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DNA methylation of IGF2, GNASAS, INSIGF and LEP and being born small for gestational age

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Abstract

Being born small for gestational age (SGA), a proxy for intrauterine growth restriction (IUGR), and prenatal famine exposure are both associated with a greater risk of metabolic disease. Both associations have been hypothesized to involve epigenetic mechanisms. We investigated whether prenatal growth restriction early in pregnancy was associated with changes in DNA methylation at loci that were previously shown to be sensitive to early gestational famine exposure. We compared 38 individuals born preterm (<32 weeks) and with a birth weight too low for their gestational age (<-1SDS; "SGA") with 75 individuals born preterm but with a birth weight appropriate for their gestational age (>-1SDS) and a normal postnatal growth (>-1SDS at 3 months post term; "AGA"). The SGA individuals were not only lighter at birth, but also had a smaller length ($P=3.3x10^{-13}$) and head circumference at birth ($P=4.1x10^{-13}$). The DNA methylation levels of *IGF2*, *GNASAS*, *INSIGF* and *LEP* were 48.5%, 47.5%, 79.4% and 25.7% respectively. This was not significantly different between SGA and AGA individuals. Risk factors for being born SGA, including preeclampsia and maternal smoking, were also not associated with DNA methylation at these loci. Growth restriction early in development is not associated with DNA methylation at loci shown to be affected by prenatal famine exposure. Our and previous results by others indicate that prenatal growth restriction and famine exposure may be associated with different epigenetic changes or non epigenetic mechanisms that may lead to similar later health outcomes.

Introduction

The developmental origins hypothesis states that adverse environmental conditions during specific time windows of mammalian development can have a lasting effect on metabolic pathways and physiology influencing chronic disease susceptibility[1]. Intrauterine growth restriction (IUGR) is considered to be the result of a poor intrauterine environment and has been associated with diverse adverse health outcomes later in life, including type 2 diabetes and hypertension[2-5]. More than 5% of all pregnancies in the Western world result in infants being born small for gestational age (SGA)[6], an often used proxy for IUGR[7].

In animal models IUGR is modeled by inducing placental insufficiency by artificially reducing placental perfusion or by limiting the maternal nutrients supply with protein or caloric restriction[8]. In humans, SGA is associated with both placental insufficiency and suboptimal prenatal nutrition. For instance, preeclampsia which changes placental perfusion[9] is one of the major risk factor for SGA[10] and the risk to develop preeclampsia is reduced by early gestational micronutrient supplementation[11]. Furthermore, micronutrient supplementation during pregnancy has been found to increase birth weight and the risk for severe SGA is decreased by iron-folic acid intake alone[12,13].

The induction of persistent epigenetic change by prenatal environmental conditions may be a mechanism contributing to the associations between early development and later life health in humans. For example, extensive work in animals has shown that placental insufficiency or restricting the maternal diet of protein, folic acid or other micronutrients can persistently alter DNA methylation and other epigenetic marks and may contribute to the development of diabetes and hypertension[14-19]. In humans, periconceptional exposure (e.g. around conception and the first trimester) to the Dutch Famine, a famine at the end of WWII, is associated with persistent differences in DNA methylation of various important loci involved in growth and metabolism, including *IGF2*, *GNASAS, INSIGF* and *LEP*[20,21]. Further work indicated that *IGF2* methylation is also sensitive to maternal periconceptional folic acid use[22].

These loci are also relevant in relation to prenatal growth restriction. *IGF2* is a major driver of embryonic growth[23] and in concordance with this role genetic variation on the paternal allele of the *IGF2-INS* region was found to influence the risk of being born SGA[24]. *GNAS* and *LEP* have both been found to be differentially expressed between placentas of IUGR and normal children[25]. Both the *GNAS* region and leptin are similarly involved in early growth and glucose metabolism as the *IGF2-INS* region[26,27].

SGA and prenatal famine exposure are associated with similar later life phenotypic consequences[3,28], but it is unclear to what extent this is due to the same mechanism. Here we investigated whether growth restriction during early and/or mid gestation (<32 weeks) is associated with differences in DNA methylation at these 4 loci we found to be sensitive to environmental conditions early in development. We selected individuals from the Dutch nation-wide Project On Preterm and Small-for-gestational age infants (POPS) cohort[29] and measured DNA methylation of the *IGF2* differentially methylated region (DMR) and *INSIGF*, *GNASAS* and *LEP* promoters. We compared levels of DNA methylation between preterm born SGA individuals with individuals born preterm but with a birth weight appropriate for their gestational age and with a normal postnatal growth. In addition we explored possible associations with major risk factors for SGA, including preeclampsia and prenatal smoking.

Results

Child, pregnancy and maternal characteristics

In this study, 38 individuals born pre-term and SGA and 75 individuals born preterm but with a birth weight appropriate for their gestational age (AGA), were compared. The SGA individuals were not only small in terms of birth weight, the selection criterion, but also smaller in terms of birth length and head circumference at birth (**Table 1**), compatible with IUGR[30]. The SGA

Table 1. Characteristics at birth and pregnancy.

Characteristics		SGA	AGA	P_{value} [1]
Number of individuals	N	38	75	
Male	%	39.5	44.0	0.65
Adult height	SDS[2](SD)	-1.02(0.99)	-0.17(0.97)	$3.3x10^{-5}$
At Birth				
Gestational age at birth	weeks(SD)	30.6 (1.1)	30.1(1.5)	0.053
Birth weight	SDS[3](SD)	-1.86(0.50)	0.31(0.73)	$1.1x10^{-31}$
Birth head circumference	SDS(SD)	-1.34(0.79)	0.20(0.92)	$4.1x10^{-13}$
Birth length	SDS(SD)	-1.83(0.87)	0.19(1.12)	$3.3x10^{-13}$
Obstetric data				
First child (parity)	%	68%	52%	0.097
Maternal age	years(SD)	27.8(4.8)	27.9(5.9)	0.92
Problematic obstetric history[4]	%	18.4	17.3	0.89
Socio-economic status[5]	SES	3.53(1.50)	3.59(1.55)	0.82
Maternal height	cm(SD)	165.6(5.4)	167.3(6.1)	0.14
Maternal diabetes mellitus	%	5.3	4.0	0.57
Chorioamnionitis[6]	%	5.3	25.3	0.009
Smoking during pregnancy	%	28.9	35.7	0.029
Pre-existing hypertension	%	10.7	2.7	0.08
Preeclampsia	%	65.8	10.7	$3.4x10^{-11}$

1. P value resulting from an unpaired T-Test between the SGA and AGA groups.
2. Standard deviation score from the reference population mean.
3. The birth weight in grams (SD) for the SGA and AGA groups were 963(149) and 1508(301) respectively.
4. Percentage of mothers with previous pregnancies and/or births with complications
5. Socio-economic status of the family given on a 1 to 6 scale. With 1 being the poorest score and 6 being the highest score
6. Intrauterine infection followed by a prolonged rupture of the membrane and preterm labor

individuals remained relatively short (-1.02 SDS), while the AGA group was similar in adult height to the Dutch reference values (-0.17 SDS). The greatest differences in the obstetric data for an SGA child as compared to an AGA child were a higher prevalence of preeclampsia (65.8% vs. 10.7%) and a lower occurrence of chorioamnionitis, an intrauterine infection followed by a prolonged rupture of the membrane and preterm labor (5.3% vs. 25.3%). In addition, smoking during pregnancy was less common in the pregnancies leading to a SGA child (28.9% vs. 35.7%).

Comparison between SGA and AGA

We measured gene specific methylation for *IGF2*, *GNASAS*, *INSIGF* and *LEP* in whole blood. The average DNA methylation levels were 48.5%, 47.5%, 79.4% and 25.7% for *IGF2*, *GNASAS*, *INSIGF* and *LEP*, respectively (**Table 2**). DNA methylation levels in the SGA group were not significantly different from the AGA group. The results were similar for the individual CpG dinucleotides (*data not shown*). The variance in DNA methylation was also not significantly different between the two groups (Levene's test P>0.14). We repeated the analyses using other frequently used cut-offs for birth weight SDS scores to define growth restriction. Using a cut-off of < -1.3 SDS (the 10th percentile, N=34 vs. N=75) or < -2SDS (frequently used by pediatric

Table 2. Methylation difference between SGA and AGA.

	AGA (SD)	SGA - AGA[1]	P_{value}[2]
IGF2	48.5% (3.5)	-0.2%	0.81
GNASAS	47.5% (4.6)	-0.7%	0.41
INSIGF	79.4% (3.2)	-0.2%	0.78
LEP	25.7% (5.3)	-1.3%	0.24

1. The difference in DNA methylation between the small for gestational age and appropriate for gestational age groups. A negative difference means that the SGA group has a lower methylation level.
2. A two-sided P-value resulting from a linear mixed model corrected for the correlation between individual CpG dinucleotides, bisulfite batch and sex between the SGA and AGA groups.

endocrinologists, N=13 vs. N=75) and stratifying all performed analyses by sex did not change the outcome (*data not shown*).

Preeclampsia and other risk factors

The risk to develop preeclampsia is influenced by nutrition in the same period of gestation[11] as our previous studies[20-22]. To reduce the influence of heterogeneity, we first restricted our analysis to the individuals born SGA after a pregnancy with preeclampsia with those born AGA and without (25 vs. 67). No significant differences were found for these loci (data not shown). Next we tested for an association in all measured individuals between DNA methylation and the factors with the greatest difference between the SGA and AGA groups. Preeclampsia and maternal smoking during pregnancy were not associated with DNA methylation at these loci (**Table 3**). A nominally significant association was observed for *LEP* and chorioamnionitis (P=0.033), which would no longer be significant after accounting for the number of tests performed. Factors reported to be associated with increased risk of developing a SGA child, but not found in the current study, namely gestational age, a first pregnancy and maternal hypertension before pregnancy, were not associated with DNA methylation.

Table 3. The relation between DNA methylation and risk factors.

	IGF2		*GNASAS*		*INSIGF*		*LEP*	
	β[1]	P[2]	*β*	P	*β*	P	*β*	P
Preeclampsia	0.8%	0.34	0.9%	0.29	0.0%	0.99	-0.2%	0.88
Chorioamnionitis	0.8%	0.36	0.8%	0.42	0.5%	0.52	2.8%	0.033
Smoking[3]	-1.5%	0.054	-0.9%	0.29	-0.4%	0.53	-1.9%	0.98

1. The beta from a linear mixed model corrected for the correlation between individual CpG dinucleotides, bisulfite batch and sex. The investigated variable was entered as a fixed effect.
2. A two-sided P-value resulting from a linear mixed model corrected for the correlation between individual CpG dinucleotides, bisulfite batch and sex.
3. Smoking during pregnancy by the mother.

Discussion

We tested for the association of being born SGA before 32 weeks of gestation with DNA methylation of *IGF2*, *GNASAS*, *INSIGF* and *LEP* genes for which we previously showed an association with prenatal famine exposure and, for *IGF2*, folic acid supplementation[20-22]. We did not observe differences in DNA methylation at these genes between individuals who were born preterm and growth restricted and individuals born preterm but with a weight appropriate for their gestational age and a normal post natal growth. Preeclampsia was also not associated with DNA methylation levels.

The loci tested for DNA methylation differences may be regarded as markers for prenatal nutritional conditions. Our results are compatible with the interpretation that SGA and preeclampsia do not have a nutritional component in our western and thus well nourished cohort. Other studies on individuals born SGA at term also did not find an association with DNA methylation around the *IGF2* locus[31,32]. Our data does not exclude the possibility that a similar study in developing countries would yield different results for the loci studied, as malnutrition can be expected to play a more prominent role in those countries[33].

In western cohorts SGA may more readily be associated with placental insufficiency and an insufficient transfer of oxygen to the child, which is known to contribute to growth restriction and prenatal programming[34] and shown to influence DNA methylation patterns in animal models[35]. Indeed, epigenetic differences may be still be present in humans born SGA, but at other loci than those influenced by prenatal famine, as is suggested by work by Einstein *et al*.[36]. Beside maternal and environmental factors, however, genetic predisposition may play a role. Twin studies show that some of the associations between birth weight and later health are confounded by genetic factors[2,37]. Indeed, genetic variation influencing birth weight also contributes to the risk of diabetes[38] and genetic variation in the glucocorticoid receptor was found to influence both growth and later glucose homeostasis in children born preterm and SGA[39].

The current study focuses on the influence of conditions during early and mid-gestation to account for the observation that DNA methylation at these loci may be less sensitive during late gestation[20-22]. One may consider the possibility that all very pre-term born children irrespective of prenatal growth experienced an adverse development. In that case, DNA methylation changes may have occurred in both groups studied. However, chorioamnionitis, which is a generally more acute complication of pregnancy, was more prevalent among children born AGA and is not associated with DNA methylation at these loci. Furthermore, the height of AGA individuals at 19 years was not different from the Dutch reference values indicating that prenatal birth per se did not compromise postnatal growth. This supports the interpretation that there are persistent phenotypic differences between SGA and AGA individuals born very preterm, which were not explained by differences in DNA methylation at the measured loci. Also, the association between birth weight and cardiovascular disease was found to be independent of gestational length, suggesting a link with prenatal growth and not preterm birth for fetal programming[3]. A comparison of very preterm children with children at term may not be sufficient to solve this issue because of the possible influence of the intensive neonatal treatments on epigenetic marks.

These results, together with the findings by others[31,32,36], suggest that SGA is not associated with similar epigenetic changes as prenatal famine exposure in Western populations. If so, the etiology of the similar later life consequences associated with these early life conditions, diabetes and cardiovascular disease, may be different. More detailed studies of the epigenetic changes associated with human and animal growth restriction are warranted to gain insight into the link between development and disease. Animal models will be important to elucidate the basic principles, but care may have to be taken when extrapolating epigenetic studies to humans, since it may be possible that animal models implementing nutritional restrictions early in gestation may better simulate human famine exposure than IUGR. Studies in humans will require extensive and detailed phenotyping of prenatal growth, maternal and environmental factors and genetic variation. Most likely such studies will require a relatively large initial study size in which homogeneous subselections

can be made to overcome the complexity and variation inherent to clinical cohorts of prenatal growth restricted humans.

Subjects and Methods

Study population

The Dutch Project On Preterm and Small-for-gestational age infants (POPS) is a nation-wide prospective study, encompassing 94% of all live born infants born very preterm (<32 weeks) and/or with a very low birth weight (<1500 gram) in 1983. The recruitment, details of measurements, and physical and psychosocial outcomes have been reported previously in detail[29,40]. The anthropometric data at birth has been transformed into standard deviation scores (SDS) based on the Swedish references for very preterm infants[41]. The Swedish references were chosen because the Dutch references lack data on birth length and head circumference, while being highly similar[42]. All other anthropometric data has been transformed using the Dutch reference values[43]. The study was approved by the medical ethics committees of all participating centers and written informed consent was obtained from all participants.

Selection for current study

From the POPS cohort we had 413 individuals available who were born before <32 weeks of gestation. We excluded non white participants (excluding 53), twins (excluding 86), individuals treated with glucocorticoids (dexa- or beclomethasone) during the prenatal and/or neonatal period (excluding 71) and individuals with chromosomal abnormalities or inborn errors in metabolism (excluding 2). We defined small for gestational age (SGA) as individuals born with a birth weight of <-1 SDS. As a control group we selected individuals with a birth weight >-1 SDS and a weight at 3 months of >-1 SDS (AGA). From the 201 remaining individuals 42 met our SGA and 92 met our

AGA definition. Four SGA and seventeen AGA individuals had not enough genomic DNA available and were excluded. This resulted in a selection of 38 small for gestational age individuals and 75 individuals with a birth weight appropriate for their gestational age and a normal postnatal growth, which extended up to age 19y (height -0.17SDS).

DNA methylation measurements

Genomic DNA was isolated from whole blood drawn at age 19 using the Qiagen mini kit. Half a microgram of genomic DNA was bisulfite treated using the EZ 96-DNA methylation kit (Zymo Research) using the standard over night bisulfite treatment protocol. The 113 individuals were bisulfite treated on two 96-well plates. SGA and AGA individuals were equally distributed on the plates. The distribution of men and women was also similar on the 2 plates. DNA methylation for individual CpG dinucleotides of *IGF2*, *GNASAS*, *INSIGF* and *LEP* was determined by a mass spectrometry based method (Epityper, Sequenom), for which the reproducibility and accuracy has been shown extensively[20,44,45]. Details of the measured amplicons, including details of functional relevance were published before[46]. In short, *IGF2* DMR hypomethylation was associated with biallelic *IGF2* expression[47] and *INSIGF* locus measured is the DMR located in the promoter of the imprinted *INSIGF* transcript which originates from the *INS* promoter[48]. The *GNASAS* amplicon is part of the *GNAS* DMR2 and is located at the proximal promoter of this imprinted RNA antisense transcript of *GNAS*[49] overlapping the binding site of several transcription factors according to ENCODE[50] CHIP-seq data. The *LEP* amplicon also covers the proximal promoter and includes several CpG sites of which the methylation status influences transcription[51]. DNA methylation was measured in samples from 19 year old individuals which were assumed to provide information on potential epigenetic differences induced during prenatal development. The stability of the methylation marks at the four loci investigated during the life course was suggested by their association with prenatal famine 60 decades post exposure[20,21]. In addition, comparing blood samples taken 10-20 years apart indicated

the stability of the methylation of *IGF2* DMR, *LEP* and, to a lesser extent, *INSIGF* (*GNASAS* was not studied)[46]. Data for the four loci was acquired and processed as previously described[20,21,46]. The PCR and the subsequent steps were performed in triplicate and performed according to the manufacturers' protocol. Each locus was measured on the same 384 well plate for all 113 individuals studied. Data quality control and filtering consisted of the removal of triplicate measurements for which less than two measurements were successful or for measurements with a standard deviation larger than 0.1. CpG dinucleotides of which the measurement could be confounded by single nucleotide polymorphisms and CpG dinucleotides of which the success rate after filtering was below 75% were removed. Details about the primers and the CpG sites included and biological relevance is provided in **Supplement I, Table S4 and Table S5**.

Statistics

Unpaired T-tests were used for the analyses of the anthropometric and pregnancy characteristics. We applied linear mixed models on the raw data without imputation of missing values to calculate differences in DNA methylation for each locus between the SGA and AGA groups. All group analyses account for bisulfite plate, sex and the correlation between CpG dinucleotides. Person identifier was added as random effect and bisulfite batch, sex and group identifier (e.g. being SGA or AGA) were entered as fixed effects. The linear mixed model is preferred above more standard tests because it allows the incorporation of multiple individual CpG dinucleotides in one test, accounts for the correlation between adjacent CpG dinucleotides, incorporates the relevant adjustments within the model on the raw data, and uses available but incomplete data for individuals. All analyses were also performed using <-1.3 SDS (the 10th percentile) and <-2 SDS birth weight as cut-offs to define SGA status. The analyses were also performed for individual CpG sites. The test for associations between birth characteristics or risk factors with DNA methylation was performed by adding the respective variable to the mixed model as a fixed effect. To test for differences in the

variance in DNA methylation between the groups we used the Levene's test statistic for homogeneity of variance from the one-way ANOVA test in PASW 17.0. All analyses were performed using PASW Statistics 17.0, previously known as SPSS. All P-values reported are two sided.

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