linear regression models for the adjustment of birth weight, 4-year BMI, and potential confounders (above). The final model included covariates which were demonstrated to alter the effect size of methylation (>10% change in coefficient) or improve the model fit (likelihood ratio test $p<0.05$). Genotypes at each of the 14 tag SNPs were considered as covariates. P-values are presented unadjusted for multiple comparisons.

For sensitivity analysis, associations between methylation of individual CpG units in each region and 4-year cardiovascular measure were considered. In addition, cellular composition of blood samples (proportions of lymphocytes, monocytes and granulocytes, adjusted for exposure to labour at birth

(any/none)) and bisulphite conversion batch were also considered in the multivariable linear regression model to determine if they altered any findings.

Results

The distribution of cohort characteristics is shown in **Table 1**, and the distribution of methylation is shown in **Figure 2**. There was a moderate negative correlation between *HIF3A.1* and *HIF3A.2* average methylation at birth (r=-0.17, p=0.006, **Supp. Tables 2** and **3**). There was no evidence of an association between birth or 12-month average *HIF3A.1* methylation and any of the four-year weight or cardiovascular measures (**Supp. Table 4**). There was modest evidence that *HIF3A.2* methylation was positively associated with systolic blood pressure (r=0.12, p=0.03) in the correlation analysis (**Table 2**). When stratified by sex, there was some evidence for a relationship between *HIF3A.2* methylation and both systolic (r=0.16, p=0.03) and diastolic (r=0.16, p=0.03) blood pressure in males, but not females (**Table 3**). In linear regression modelling, none of the prenatal maternal factor appeared to confound this relationship, and similarly, adjusting for birth outcomes or SNP genetic covariates did not improve the fit of the model or alter the effect size of *HIF3A.2* methylation on systolic blood pressure, with the exception of birth weight (z-score), which modestly increased the methylation coefficient and improved the fit of the model. BMI at four years was associated with systolic blood pressure, but adjusting for BMI did not attenuate the association between methylation and systolic blood pressure (**Table 4**).

In sensitivity analyses, methylation of most, but not all, individual *HIF3A.2* CpG units were positively associated with systolic blood pressure, while several individual *HIF3A.2* CpG units were also positively associated with diastolic blood pressure (**Supp. Table 5**). There was no evidence for individual CpG units in *HIF3A.2* or in *HIF3A.1* at birth or 12-months associating with other 4-year cardiovascular measures (data not shown). Adjustment for bisulphite conversion batch, cellular

composition of blood samples and any exposure to labour did not alter the findings (data not shown).

Discussion

In this study, we investigated the potential for blood *HIF3A* methylation in early life to associate with four-year weight and cardiovascular measures. We found some evidence that higher *HIF3A.2* methylation in cord blood correlates with higher systolic and diastolic blood pressure, primarily in males. This association persisted following adjustment for birth weight. To our knowledge, this is the first study to investigate the link between *HIF3A* methylation and cardiovascular health measures, and the first to report potential evidence of early life *HIF3A* methylation associating with health measures later in childhood. In light of previous findings for *HIF3A.1,* these findings suggest that methylation patterns at the two different *HIF3A* promoter regions may have differing relevance for cardiovascular and metabolic health outcomes.

We have found stronger evidence of *HIF3A.2* methylation associating with blood pressure in males than females. While potential relationship between DNA methylation and childhood blood pressure is currently uncharacterised, there are well-established sex differences in vascular and heart physiology and blood pressure regulation [28]. Our findings may relate to this sexual dimorphism. However, it is important to note that the sex-stratified analysis is performed on a reduced sample size and as such, has greater potential to generate false positives, particularly when outlier values are included. Also, by including additional sex-stratified analysis the number of tests performed has increased, which should also be considered when interpreting the overall strength of evidence arising from association analyses.

There is considerable evidence that both systolic and diastolic blood pressure in childhood are predictive of cardiovascular risk in later life. In particular, elevated blood pressure in childhood is

associated with increased risk of hypertension, metabolic syndrome [4] and altered heart structure [5] in adulthood. Based on our findings, it is unlikely that early life *HIF3A.2* methylation has predictive utility in isolation, but could potentially improve prediction in combination with other predictive measures.

The effects of methylation on *HIF3A* gene expression are poorly characterised. There is evidence for *HIF3A* producing up to eight alternatively spliced transcripts across multiple promoter regions [29]. Methylation of the *HIF3A.1* promoter region has been reported to decrease total *HIF3A* expression in adipose tissue [13], whereas no association between any *HIF3A* methylation probe and total expression was found in blood or fibroblasts [30]. It is possible that *HIF3A.2* methylation may relate to later blood pressure through regulation of specific *HIF3A* isoforms, rather than necessarily altering total expression levels, but current evidence linking promoter-specific methylation to *HIF3A* isoforms is limited. However, it has been shown that splice variants starting from the *HIF3A.2* promoter region are more highly expressed in adult heart tissue compared to other organs, and also more highly expressed compared to splice variants starting from *HIF3A.1* [29]. As such, one or more splice variants starting from *HIF3A.2* may potentially be involved in pathways regulating cardiac function or development. More studies investigating this aspect of *HIF3A* gene regulation are required in this regard.

This study is the first to investigate the association between early *HIF3A* methylation and measures of cardiovascular health in childhood, and one of the few to address methylation across multiple *HIF3A* promoter regions. We have also considered a range of potential confounders, including abnormal metabolic prenatal exposures, birth outcomes, and genetic variation. A limitation is missing data for individual CpG units, reducing the number of infants with complete methylation data, and missing data on some of the four-year cardiovascular measures reducing our sample size in some analyses, and consequently decreasing our power to detect more subtle effect sizes. There may also be additional unknown and unmeasured confounders. Replication of our findings in other

longitudinal populations is warranted, with concomitant cardiovascular measures. Such measures at age 7 in BIS are currently underway, which will be valuable for testing the observed relationships later in childhood.

In conclusion, we provide some evidence for an association of cord blood methylation at a specific *HIF3A* promoter region with measures of four-year cardiovascular health independently of child anthropometry at birth and four years of age, with stronger evidence for a relationship in males. Our findings suggest the importance of considering promoter-specific *HIF3A* methylation status in broader association studies. Further evidence from paediatric and adult cohorts is required to characterise the extent to which earlier *HIF3A* methylation might be associated with later cardiovascular health throughout life course and also to understand the potential underlying functional mechanisms.

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Barwon Infant Study Investigator Team

The members of the Barwon Infant Study Investigator Team are the following: Peter Vuillermin and Fiona Collier, Barwon Health, Deakin University, the Murdoch Children's Research Institute; Anne-Louise Ponsonby, John Carlin, Katie Allen, Mimi Tang, Richard Saffery, Sarath Ranganathan, and David Burgner, the Murdoch Children's Research Institute, University of Melbourne; Terry Dwyer, the Murdoch Children's Research Institute and the George Institute for Global Health; and Peter Sly, University of Queensland, Queensland Children's Medical Research Institute.

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Disclosures

None.

References

1. Gluckman, P.D. and M.A. Hanson, *The developmental origins of the metabolic syndrome.* Trends in Endocrinology & Metabolism, 2004. **15**(4): p. 183-187.
2. Kelly, R.K., et al., *Factors affecting tracking of blood pressure from childhood to adulthood: the childhood determinants of adult health study.* The Journal of pediatrics, 2015. **167**(6): p. 1422-1428. e2.
3. Theodore, R.F., et al., *Childhood to early-midlife systolic blood pressure trajectories: early-life predictors, effect modifiers, and adult cardiovascular outcomes.* Hypertension, 2015. **66**(6): p. 1108-1115.
4. Sun, S.S., et al., *Systolic blood pressure in childhood predicts hypertension and metabolic syndrome later in life.* Pediatrics, 2007. **119**(2): p. 237-246.
5. Lai, C.-C., et al., *Impact of long-term burden of excessive adiposity and elevated blood pressure from childhood on adulthood left ventricular remodeling patterns: the Bogalusa Heart Study.* Journal of the American College of Cardiology, 2014. **64**(15): p. 1580-1587.
6. Hong, Y.M., *Atherosclerotic cardiovascular disease beginning in childhood.* Korean circulation journal, 2010. **40**(1): p. 1-9.
7. McCloskey, K., et al., *Aortic intima-media thickness measured by trans-abdominal ultrasound as an early life marker of subclinical atherosclerosis.* Acta Paediatr, 2014. **103**(2): p. 124-30.
8. Intapad, S., et al., *Sex differences in the developmental origins of cardiovascular disease.* Physiology, 2014. **29**(2): p. 122-132.
9. Dasinger, J.H. and B.T. Alexander, *Gender differences in developmental programming of cardiovascular diseases.* Clinical Science, 2016. **130**(5): p. 337-348.
10. Novakovic, B. and R. Saffery, *The importance of the intrauterine environment in shaping the human neonatal epigenome.* Epigenomics, 2013. **5**(1): p. 1-4.
11. Gluckman, P.D., et al., *Effect of In Utero and Early-Life Conditions on Adult Health and Disease.* The New England journal of medicine, 2008. **359**(1): p. 61-73.
12. Dengler, V.L., M.D. Galbraith, and J.M. Espinosa, *Transcriptional regulation by hypoxia inducible factors.* Critical reviews in biochemistry and molecular biology, 2014. **49**(1): p. 1-15.
13. Dick, K.J., et al., *DNA methylation and body-mass index: a genome-wide analysis.* Lancet, 2014. **383**(9933): p. 1990-8.
14. Pan, H., et al., *HIF3A association with adiposity: the story begins before birth.* Epigenomics, 2015. **7**(6): p. 937-50.
15. Richmond, R.C., et al., *DNA Methylation and BMI: Investigating Identified Methylation Sites at HIF3A in a Causal Framework.* Diabetes, 2016. **65**(5): p. 1231-44.
16. Mansell, T., et al., *Early-life determinants of hypoxia-inducible factor 3A gene (HIF3A) methylation: a birth cohort study.* Clinical Epigenetics, 2019. **11**(1): p. 96.
17. Davis, E.F., et al., *Cardiovascular risk factors in children and young adults born to preeclamptic pregnancies: a systematic review.* Pediatrics, 2012. **129**(6): p. e1552-e1561.
18. Kajantie, E., et al., *Pre-eclampsia is associated with increased risk of stroke in the adult offspring: the Helsinki birth cohort study.* Stroke, 2009. **40**(4): p. 1176-1180.
19. Leybovitz-Haleluya, N., et al., *Maternal gestational diabetes mellitus and the risk of subsequent pediatric cardiovascular diseases of the offspring: a population-based cohort study with up to 18 years of follow up.* Acta Diabetologica, 2018. **55**(10): p. 1037-1042.
20. Vuillermin, P., et al., *Cohort Profile: The Barwon Infant Study.* Int J Epidemiol, 2015.
21. McCloskey, K., et al., *Reproducibility of aortic intima-media thickness in infants using edge-detection software and manual caliper measurements.* Cardiovascular ultrasound, 2014. **12**(1): p. 18.
22. Collier, F.M., et al., *The ontogeny of naïve and regulatory CD4+ T-cell subsets during the first postnatal year: a cohort study.* Clinical & translational immunology, 2015. **4**(3): p. e34.

23. Cole, T.J., A.F. Williams, and C.M. Wright, *Revised birth centiles for weight, length and head circumference in the UK-WHO growth charts.* Ann Hum Biol, 2011. **38**(1): p. 7-11.
24. Haertle, L., et al., *Epigenetic signatures of gestational diabetes mellitus on cord blood methylation.* Clinical Epigenetics, 2017. **9**(1): p. 28.
25. Nankervis, A., et al., *Testing for gestational diabetes mellitus in Australia.* Diabetes Care, 2013. **36**(5): p. e64.
26. Tranquilli, A., et al., *The classification, diagnosis and management of the hypertensive disorders of pregnancy: a revised statement from the ISSHP.* Pregnancy hypertension, 2014. **4**(2): p. 97.
27. Pink, B., *Socio-economic indexes for areas (SEIFA) 2011.* Canberra: Australian Bureau of Statistics, 2013.
28. Mendelsohn, M.E. and R.H. Karas, *Molecular and cellular basis of cardiovascular gender differences.* Science, 2005. **308**(5728): p. 1583-1587.
29. Pasanen, A., et al., *Hypoxia-inducible factor (HIF)-3alpha is subject to extensive alternative splicing in human tissues and cancer cells and is regulated by HIF-1 but not HIF-2.* Int J Biochem Cell Biol, 2010. **42**(7): p. 1189-200.
30. Gutierrez-Arcelus, M., et al., *Passive and active DNA methylation and the interplay with genetic variation in gene regulation.* elife, 2013. **2**.

Figure Legends

Figure 1. Flowchart summarising the BIS participants included in this analysis (grey-bordered box).

Figure 2. Distribution of methylation of individual CpG units and the average methylation across *HIF3A.1* in cord blood and 12-month blood and *HIF3A.2* in cord blood. Error bars are mean ± standard deviation.

Table Legends

Table 1. Cohort characteristics for the full sample (any BIS infant with both any methylation data and any four-year measure), and the sex-stratified sample.

Table 2. Correlations between cord blood *HIF3A.2* methylation and cardiovascular and weight measures at four years.

Table 3. Correlations between cord blood *HIF3A.2* methylation and cardiovascular and weight measures at four years, stratified by sex.

Table 4. Final linear regression models with four-year blood pressure as outcome, unadjusted and adjusted models in both combined-sexes and sex-stratified analysis.

Supplementary Data

Supplementary Table 1. Details on the CpG units in each analysed region of *HIF3A*.

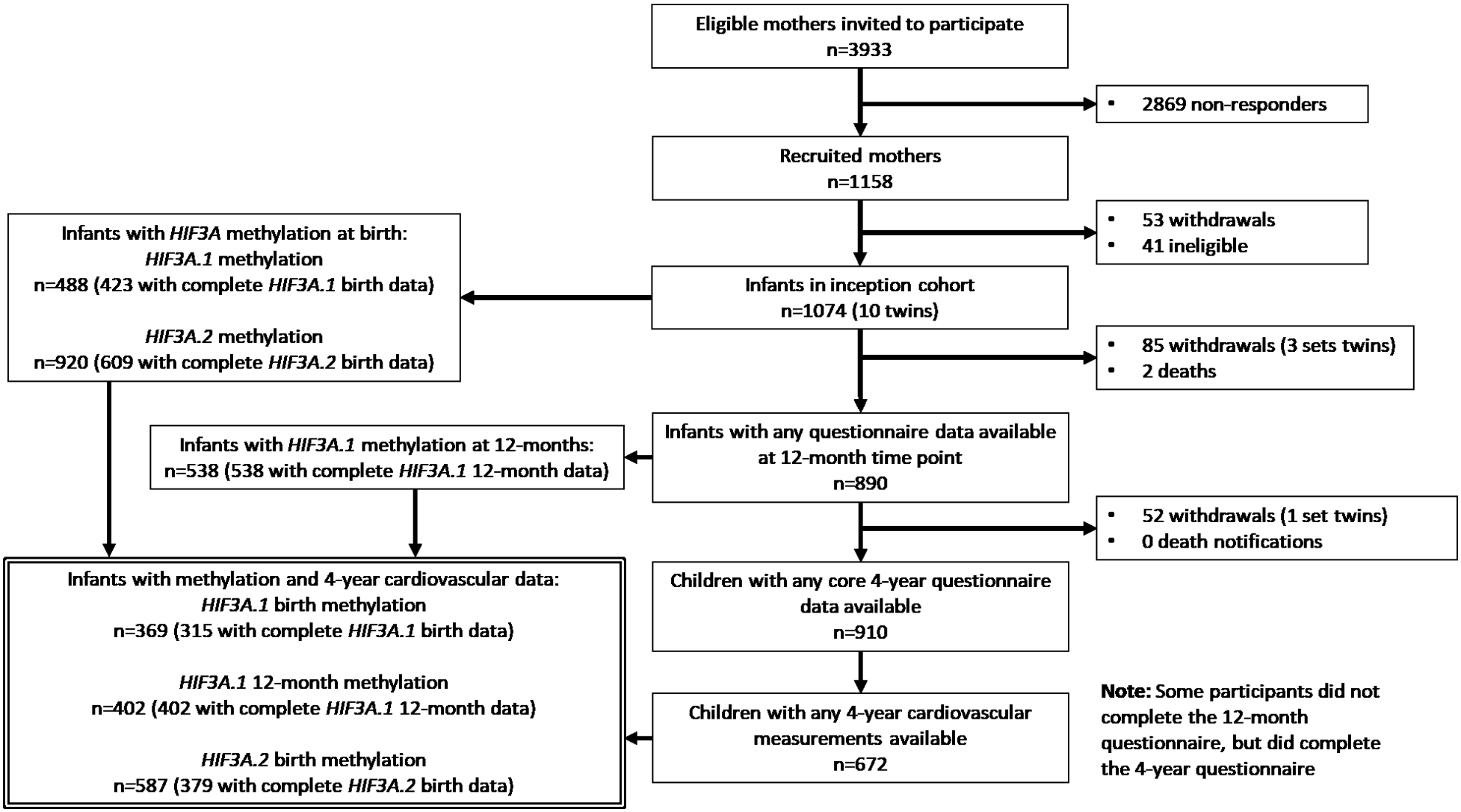
Supplementary Table 2. Pairwise correlation coefficients for methylation of each CpG unit in both regions (HIF3A.1 at birth and 12 months, and HIF3A.2 at birth).

Supplementary Table 3. P-values for the pairwise correlations of methylation of each CpG unit in both regions (*HIF3A.1* at birth and 12 months, and *HIF3A.2* at birth).

Supplementary Table 4. Correlations between *HIF3A.1* methylation in cord blood and 12-month peripheral blood and cardiovascular and weight measures at four years.

Supplementary Table 5. Correlations between cord blood methylation of individual CpG units in *HIF3A.2* and blood pressure at four years.

Figure 1. Flowchart summarising the BIS participants included in this analysis (grey-bordered box).



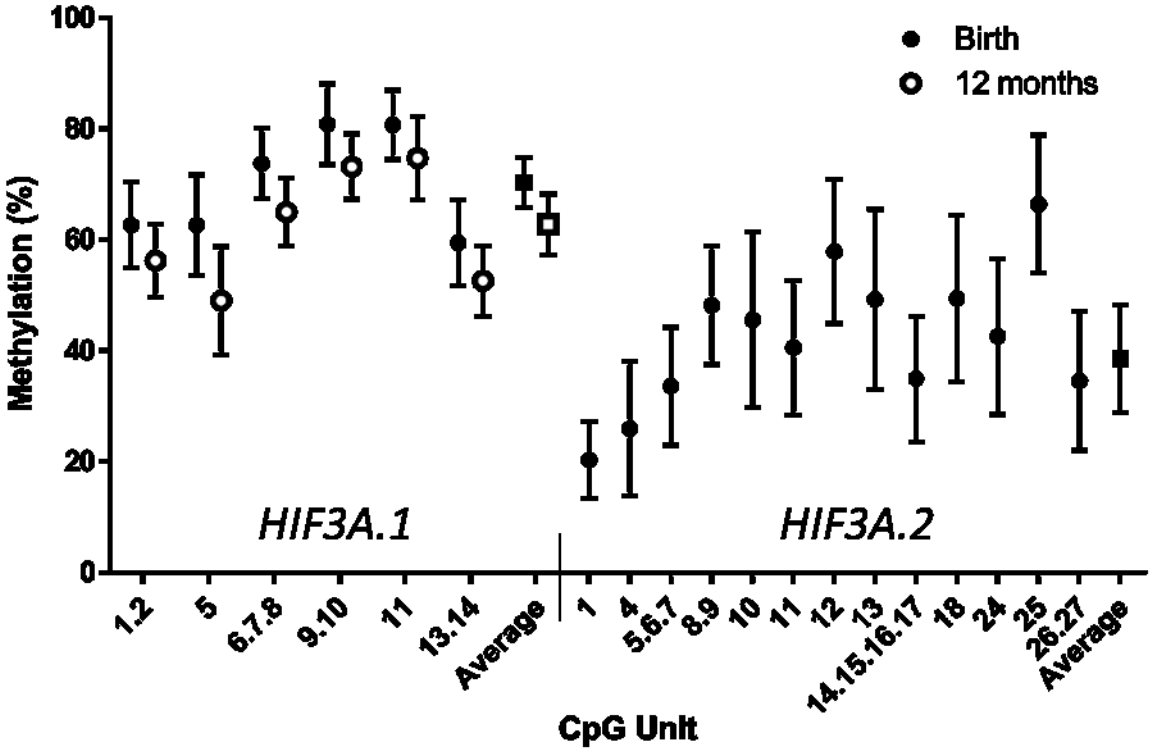


Figure 2. Distribution of methylation of individual CpG units and the average methylation across *HIF3A.1* in cord blood and 12-month blood and *HIF3A.2* in cord blood. Error bars are mean ± standard deviation.

Table 1. Cohort characteristics for the full sample (any BIS infant with both any methylation data and any four-year measure), and the sex-stratified sample.

Measure	Combined sexes		Males (n=506)		Females (n=476)	
Maternal	**N**	**Mean (SD)**	**N**	**Mean (SD)**	**N**	**Mean (SD)**
Age (years)	982	31.40 (4.74)	506	31.47 (4.74)	476	31.34 (4.74)
Pre-pregnancy BMI (kg/m^2)	849	25.38 (5.36)	443	25.20 (5.14)	406	25.57 (5.59)
	N	**n (%)**	**N**	**n (%)**	**N**	**n (%)**
Socio-economic status (SEIFA tertiles)	974		501		473	
Low SEIFA (most disadvantaged)		325 (33.38)		176 (35.12)		149 (31.50)
Medium SEIFA		323 (33.16)		175 (34.93)		148 (31.29)
High SEIFA (least disadvantaged)		326 (33.47)		150 (29.94)		176 (37.21)
GDM (yes)	839	42 (5.01)	438	19 (4.34)	401	23 (5.74)
Pre-eclampsia (yes)	879	28 (2.86)	505	15 (2.97)	473	13 (2.75)
Birth	**N**	**Mean (SD)**	**N**	**Mean (SD)**	**N**	**Mean (SD)**
Gestational age (weeks)	982	39.49 (1.44)	506	39.51 (1.41)	476	39.48 (1.46)
Weight (z-score)	982	0.38 (0.95)	506	0.38 (0.94)	476	0.38 (0.95)
Four-year measure	**N**	**Mean (SD)**	**N**	**Mean (SD)**	**N**	**Mean (SD)**
Actual age (years)	633	4.21 (0.29)	331	4.21 (0.28)	302	4.22 (0.29)
Weight (kg)	626	17.64 (2.54)	327	17.79 (2.30)	299	17.47 (2.78)
BMI (kg/m^2)	624	15.59 (1.50)	326	15.55 (1.29)	298	15.65 (1.71)
Systolic BP (mmHg)	580	106.68 (8.18)	298	106.75 (7.97)	282	106.61 (8.40)
Diastolic BP (mmHg)	580	64.08 (6.36)	298	64.12 (6.48)	282	63.94 (6.23)
Heart rate (BPM)	577	89.73 (9.53)	297	89.10 (9.61)	280	90.42 (9.41)
Pulse wave velocity (m/sec)	546	3.97 (0.44)	281	3.99 (0.45)	265	3.96 (0.44)
aIMT mean (mm)	429	0.54 (0.04)	221	0.54 (0.04)	208	0.54 (0.04)
cIMT mean (mm)	479	0.51 (0.05)	253	0.51 (0.05)	226	0.51 (0.05)

N = number of participants with data for specified measure and any methylation data, SD = standard deviation, n = number of participants in specified category

Table 2. Correlations between cord blood *HIF3A.2* methylation and cardiovascular and weight measures at four years.

Four-year measure	N	Mean (SD)	Correlation with *HIF3A.2* (r)	p
Weight (kg)	380	17.66 (2.47)	0.03	0.63
BMI (kg/m^2)	378	15.52 (1.46)	0.02	0.69
Systolic BP (mmHg)	353	106.66 (7.85)	0.12	0.03
Diastolic BP (mmHg)	353	63.94 (6.41)	0.08	0.13
Heart rate (BPM)	352	89.78 (9.37)	-0.03	0.65
Pulse wave velocity (m/sec)	339	3.97 (0.45)	0.05	0.38
aIMT mean (mm)	260	0.54 (0.04)	0.04	0.55
cIMT mean (mm)	294	0.51 (0.05)	-0.03	0.58

Semi-partial correlations adjusted for actual age at four-year time point and Sequenom batch. Systolic and diastolic blood pressure correlations were additionally adjusted for child sex and height. Mean aIMT correlation was additionally adjusted for aIMT diameter.

Table 3. Correlations between cord blood *HIF3A.2* methylation and cardiovascular and weight measures at four years, stratified by sex.

	Female				Male			
Four-year measure	n	Mean (SD)	r	p	n	Mean (SD)	r	p
Weight (kg)	172	17.46 (2.64)	0.01	0.85	208	17.83 (2.33)	0.10	0.13
BMI (kg/m^2)	171	15.55 (1.60)	0.00	0.96	207	15.50 (1.34)	0.05	0.47
Systolic BP (mmHg)	163	105.97 (7.74)	0.07	0.39	190	107.24 (7.93)	0.16	0.03
Diastolic BP (mmHg)	163	63.54 (6.41)	-0.02	0.86	190	64.28 (6.41)	0.16	0.03
Heart rate (BPM)	162	89.82 (9.23)	0.01	0.90	190	89.75 (9.51)	-0.05	0.51
Pulse wave velocity (m/sec)	156	3.94 (0.43)	0.12	0.14	183	3.98 (0.47)	0.00	0.96
aIMT mean (mm)	117	0.54 (0.04)	-0.02	0.81	143	0.54 (0.04)	0.06	0.44
cIMT mean (mm)	128	0.51 (0.04)	-0.08	0.35	166	0.51 (0.05)	-0.05	0.55

Semi-partial correlations adjusted for actual age at four-year time point and Sequenom batch. Systolic and diastolic blood pressure correlations were additionally adjusted for height. Mean aIMT correlation was additionally adjusted for aIMT diameter.

Table 4. Final linear regression models with four-year blood pressure as outcome, unadjusted and adjusted models in both combined-sexes and sex-stratified analysis.

4-year systolic blood pressure												
	Combined sexes				**Male-only**				**Female only**			
	Unadjusted[1] model (n=346)				**Unadjusted[1] model** (n=187)				**Unadjusted[1] model** (n=159)			
Measure	β (mmHg)	p	95% CI	R^2	β (mmHg)	p	95% CI	R^2	β (mmHg)	p	95% CI	R^2
Average *HIF3A.2* (%)	0.10	0.03	0.01 to 0.19	2.33%	0.14	0.03	0.01 to 0.27	2.90%	0.05	0.39	-0.07 to 0.18	3.22%
	Adjusted model (n=346)				**Adjusted model** (n=187)				**Adjusted model** (n=159)			
Measure	β (mmHg)	p	95% CI	R^2	β (mmHg)	p	95% CI	R^2	β (mmHg)	p	95% CI	R^2
Average *HIF3A.2* (%)	0.11	0.01	0.02 to 0.19	3.93%	0.14	0.03	0.02 to 0.26	5.25%	0.08	0.21	-0.04 to 0.20	4.38%
4-year BMI (kg/m^2)	1.77	<0.001	1.21 to 2.33	9.99%	2.13	<0.001	1.28 to 2.98	11.75%	1.44	<0.001	0.69 to 2.20	8.03%
Birth weight (z-score)	-1.02	0.02	-1.86 to -0.18	4.60%	-0.86	0.17	-2.09 to 0.37	0.91%	-1.05	0.08	-2.23 to 0.13	1.73%
4-year diastolic blood pressure												
	Combined sexes				**Male-only**				**Female only**			
	Unadjusted[1] model (n=346)				**Unadjusted[1] model** (n=187)				**Unadjusted[1] model** (n=159)			
Measure	β (mmHg)	p	95% CI	R^2	β (mmHg)	p	95% CI	R^2	β (mmHg)	p	95% CI	R^2
Average *HIF3A.2* (%)	0.05	0.15	-0.02 to 0.13	1.89%	0.11	0.30	0.01 to 0.22	3.28%	-0.01	0.85	-0.12 to 0.10	2.98%
	Adjusted model (n=346)				**Adjusted model** (n=187)				**Adjusted model** (n=159)			
Measure	β (mmHg)	p	95% CI	R^2	β (mmHg)	p	95% CI	R^2	β (mmHg)	p	95% CI	R^2
Average *HIF3A.2* (%)	0.06	0.11	-0.01 to 0.13	2.18%	0.11	0.03	0.01 to 0.21	4.59%	0.00	0.97	-0.11 to 0.11	2.82%
4-year BMI (kg/m^2)	0.94	<0.001	0.46 to 1.41	4.12%	1.11	0.002	0.41 to 1.81	4.85%	0.89	0.01	0.21 to 1.56	4.24%
Birth weight (z-score)	-0.55	0.14	-1.27 to 0.17	0.62%	-0.65	0.21	-1.66 to 0.37	0.79%	-0.29	0.58	-1.35 to 0.76	0.19%

[1]All models were adjusted for child sex, age and height at four-year time point, and Sequenom batch.

Supplementary Table 1. Details on the CpG units in each analysed region of *HIF3A*.

HIF3A.1					
CpG unit	**N (birth)**	**N (12-months)**	**CpG site**	**Genomic location (chromosome 19, hg38)**	**cg ID**
1.2	430	538	1	46,298,300	cg27146050
			2	46,298,305	-
5	479	538	5	46,298,385	cg22891070
6.7.8	486	538	6	46,298,412	-
			7	46,298,415	cg16672562
			8	46,298,419	-
9.10	488	538	9	46,298,429	-
			10	46,298,436	-
11	484	538	11	46,298,442	-
13.14	484	538	13	46,298,498	-
			14	46,298,510	-
HIF3A.2					
CpG unit	**N (birth)**	**N (12-months)**	**CpG site**	**Genomic location (chromosome 19, hg38)**	**cg ID**
1	920	-	1	46,304,170	-
4	782	-	4	46,304,113	-
5.6.7	875	-	5	46,304,103	-
			6	46,304,101	-
			7	46,304,097	-
8.9	911	-	8	46,304,084	-
			9	46,304,081	-
10	863	-	10	46,304,073	-
11	847	-	11	46,304,064	-
12	814	-	12	46,304,043	-
13	870	-	13	46,304,015	cg26749414
14.15.16.17	899	-	14	46,304,009	-
			15	46,304,006	-
			16	46,304,004	-
			17	46,304,000	-
18	865	-	18	46,303,990	-
24	861	-	24	46,303,929	-
25	878	-	25	46,303,914	-
26.27	893	-	26	46,303,906	-
			27	46,303,900	-

N = number of participants with successfully-measured methylation data for that CpG unit following quality control.

Supplementary Table 4. Correlations between *HIF3A.1* methylation in cord blood and 12-month peripheral blood and cardiovascular and weight measures at four years.

	Birth *HIF3A.1* methylation				12-month *HIF3A.1* methylation			
Four-year measure	N	Mean (SD)	r	p	N	Mean (SD)	r	p
Weight (kg)	317	17.42 (2.55)	0.01	0.84	404	17.47 (2.53)	-0.07	0.14
BMI (kg/m^2)	315	15.48 (1.52)	0.03	0.65	402	15.53 (1.51)	-0.06	0.25
Systolic BP (mmHg)	300	106.48 (7.87)	0.04	0.48	381	106.48 (7.98)	-0.04	0.40
Diastolic BP (mmHg)	300	64.13 (6.11)	0.05	0.36	381	64.15 (6.28)	-0.02	0.74
Heart rate (BPM)	300	89.62 (9.81)	0.00	0.96	379	89.65 (9.92)	-0.02	0.68
Pulse wave velocity (m/sec)	287	3.97 (0.43)	-0.02	0.78	362	3.96 (0.43)	0.03	0.56
aIMT mean (mm)	235	0.54 (0.04)	-0.01	0.80	302	0.54 (0.04)	0.00	0.99
cIMT mean (mm)	262	0.51 (0.04)	-0.06	0.31	335	0.51 (0.45)	0.07	0.22

Correlations are semi-partial correlations adjusted for actual age at four-year time point and Sequenom batch. Systolic and diastolic blood pressure correlations were additionally adjusted for infant sex and height. Mean aIMT correlation was additional adjusted for aIMT diameter. 12-month associations were adjusted for actual age at 12-month time point.

Supplementary Table 5. Correlations between cord blood methylation of individual CpG units in *HIF3A.2* and blood pressure at four years.

HIF3A.2 CpG unit	N	Systolic blood pressure		Diastolic blood pressure	
		r	p	r	p
CpG 1	452	0.11	0.008	0.05	0.25
CpG 4	507	0.10	0.03	0.06	0.18
CpG 5.6.7	540	0.11	0.01	0.05	0.22
CpG 8.9	507	0.08	0.06	0.05	0.27
CpG 10	494	0.11	0.01	0.04	0.32
CpG 11	478	0.12	0.008	0.10	0.03
CpG 12	511	0.07	0.15	0.09	0.04
CpG 13	526	0.12	0.005	0.07	0.13
CpG 14.15.16.17	506	0.11	0.01	0.06	0.19
CpG 18	506	0.13	0.003	0.10	0.03
CpG 24	519	0.11	0.02	0.06	0.17
CpG 25	525	0.08	0.09	0.07	0.10
CpG 26.27	538	0.11	0.01	0.07	0.11

Semi-partial correlations adjusted for actual age at four-year time point, child sex, height, and Sequenom batch.