Birthweight *DNA* Methylation Signatures in Infant Saliva

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Abstract

Background Low birthweight has been repeatedly associated with long-term adverse health outcomes and many non-communicable diseases. Our aim was to investigate whether cord blood birthweight-associated CpG sites identified by the PACE Consortium replicate in infant saliva, and to explore saliva-specific DNA methylation signatures of birthweight.

Methods DNA methylation was assessed using Infinium HumanMethylation450K array in 141 saliva samples collected from children of the NINFEA birth cohort at an average age of 10.8 (range 7-17) months. The association analyses between birthweight and DNA methylation variations were carried out using robust linear regression models both in replication and exploratory EWAS analyses.

Results None of the cord blood birthweight-associated CpGs identified by the PACE Consortium was replicated in infant saliva. In saliva EWAS analyses, birthweight as continuous variable was associated with DNA methylation variation in 44 CpG sites, while being born small for gestational age (SGA, lower 10^{th} percentile of birthweight for gestational age according to WHO reference charts) was associated with DNA methylation variation in 44 CpGs (all false discovery rate p-values<0.05), with only one overlapping CpG between the two analyses. Despite no overlap with PACE results at the CpG level, two of the top saliva birthweight CpGs mapped at genes identified also by the PACE consortium (*MACROD1* and *RPTOR)*.

Conclusion Our study provides an indication of the birthweight and SGA epigenetic salivary signatures in children around 10 months of age. DNA methylation signatures in cord blood may not be comparable with saliva DNA methylation signatures at about 10 months of age, suggesting that the birthweight epigenetic marks are likely time and tissue specific.

Background

The existence of a relationship between intrauterine or early life exposures and health during the lifecourse has come to attention in the 90's [1], and is nowadays recognized as the developmental-origins of health and diseases (DOHAD) hypothesis[2]. The intrauterine life is a critical period for adverse exposures to exert their effect[3], as fetal organs start developing and are sensitive to environmental stimuli that may cause an indelible imprint on future development and function.

In a hostile uterine environment caused by insults such as for example poor nutrition, the fetus responds by developing adaptations such as down-regulation of metabolic function, or organs function to slow down its growth rate to match the nutrient supply. The adaptive process, however, may cause irreversible changes in the development of some tissues and organs and predispose the individual to a higher risk of diseases not only early in life but also during the lifecourse. [2]

Low birthweight may be associated with accumulation of adipose tissue and rapid weight gain during childhood[3], and the risk of respiratory [4][5], metabolic[6][7] and cardiovascular diseases[8][9],

hypertension[10], and neurobehavioral disorders[11]. Low birthweight has also been associated with an increased overall mortality[12] while cancer incidence rises with increasing birthweight for most type of cancer[13]–[17].

The PACE consortium analyzed the epigenome-wide DNA methylation in neonatal blood of 8825 newborns from 24 birth cohorts[18] and found that 914 CpGs, located in or near 729 genes, were associated with birthweight treated as a continuous variable. In the same study, methylation variation in 51 CpG sites was associated with high birthweight, as compared to normal weight, and 4 CpGs appeared to be associated with low versus normal birthweight. In additional analyses conducted on blood from 7,278 children at later ages, < 1.3% of 914 birthweight-associated differentially methylated CpGs at birth remained associated in childhood (2–13 years; n = 2756 from ten studies) and adolescence (16–18 years; n = 2906 from six studies), and none in adulthood (30–45 years; n = 1616 from three studies). Only a minority of CpGs survived the Bonferroni correction for 914 tests ($p < 5.5 \times 10-5$): 12 (1.3%), 1 (0.1%) and 0 CpGs in childhood, adolescence and adulthood, respectively.

Although it seems that cord blood DNA methylation markers of birthweight do not persist at later ages, the associations observed at birth are extensive and it is important to confirm their persistence or variations over time. Also DNA methylation profiles are tissue-specific, and it would be optimal to analyse the DNA profile linked to birthweight in different tissues. Most tissues are not accessible with non-invasive methods, and blood is typically used as a surrogate, with the assumption that, being a universal body fluid, it may capture epigenetic changes of target tissues[19]. Saliva and nasal brushes are other easily accessible tissues, especially in infants and children, but to date they have been much less studied. [20], [21]

Our aim was to investigate if the associations observed between birthweight and DNA methylation variation in the cord blood replicate in saliva samples from the NINFEA birth cohort, and to conduct an EWAS analysis to identify methylation markers of birthweight that are specific for saliva obtained in infancy.

Methods

2.1 Study population

The data were derived from an epigenome-wide case-control study on wheezing nested within the NINFEA birth cohort[22]. The NINFEA study is an Italian web-based multi-purpose mother-child cohort, aimed at exploring the relationship between early-life exposures and long-term health outcomes[23]. Members of the cohort are children born from approximately 7500 pregnant women who between 2005 and 2016 volunteered to participate in the study, had Internet access and had enough knowledge of Italian to complete web-based questionnaires. The children are followed up with six questionnaires completed by their mothers 6 and 18 months after delivery, and when they turn 4, 7, 10 and 13 years of age. When the child was aged approximately 6 months, mothers were asked to donate their and their children's saliva

samples using a mailed sponge Oragene™ DNA self-collection kits (OG-250; DNA Genotek, Inc, Ottawa, Ontario, Canada).

The case-control study was designed as an EWAS of early childhood wheezing, consisting of 72 cases with at least one episode of wheezing between 6 and18 months of age, and 72 infants without wheezing matched to cases by sex, age and seasonality/calendar year of saliva sampling. Cases and controls were singletons, residents in the City of Turin, Italy, and born to mothers who did not report having asthma active during pregnancy. The baseline NINFEA questionnaire, completed at any time during pregnancy, was used to derive information on maternal and pregnancy factors, while child-related variables, were collected at the first follow-up questionnaire completed approximately 6 months after delivery. Although information on children ethnic background was not available in the NINFEA cohort, almost the entire study population has both parents born in Italy, and only few study children have one of the parents born in other European countries. Therefore, the ethnic background of the children included in the study is, if not entirely, largely European. In this study, we used the following variables: maternal age at delivery (years), maternal education (low = no education, primary or secondary school vs. high = university degree or higher), parity (nulliparous vs. at least one previous pregnancy > 22 gestational weeks), maternal pre-pregnancy body mass index (BMI; kg/m^2), sex, gestational age at birth (weeks), birthweight (grams), small for gestational age (yes vs. no), age at saliva sampling (< 10.4 months vs. ≥10.4 months) (see Table 1 for detail). Saliva samples are stored in a biobank at -80C.

Table 1
Descriptive table for the NINFEA population under study (N = 141)

Characteristics	N or Mean	% o SD
Maternal pre-pregnancy BMI[a]	22	3.0
Missing values	1	-
Maternal age at delivery (years)	35	4.1
Maternal education		
Low/medium (no university degree)	38	27%
High (university degree or higher)	102	72%
Missing values	1	1%
Parity		
First born	45	32%
Non first born	92	65%
Missing values	4	3%
Gestational duration (weeks)		
< 37 weeks	7	5%
37 + weeks	134	95%
Child sex		
Female	61	43%
Male	80	57%
Birthweight (grams)	3242	447.1
Low (< 2500 grams)	5	3.5%
Medium (2500–4000 grams)	128	90.8%
High (> 4000 grams)	8	5.7%
Small-for-gestational-age		
Yes	22	16%
No	119	84%
Age at saliva sampling (months)	10.78	2.2

[a] *BMI: body mass index (Kg/m^2)*

Characteristics	N or Mean	% o SD
< 10.4	69	49%
≥ 10.4	72	51%
[a] *BMI: body mass index (Kg/m^2)*		

The IlluminaInfinium ® HumanMethylation450 BeadChip (Illumina,Inc, San Diego, CA, USA) was employed to evaluate DNA methylation status of over 485 000 probes in saliva samples. Details on pre-processing of samples and data quality control can be found in the Additional File 1(Methods, DNA methylation measurement, data pre-processing and quality control). Quality controls and probes filtering led to the exclusion of three samples and 63218 probes, leading to a total of 141 saliva samples and 421,782 probes for analyses.

NINFEA study was approved by Ethical Committee of the San Giovanni Battista and CTO/CRF/Maria Adelaide Hospital of Turin and all participating mothers gave informed consent at enrolment and at saliva donation.

2.2 Statistical analyses

For all analyses we pooled together cases and controls, leading to 141 subjects in the analyses. Methylation levels were analysed as β-values (ratio of methylated probe intensity to overall intensity, representing 0 to 100% methylation at each probe). All the analyses were performed using R statistical computing software (version 3.6.0) and RStudio (version 1.2.1335)[24].

2.2.1Replication analysis

Out of the 914 birthweight-associated CpGs identified by the PACE consortium, 891 (97.5%) were available in the NINFEA study after quality checks and probes filtering. We used the same confounding variables of the PACE analysis, but, differently than in the PACE study, we used birthweight as the exposure and DNA methylation variation as the outcome to meet the biological temporality from birth to infancy. Both birthweight and DNA methylation were modeled as continuous variables. We used robust linear regression adjusted for maternal age at delivery, maternal education, parity, maternal pre-pregnancy BMI, child sex, gestational age at birth, age at saliva sampling, batch and case-control status of the original nested case-control study (wheezing between 6 and 18 months of age). Using *vcovHC* function in the sandwich R package [25], we calculated heteroscedasticity-consistent standard errors.

Although maternal smoking during pregnancy affects both birthweight and offspring DNA methylation, we did not adjust for smoking, as it was rather infrequent in our study sample (2% prevalence).In order to account for residual technical variability and for cell-type heterogeneity, we performed surrogate variables analysis (*sva*) [26], and estimated 7 surrogate variables that were also included as covariates in the model. Cell composition was additionally estimated using the reference-based projections for saliva proposed by Zheng[27], and given a high correlation between the epithelial tissue component estimate

and the first sva component (rho = 0.99) we used only the 7 estimated surrogate variables in the analyses. P-values adjusted for multiple comparisons were calculated using the Bonferroni correction and the Benjamini and Hochberg false discovery rate (FDR), while histograms and quantile–quantile (QQ) plots were used to graphically compare the observed distribution of p-values versus the expected uniform distribution under the null hypothesis. Given that the direction of the association was determined by the PACE study, we also calculated one sided p-values for each CpG used in the replication analysis. To account for the correlation between the methylation levels of the 891 selected CpGs, we also calculated permutation-based p-values.

2.2.2 Epigenome-wide association analyses

Two exploratory epigenome-wide association analyses were conducted, first with birthweight as a continuous exposure variable and, second, with small for gestational age as a binary exposure variable. The latter was defined as the lowest 10th percentile of the World Health Organization birthweight for sex and gestational age charts[28].

In both EWAS analyses we used robust linear regression models adjusted for sex, age at saliva sampling, gestational age, maternal age, parity, maternal pre-pregnancy BMI, maternal education, batch, estimated surrogate variables and case-control status in the original nested case-control study. P-values adjusted for multiple comparisons were calculated using the Benjamini and Hochberg false discovery rate (FDR), and Volcano plots were used to visually present the results. In the EWAS of SGA, as a sensitivity analysis we provisionally excluded children born pre-term (< 37 gestational weeks at birth).

To assess whether the age at saliva sampling could have influenced the findings on the top CpG sites identified in the EWAS analyses, we tested associations of the age at saliva sampling with the methylation levels in the top CpG sites using the robust linear regression models adjusted for sex, batch and cell type composition estimated with the reference-based projections for saliva proposed by Zheng[27].

2.3 CpGs annotation and functional analysis

Gene Ontology (GO) and Kyoto Encyclopedia of gene and Genomes (KEGG) enrichment analyses were carried out to identify possible functional pathways in the saliva birthweight associated CpGs set.

In order to compare previously reported associations of epigenome-wide birthweight-associated CpGs and our own results, we searched for findings reported in the EWAS Catalog (http://www.ewascatalog.org; accessed on 8 April 2020) and EWAS Atlas (https://bigd.big.ac.cn/ewas/tools; accessed on 8 April 2020), looking for a CpG level match.

After this first step based on the overlap between our results and those of other EWAS on the same trait, we further accessed EWAS Atlas to examine if CpGs identified in our study were previously associated with traits other than birthweight.

Lastly, if CpGs identified in our study were not associated with any trait EWAS Atlas, we looked for traits associated with specific genes on which these CpGs mapped using the GWAS Catalog.

Results

Table 1 shows the characteristics of the study population. The mean maternal age was 35 years; 72% of the mothers had a high educational level. The mean birthweight was 3242 grams, 5% of the children were born pre-term and 15.6% of children were born small for gestational age. The mean age at saliva sampling was 10.8 months (median 10.4, range 7–17).

3.1 Replication analysis

Of the 891 CpG sites associated with birthweight in the PACE study, 81 (9.1%) had a nominal p-value < 0.05 in our study (Fig. 1, Table 2).

Table 2
Replication analysis results

Results	N or %
Number of CpGs tested	891
Number of CpGs with p-value < 0.05	81
Percentage of concordance in the direction of the effect between the PACE and replication NINFEA study	47.25%
Family-wise error rate (Bonferroni-corrected p-values) < 5.61 × 10 − 5	0
Number of CpGs with permutation-corrected p-value < 0.05	0

However, none of these 81 CpGs survived the Bonferroni correction ($p < 5.61 \times 10 - 5$) or had a FDR < 0.05 (Table 2, S1 Table). There was a 47% concordance in the direction of the coefficients between the PACE and our study (binomial sign test, p value = 0.11, 95% confidence intervals (CI): 0.44; 0.51). In the NINFEA saliva samples, there was a strong mean pairwise correlation between the DNA methylation values of the 891 CpG sites (mean absolute Pearson correlation coefficient between the CpGs: 0.48, results not shown in Tables). When we carried out permutation tests to take into account this correlation structure, the minimum p-value was 0.56.

3.2 EWAS of continuous birthweight

Out of the 421,782 probes analysed, 8.9% (N = 37365) were associated with birthweight with a nominal p-value < 0.05. After correction for multiple testing, 44CpG sites had a FDR < 0.05 (Table 3 and Fig. 2A). Their coefficient estimates ranged from − 0.31 to 0.57, which corresponds to a methylation increase of 0.57% with a 100 gr increase in birthweight. None of the 44 saliva birthweight-associated CpGs overlapped with the birthweight related CpGs in the cord blood. The average absolute pairwise Pearson's

correlation coefficient between the β-values of the identified 44CpGs was 0.23, which is similar to the mean pairwise genome-wide correlation coefficient in the NINFEA saliva samples [29].

Table 3

Top 44 CpGs from EWAS study with Benjamini and Hochberg false discovery rate (FDR)-adjusted p-values < 0.05. The effect is estimate as the difference in % of methylation per 100 grams in birthweight difference. In bold the CpG with the strongest effect.

CpG	Effect estimate	Nominal p-value	BH corrected p-value
cg03045325	-0.266	3.53E-07	0.046
cg19854704	0.165	3.56E-07	0.046
cg07728793	-0.155	4.91E-07	0.046
cg18072629	-0.112	7.03E-07	0.046
cg05005073	0.233	8.01E-07	0.046
cg09516627	0.155	8.07E-07	0.046
cg02727104	0.569	8.7E-07	0.046
cg26725813	-0.136	9.99E-07	0.046
cg05931551	-0.290	1.08E-06	0.046
cg26392737	0.268	1.21E-06	0.046
cg04512603	-0.072	1.24E-06	0.046
cg23218354	**0.565**	**1.4E-06**	**0.046**
cg09855212	0.169	1.43E-06	0.046
cg04305601	-0.215	1.72E-06	0.046
cg13590166	-0.211	1.86E-06	0.046
cg01807862	-0.159	1.91E-06	0.046
cg14781041	0.105	2.25E-06	0.046
cg15915658	0.307	2.3E-06	0.046
cg03466415	0.142	2.32E-06	0.046
cg22453818	0.254	2.43E-06	0.046
cg07175848	-0.204	2.7E-06	0.046
cg18971416	0.129	2.72E-06	0.046
cg18152712	0.115	2.76E-06	0.046
cg08060902	-0.151	2.8E-06	0.046

CpG	Effect estimate	Nominal p-value	BH corrected p-value
cg04963607	-0.135	2.87E-06	0.046
cg00483825	-0.157	2.99E-06	0.046
cg18417562	-0.198	3.07E-06	0.046
cg09361653	-0.192	3.18E-06	0.046
cg22896429	0.165	3.33E-06	0.046
cg19794939	0.101	3.48E-06	0.046
cg03113121	0.178	3.49E-06	0.046
cg23114964	-0.229	3.67E-06	0.046
cg06527318	-0.313	3.77E-06	0.046
cg20100049	-0.204	3.84E-06	0.046
cg20515787	0.108	3.91E-06	0.046
cg13680864	0.235	3.96E-06	0.046
cg23096644	0.167	4.37E-06	0.047
cg03722643	-0.044	4.43E-06	0.047
cg19857227	-0.215	4.49E-06	0.047
cg08707988	-0.258	4.51E-06	0.047
cg09749788	0.229	4.7E-06	0.047
cg06655187	0.125	4.79E-06	0.047
cg10800369	0.327	4.91E-06	0.047
cg24864887	0.347	4.92E-06	0.047

3.3 EWAS of small for gestational age

We found 44 CpGs associated with SGA at a FDR of less than 0.05 (Table 4 and Fig. 2B). The largest coefficient showed a 4.1% difference in methylation when comparing small with non-small for gestational age (cg12322146), the largest negative association was − 2.3% (cg03066788). Only one CpG (cg18072629) overlapped with the 44CpGs found to be associated with continuous birthweight in our sample, and none overlapped with the 914 CpGs associated with continuous birthweight or with the 4 CpGs associated with low birthweight (< 2500 gr) in the PACE study conducted on cord blood samples.

Table 4

44 CpGs associated with AGA vs. SGA when treats birthweight as categorical variable. The coefficient estimates are the difference in % of methylation per AGA vs. SGA. In bold the CpG with the strongest effect.

CpG	Effect estimate	Nominal p-value	BH corrected p-value
cg26168577	1.374	5.36E-09	0.002
cg03066788	-2.371	2.07E-08	0.004
cg26615232	-1.304	1.3E-07	0.017
cg12055114	0.898	2.18E-07	0.017
cg04873627	1.771	2.3E-07	0.017
cg15225594	2.016	2.76E-07	0.017
cg01286950	-0.747	3.4E-07	0.017
cg18072629	0.715	3.62E-07	0.017
cg13984701	4.051	3.88E-07	0.017
cg26332310	-1.867	4.39E-07	0.017
cg24391471	-0.829	4.84E-07	0.017
cg02547025	-0.517	5.26E-07	0.017
cg23508813	2.372	5.33E-07	0.017
cg26589351	-1.370	5.97E-07	0.018
cg06234201	2.202	6.49E-07	0.018
cg23954819	-2.136	7.26E-07	0.019
cg19445996	-0.744	7.85E-07	0.019
cg02980127	1.402	9.4E-07	0.022
cg22869025	0.629	1.14E-06	0.025
cg19403534	1.688	1.22E-06	0.025
cg12322146	**4.106**	**1.31E-06**	**0.025**
cg06458489	-0.999	1.38E-06	0.025
cg00383136	1.370	1.39E-06	0.025
cg16899265	-1.257	1.59E-06	0.027
cg15847996	2.390	1.61E-06	0.027
cg08715720	0.780	1.83E-06	0.030

CpG	Effect estimate	Nominal p-value	BH corrected p-value
cg20388707	1.510	2.31E-06	0.036
cg01504555	-0.766	2.4E-06	0.036
cg07965823	-0.820	2.64E-06	0.036
cg20244295	1.049	2.65E-06	0.036
cg09311778	-0.795	2.78E-06	0.036
cg05754929	-1.993	2.81E-06	0.036
cg18246134	-0.767	2.85E-06	0.036
cg21688288	1.994	3.08E-06	0.038
cg22159939	-1.137	3.49E-06	0.042
cg25271404	-0.617	3.62E-06	0.042
cg24745895	0.669	4.01E-06	0.044
cg17836487	-1.328	4.07E-06	0.044
cg24113784	1.050	4.08E-06	0.044
cg00701706	-1.747	4.57E-06	0.048
cg24663455	-1.615	4.73E-06	0.048
cg08935613	-0.720	4.83E-06	0.048
cg22213242	1.191	4.91E-06	0.048
cg24420742	1.994	4.96E-06	0.048

Findings were practically unchanged when we excluded preterm infants from the EWAS analysis (data not shown). We found no association between age at saliva sampling and methylation status in none of the top CpGs associated with continuous birthweight or with SGA in our two EWAS analyses.

3.4 CpGs Annotation and functional analysis

None of the two sets of CpGs identified in our study, the 44 birthweight-associated CpGs and the 44-SGA-associated CpGs, showed functional enrichment of GO or KEGG terms.

Also, there was no overlap between CpGs identified in our EWAS on continuous birthweight and the 995 birthweight-related CpGs reported in 4 cord-blood and subcutaneous adipose tissue studies from the EWAS Atlas data[30]–[32], including the PACE study[18]. However, DNA methylation in two genes, *MACROD1* and *RPTOR*, found in our study (cg03045325 and cg09361653) were also associated with birthweight in the PACE study, with the same direction of the effect, but with CpGs different than ours.

These two genes have been previously associated with obesity[33], adult BMI[34]–[40], BMI-adjusted waist-hip ratio[35], [36], [41], [42], [39], high density lipoprotein cholesterol measurement[43]–[45], and visceral adipose tissue measurement[46].

In the EWAS Catalog there were 34 CpGs associated with birthweight in four studies using DNA methylation in cord blood[47][48][49][50], but none of these overlapped with 87 CpGs found to be associated with birthweight in our study. In the EWAS Atlas, 29 out of 87 birthweight- and SGA-associated CpGs in our study were associated with different traits (Additional file 1 - S2 and S3 Tables). DNA methylation variation at eleven of them has been previously associated with **insulin resistance**[51] (cg03045325), **colorectal cancer**[52] (cg02727104, cg26332310, cg12322146), **obesity**[53](cg03066788), **bariatric surgery**[54](cg20515787, cg02547025), **mortality**[55] (cg06234201), **gestational diabetes mellitus**[56] (cg00383136), **amount of visceral adipose tissue**[57] (cg20388707), and **gestational age**[32] (cg00701706).

Some of the birthweight- and SGA-associated CpGs in our study map in genes which variants were associated with the following traits: **birthweight** (*USH2A*), **BMI** (*CENPO, E2F3, RPTOR, SNTB2, PNOC,LGR4*), **body fat distribution** (*CENPO*), **waist-hip ratio** (*SYTL2, ZNF423, FOXA3, LMNB2, COL5A1,LGR4*), **BMI-adjusted waist hip ratio** (*ZNF423, AFF3*), **cardiovascular disease** (*SIPA1L2, FOXA3, RAB37, INPP5A*), **subcutaneous adipose tissue measurement** (*FOXA3, RPTOR*), **gestational age**(*CFAP46, INPP5A*), **lipoprotein cholesterol measurement** (*DMTN, SNTB2*), **total cholesterol measurement** (*E2F3,DMTN*), **type I diabetes nephropathy**(*AFF3*), **type II diabetes mellitus** (*RAMP1, SYCE1L, ZNF710*).

Discussion

In this study we investigated the association between birthweight and methylation patterns in saliva samples taken at around 10 months of age. The cord blood methylation signatures of birthweight found by a large study of the PACE consortium was not replicated in infant saliva.

However, we identified 87 infant saliva-specific signatures of birthweight and SGA, of which two overlap with PACE results at gene level (*MACROD1* and *RPTOR)*. Single nucleotide polymorphisms in these two genes have been previously associated with obesity, adult BMI, BMI-adjusted waist-hip ratio, high density lipoprotein cholesterol and visceral adipose tissue levels. DNA methylation variation at some of the 87 loci identified in our study has been previously associated with multiple traits, such as insulin resistance, colorectal cancer, obesity, and gestational diabetes mellitus. Moreover, the SGA-associated locus cg26615232 maps within *USH2A*,which variant has been associated with birthweight [58].

Although we did not have both saliva and cord blood samples, our results suggest that birthweight-related methylation patterns likely differ between cord-blood and infant saliva. In addition to tissue specificity of DNA methylation, there are also age-related changes (birth vs. infancy) that could explain differences between our and the PACE Consortium findings.

It has been repeatedly shown that DNA methylation at many sites is not temporally stable and that each tissue has its unique epigenetic landscape that likely reflects its specific function and response to environmental exposures[59][21].For example, a study on 1019 infants [60]compared the associations between preterm birth and genome-wide DNA methylation profiles using both cord tissue and cord blood samples. Cord tissue DNA methylation data was available for all 1019 infants, while cord blood analyses were performed inonly a subset of infants. The results highlighted differences between the two tissues inDNAm variation associated with preterm birth, with only a minority of overlapping CpGs. In DNA from cord tissue, DNA methylation analysis showed enrichment of differentially methylated regions in genes involved in molecular pathways related to fetal growth and development (i.e., Wnt signaling, bone remodeling, and extracellular matrix organization), while in cord blood immune response pathways (i.e. regulation of T cell differentiation, inositol lipid-mediated signaling, and regulation of RNA stability) were enriched.

Therefore, it is reasonable to speculate that saliva and blood, which have different functions, include different cell types, have different embryonic origin, and different mechanisms of exposure to environmental factors, do not share the same DNA methylation response to fetal growth, birthweight, and their risk factors.

In most tissues, DNA methylation may also vary substantially with time, especially during periods of life associated with high plasticity and fast development. The PACE study found that differential methylation associated with birthweight in neonates persisted only minimally across childhood and disappears by adulthood[18].

This result is consistent with another EWAS of birthweight and gestational age[61],where birth-related methylation changes were investigated in cord blood and peripheral blood at ages 7 and 17 in the same children. Across the majority of CpG sites that showed differential methylation in cord blood, a pattern of fast evolution was observed during early childhood that stops with adolescence, providing evidence for the lack of persistence of early life methylation differences.

This is evident also in two studies that analysed infant saliva samples. A longitudinal study with repeated saliva samples collected at birth and at 1 year of age from 50 preterm and 40infants born at term showed that DNA methylation at the differentially methylated region (DMR) of *IGF2* and *FKBP5* at birth was lower in preterm infants compared with term infants, but these differences did not persist at 1 year of age[62].Also, another study showed a clear age-dependent variation of DNA methylation, with a clear difference between saliva DNA methylation at 6 and 52 weeks of age. [63]

In our study, we could not distinguish between the tissue- and the time-related differences in DNA methylation of birthweight-associated CpG sites. However, our findings are in line with previous studies supporting the time- and tissue- dependent DNA methylation changes related to early life outcomes. Even though the age at saliva sampling varied between 7 and 17 months in our study, it was not associated with DNA methylation variation in none of the CpG sites associated with continuous birthweight and SGA, suggesting that the observed associations are unlikely influenced by the different age of saliva

collection.We supplemented the analysis of birthweight with the analysis on SGA and found different CpG sites associated with the two exposures, with only 1 overlapping CpG. This may suggest that birthweight and SGA capture different aspects of growth and/or could be proxies for different intrauterine exposures. Consistently with our findings, also in the PACE analyses, the 4 CpGs associated with low birthweight (< 2500gr) did not overlap with the 914 associated with continuous birthweight. We could not analyze the same birthweight subgroups as in PACE because of the small number of low birthweight children (N = 5).

The main strength of our study was the possibility to analyse DNA methylation in saliva samples, which are easy to collect at any age in childhood and are, therefore, good candidates for future large DNA methylation studies. Moreover, while it is probably unfeasible to collect repeated blood samples over relatively short time periods, it is possible to use saliva to monitor changes in DNA methylation over time. As salivary DNA methylation is poorly studied, especially in newborns and infants, it would be important to understand if, and for which specific traits and exposures, salivary and blood DNA methylation can be used interchangeably, and when they clearly show distinct methylation signatures. Previous DNA methylation studies using saliva samples focused on neurobehavioural conditions[64], [65],[66], respiratory traits[22], and cancer research[67], but to our knowledge the associations between birthweight and saliva DNA methylation has not been studied so far.

The sample size of our study was relatively small. This does not have a strong impact on the replication analyses; for example, the estimate of a 47% concordance in the direction of the associations (similar as expected by chance alone) is fairly precise. The small sample size may however have had an impact on EWAS analyses. Despite this, after an FDR-correction, we identified some novel CpGs associated with birthweight, which are likely to be saliva-specific birthweight signatures. These findings need replication in independent saliva EWAS.

Conclusion

In conclusion, our study provides an indication of the birthweight and small for gestational age epigenetic salivary signatures in children around 10 months of age, and suggests that DNA methylation signatures of birthweight likely differ between cord blood and infant saliva. Further insights are needed to understand whether these differences are due to biological differences between the two tissues or could be attributed to age-related DNA methylation changes.

Abbreviations

CpG, cytosine
guanine dinucleotide; AGA, Adequate-for-Gestational-Age; SGA, Small-for-Gestational-Age; FDR, False Discovery Rate; EWAS, epigenome-wide association study

Declarations

Ethics approval and consent to participate

NINFEA study was approved by Ethical Committee of the San Giovanni Battista and CTO/CRF/Maria Adelaide Hospital of Turin and all participating mothers gave informed consent at enrolment and at saliva donation.

Consent for publication

Not applicable

Availability of data and materials

Data from the NINFEA cohort underlying the findings reported in this study is available to researchers who meet the criteria for access to confidential data and upon reasonable request. Data availabilitycontact: Prof. Lorenzo Richiardi (lorenzo.richiardi@unito.it)

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

Study conception and design:Richiardi,Popovic,Moccia

Acquisition of data: Fiano, Trevisan,Polidoro, Rusconi, Richiardi,

Data Analysis: Moccia, Popovic, Isaevska

Data interpretation: Moccia, Popovic, Isaevska, Richiardi, Fiano, Trevisan, Polidoro, Rusconi,

Drafting of manuscript: Moccia, Richiardi, Popovic

Critical revision: Moccia, Richiardi, Popovic, Rusconi, Isaevska, Fiano, Trevisan, Polidoro

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Figures

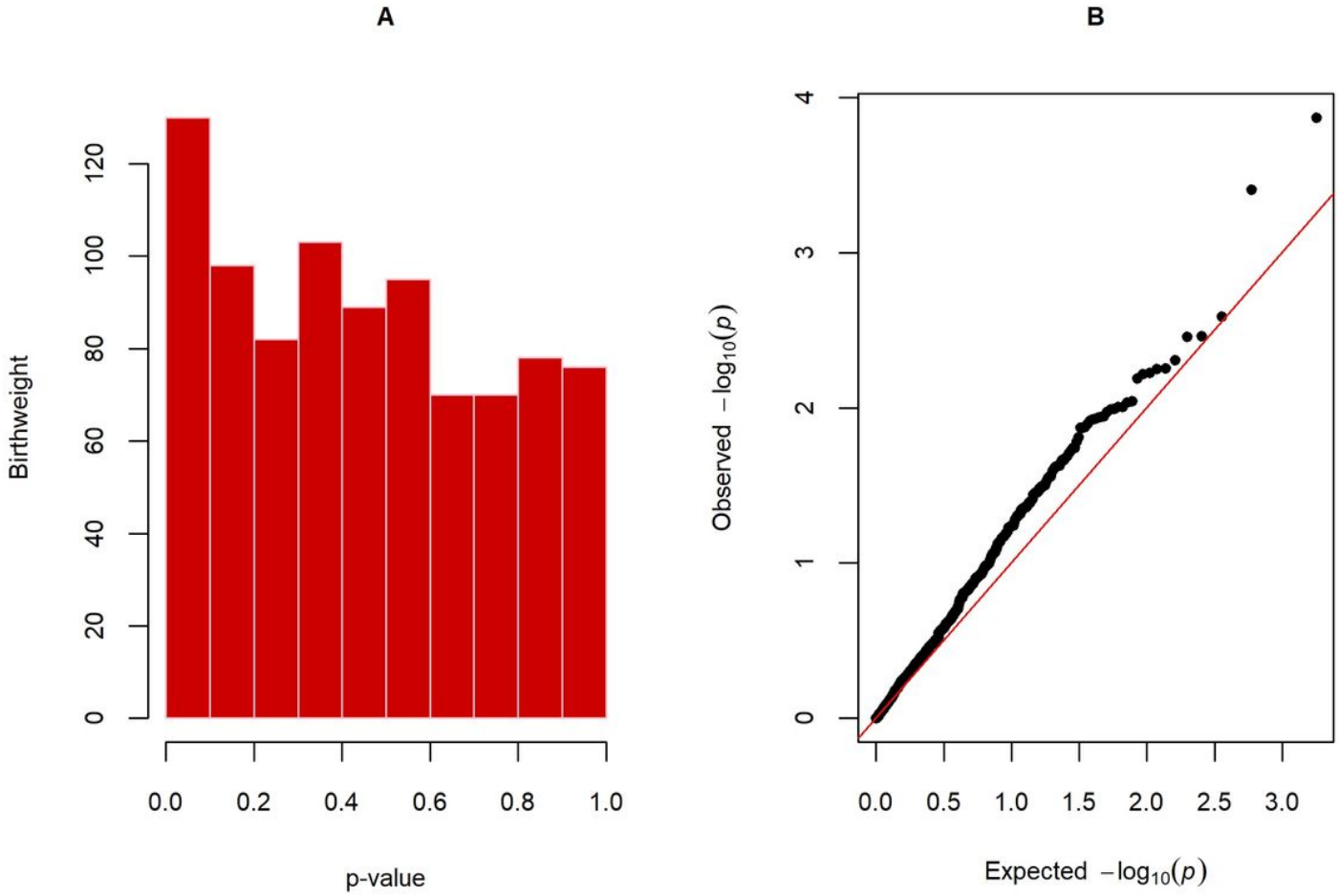

Figure 1

Histogram and qq-plot of the replication p-values

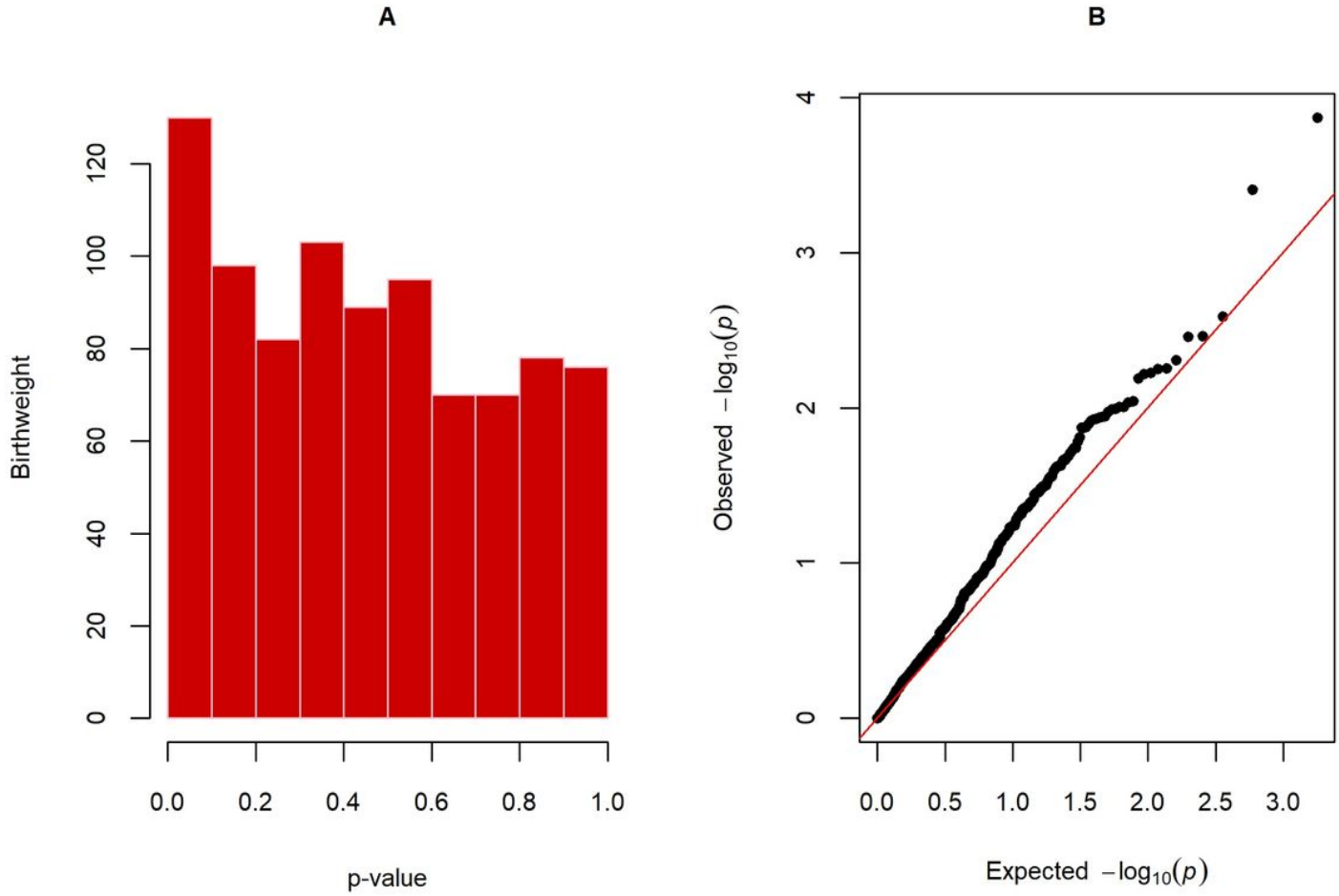

Figure 1

Histogram and qq-plot of the replication p-values

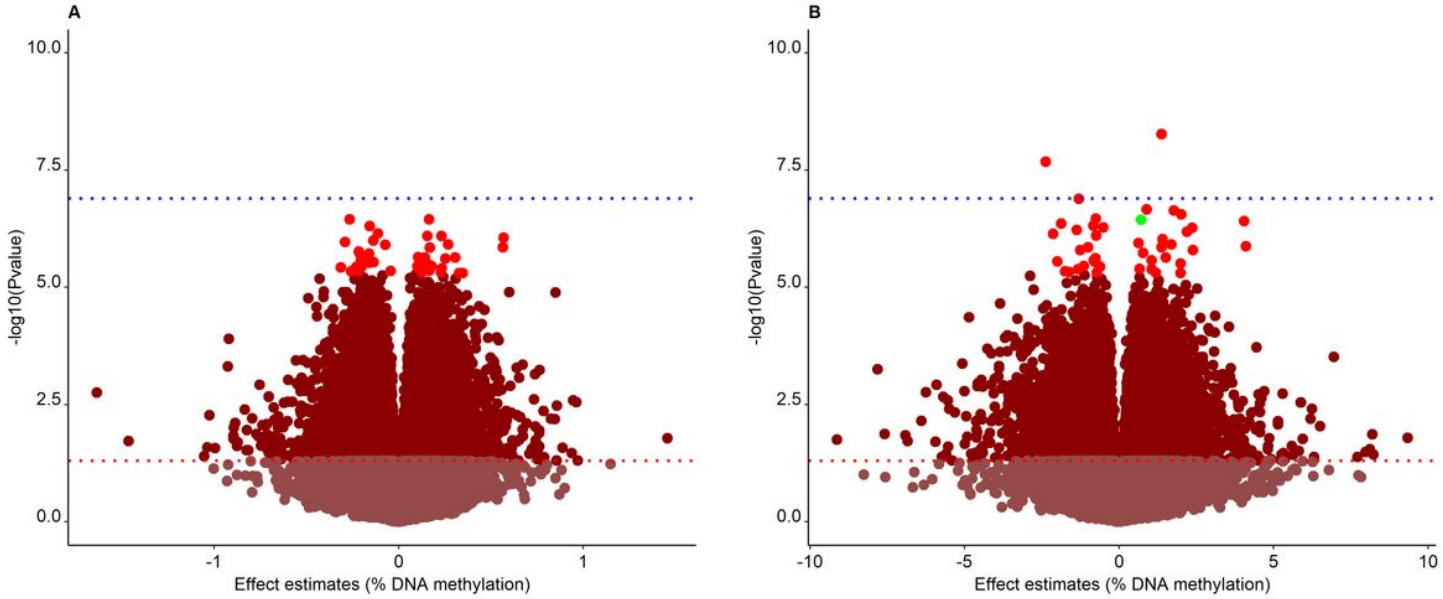

Figure 2

Volcano plot of the two EWAS.

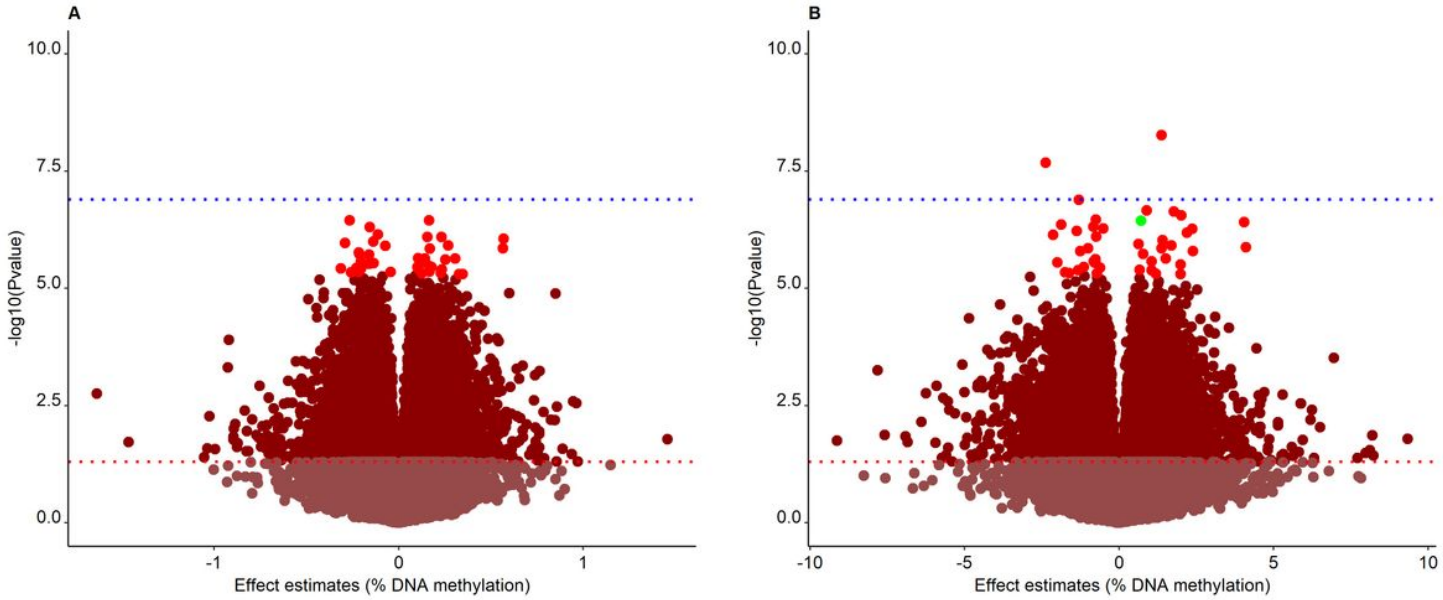

Figure 2

Volcano plot of the two EWAS.

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