Determination of saliva epigenetic age in infancy, and its association with parental socio-economic characteristics and pregnancy outcomes

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Abstract

Epigenetic age acceleration (AA) has been associated with adverse environmental exposures and many chronic conditions. We estimated, in the NINFEA birth cohort, infant saliva epigenetic age, and investigated whether parental socioeconomic position (SEP) and pregnancy outcomes are associated with infant epigenetic AA. A total of 139 saliva samples collected at on average 10.8 (range 7-17) months were used to estimate Horvath's DNA methylation age. Epigenetic AA was defined as the residual from a linear regression of epigenetic age on chronological age. Linear regression models were used to test the associations of parental SEP and pregnancy outcomes with saliva epigenetic AA. A moderate positive association was found between DNA methylation age and chronological age, with the median absolute difference of 6.8 months (standard deviation 3.9). The evidence of the association between the indicators of low SEP and epigenetic AA was weak; infants born to unemployed mothers or with low education had on average 1 month higher epigenetic age compared with infants of mothers with high education and employment (coefficient 0.78 months, 95% confidence intervals (CI): -0.79, 2.34 for low/medium education; 0.96, 95% CI:-1.81, 3.73 for unemployment). There was no evidence for association of gestational age, birthweight or caesarean section with infant epigenetic AA. Using the Horvath's method, DNA methylation age can be fairly accurately predicted from saliva samples already in the first months of life. This study did not reveal clear associations between either pregnancy outcomes or parental socioeconomic characteristics and infant saliva epigenetic AA.

Key Words: Epigenetic age, epigenetic age acceleration, saliva, DNA methylation, birth cohort

Introduction

A person's chronological age is not always closely related to his/her biological age. Biological ageing occurs predominantly at cellular level as a result of accumulating cellular damages, caused by several molecular mechanisms, including mitochondrial dysfunction, oxidative stress, accumulation of aberrant proteins, somatic mutations, DNA damage and telomere shortening.[1,2] These mechanisms are strongly regulated by the defence and repair systems, which maintain the cellular balance and functionality.[3] Impairment of cellular maintenance pathways or increased accumulation of cellular damage due to extrinsic hazards compromise the cell function, leading to an increased velocity of biological ageing.[1]

Chronological age is the strongest unmodifiable risk factor for mortality and major non-communicable diseases, including cancer, cardiovascular and neurodegenerative diseases.[4] Evidence on environmental influences on the rate of accumulation of cellular damage, and therefore, biological ageing suggests that some of the adverse effects of aging could be, at least partially, modified.[5] To date, numerous biomarkers to predict the biological age have been developed, starting from physical functions and anthropometric measurements to molecular and DNA-based biomarkers.[6,7] Two of the most promising DNA-based age biomarkers are DNA methylation and telomere length, and several different methods to quantify the biological age using these biomarkers have been developed.[7-10]

Horvath's multi-tissue DNA methylation clock[8] is the most widely used age biomarker in humans, with a strong correlation with chronological age (Pearson rho > 0.90 in studies with a wide age range). The epigenetic age acceleration (AA), i.e. the difference between epigenetic and chronological age, has been consistently associated with overall mortality and many chronic conditions, including cardiovascular diseases and cancer.[11-14] Although the molecular mechanisms behind the epigenetic AA are largely unknown, epigenetic AA is one of the most popular measures of biological ageing.

Early life programming, induced by environmental factors at different stages of prenatal and early postnatal life, likely involves life-long alterations in the epigenetic programming and the regulation of gene transcription.[15] In fact, emerging evidence supports the role of environmentally induced epigenetic variations in linking early life exposures to long-term outcomes. Epigenetic age and gestational age accelerations at birth and in childhood have been associated with both early life environment, including maternal characteristics, socio-economic conditions and perinatal outcomes,[16-20] and with numerous childhood health outcomes.[20-24]

Socio-economic differences in health have been widely documented, and low socioeconomic position (SEP) is known to be one of the most important risk factors for ageing-related chronic diseases. Although parental SEP, early life and current socio-economic disadvantage have been reported to leave epigenetic signatures at birth,[25] during childhood and in adulthood,[25-27] findings on epigenetic AA are less consistent. For example, epigenetic AA has been associated with a low educational level in adulthood in a multi-cohort study,[28] but not with low SEP in two UK cohorts of adult women[17] and in two studies based on children from the UK ALSPAC birth cohort.[16,20]

In addition to SEP, adverse pregnancy outcomes, such as low birthweight, preterm birth and delivery by caesarean section, are known to leave long-term health effects, and are found to be associated with specific epigenetic marks.[29-31] The ALSPAC study, in their cord blood analysis, reported epigenetic AA associated with caesarean delivery, but not with birthweight and gestational age.[20] In the same study, peripheral blood epigenetic AA in childhood and adolescence was associated with birthweight, but with the opposite direction of the association. Also, gestational AA of the offspring at birth in a Finnish study of 814 mother-newborn pairs was associated with several maternal risk factors and birth outcomes, including lower birth size and 1-min Apgar score.[18] On the contrary, another study conducted

within the ALSPAC cohort (N=863) found greater cord blood gestational AA to be associated with larger birth size.[16]

As few studies used biological samples other than blood to estimate epigenetic age in infancy, and given conflicting findings on the associations of SEP and pregnancy outcomes with early life epigenetic AA, we estimated, in the NINFEA (Nascita ed INFanzia: gli Effetti dell'Ambiente) birth cohort, saliva epigenetic age in children aged 7-17 months, and then, investigated whether parental socioeconomic status, pregnancy outcomes and caesarean section are associated with infant saliva epigenetic AA.

Method

Study population

Data were taken from an epigenome-wide case-control study on early childhood wheezing, nested within the NINFEA birth cohort.[32] The NINFEA study is an Italian web-based birth cohort that, during the period 2005-2016, recruited approximately 7500 pregnant women who had access to the Internet, enough knowledge of Italian to complete online questionnaires, and volunteered to participate (https://www.progettoninfea.it).[33] Women completed the baseline questionnaire at enrolment, and children are currently followed up with six questionnaires completed by mothers 6 and 18 months after delivery, and when children turn 4, 7, 10 and 13 years of age. At the end of the 6-month questionnaire, participating mothers were invited to collect their and their child's saliva samples, using the OrageneTM DNA self-collection kit (Infant Sponge Kit, CS-1, and OG-250, DNA Genotek, Inc., Ottawa, Ontario, Canada). Mothers who did not respond to this initial invitation were invited again at the end of the 18-month and 4-year questionnaire. Only children with saliva samples collected between 7 and 17 months of age were considered in this study.

The original case-control study was conducted within the subset of the NINFEA children who met the following criteria: i) singleton child, ii) saliva sample collected between 6 and 18 months after birth, iii) residence in the Province of Turin, and iv) born to mother who did not have asthma during the index pregnancy. A total of 72 cases with at least one reported episode of wheezing between 6 and 18 months of age were matched to 72 controls by sex, age at sampling and seasonality/calendar year of sampling. 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.

Saliva DNA methylation

Saliva samples are stored in a biobank at -80°C. Genomic DNA was extracted from the selected 144 saliva sponge samples by using the OrageneTM Purifier Solution (DNA Genotek, Inc., Ottawa, Ontario, Canada). Genomic DNA was bisulphite-converted using the EZ DNA Methylation-GoldTM Kit (Zymo Research, CA, USA) following the manufacturer's protocol. The methylation status of over 485,000 probes was measured using the Infinium HumanMethylation450 BeadChip array (Illumina, Inc., San Diego, CA). The matched pairs were placed on the same chip to minimize confounding by batch. DNA methylation for each probe was expressed as beta-value (ratio of methylated probe intensity to overall intensity, representing 0 to 100% methylation at each probe). Pre-processing steps and quality control were described before.[32,34] After quality control checks and probes filtering three samples with more than 1% of the CpGs with detection p-value>0.01 were excluded, resulting in a total of 141 samples and 421,782 probes included in this study.

Saliva is known to have a heterogeneous cellular composition, mostly composed of buccal epithelial cells and leucocytes.[35,36] In the absence of saliva reference methylomes, we used the

Houseman's reference free method (RefFreeEWAS package in R)[37] to estimate proportions of putative cell types based on their underlying methylomes. From the 10,000 most variable CpGs, we identified two latent variables as the optimal number of surrogates for cell-type mixture, which is in accordance with previously reported saliva cell heterogeneity profiles in older children.[35,36] We then used the 421,782 CpGs to estimate the proportion of the two cell types per sample.

Exposures

We analysed the following socioeconomic factors: parental educational level (low—primary school or less / medium—secondary school, and high—university degree), parental unemployment (employed and unemployed) at the time of conception, family size (including the index child; 2 members, 3-4 members, and 5 and more members), maternal age at delivery and an indicator of the equivalised total disposable household income at birth (the highest three quintiles vs. the lowest two quintiles of the entire NINFEA cohort distribution). The equivalised total disposable household income indicator at birth has been developed within the European Horizon 2020 LifeCycle project and it uses external data from the pan-European surveys "European Union Statistics on Income and Living Conditions" (EUSILC) and internal household and parental characteristics available within cohorts.[38] For the NINFEA cohort, the income indicator has been constructed using the following parental and household baseline characteristics: cohabitation with partner, family size (number of children and adults in the household), dwelling type, number of rooms in the house, maternal age and country of birth, parental education and occupation and maternal job coded using the ISCO-88 (International Standard Classification of Occupations) classification.[38]

Pregnancy outcomes included birthweight (grams), gestational age (weeks), size for gestational age (small, appropriate and large for gestational age, based on the 10th and the 90th percentile of the World Health Organization birthweight for sex and gestational age charts),[39]

and mode of delivery (vaginal delivery vs. caesarean section). All variables were measured through questionnaires completed by mothers either during pregnancy (socioeconomic factors) or six months after delivery (pregnancy outcomes).

Statistical analysis

We estimated DNA methylation age for each infant saliva sample by applying the Horvath's method to raw beta-values.[8] Horvath used 8000 samples from 82 Illumina DNA methylation array data sets, across an extensive range of ages and tissue samples, to identity 353 DNA methylation sites predictive of chronological age.[8] Two outliers in the NINFEA dataset were removed as their predicted epigenetic age estimates were 4 standard deviations above the sample mean, which resulted in a total of 139 samples for the subsequent analyses.

As the Horvath's age estimation method was developed using samples across wide age ranges, a new method for measuring DNA methylation age in children, named the Pediatric-Buccal-Epigenetic (PedBE) clock, has been recently developed.[40] This method was developed using buccal samples of individuals between 0 and 20 years of age, and it uses weighted DNA methylation values at 94 CpG sites to predict chronological age.[40] Given the large predominance of buccal epithelial cells in saliva samples, especially in young children,[35,36] we additionally estimated DNA methylation age using the PedBE clock, but decided to present these results only as a supplementary analysis (**Supplementary Table S1, Supplementary Figure S1**), as in the NINFEA dataset there was an extremely high correlation between the age estimated using the PedBE clock and the cell type proportions estimated using the Houseman's reference free method (**Supplementary Figure S2**), Thus, studying the epigenetic age would have been the same as studying the proportion of buccal cells in the saliva.

The Pearson's correlation, the R^2 and the median absolute difference ("median error") between the predicted and chronological age were used to assess the performance of the Horvath's age predictor. The individual epigenetic AA was defined as the residual from the linear regression of epigenetic age on chronological age. This measure of AA is independent of chronological age, with, relative to the chorological age, positive values indicating epigenetic age acceleration and negative values indicating epigenetic age deceleration.

Using linear regression models, we tested the associations of parental SEP and pregnancy outcomes with the epigenetic AA (in months). For all analyses we fitted two models for each independent variable: i) *Model 1* adjusted for child's sex, technical batch (chip), the two estimated saliva cell type proportions, and child wheezing at age 6 to 18 months, as the indicator of being a case or a control in the original case-control study; and ii) *Model 2* additionally adjusted for maternal age and parity in the analyses of socio-economic factors, and for maternal age, parity, maternal education and pre-pregnancy body mass index (BMI) in the analyses of pregnancy outcomes. The *Model 2* for maternal age at delivery was adjusted for maternal education, occupation, parity and pre-pregnancy BMI. Birthweight and gestational age were also mutually adjusted in *Model 2*. In the NINFEA cohort an equivalised total disposable household income indicator was predicted using, as one of the predictors, maternal age, so we excluded maternal age from the adjusted model of household income. Although maternal smoking during pregnancy is strongly associated with pregnancy outcomes and with offspring DNA methylation, the prevalence of smoking in our sample was rather low (N=3; 2.2%), so we did not adjust for maternal smoking in our analyses.

Finally, to assess the relative contribution of individual CpG sites from the Horvath's DNA methylation predictor to infant saliva epigenetic age estimates we ranked all 353 CpG sites according to their weight metric (%) calculated by multiplying each CpG interquartile range with the absolute value of the Horvath's training coefficient for that CpG site. We compared

the top ranked CpG sites with those reported in another epigenetic age study based on newborn saliva samples.[41]

All the analyses were performed using the computing environment R version 3.6.1 (R Development Core Team, 2019).

Results

The characteristics of the 139 children included in the analyses are shown in **Table 1**. The mothers were on average 34.5 years old at delivery, the two thirds were nulliparous and 14.5% were obese or overweight before pregnancy. In the study sample 72.5% of the mothers were highly educated (University degree or higher), more than 90% of the mothers and almost all the fathers (97.8%) were employed, while the average predicted equivalised total disposable household income was 1768.6 euros per month. Among children, the mean gestational age at delivery was 39.5 weeks, the mean birthweight was 3241 grams, and 36.7% were delivered by caesarean section.

The mean age at saliva sampling was 10.8 months (standard deviation (SD) 2.2; range 7-17), while the mean Horvath's DNA methylation age was 17.5 months (SD 4.4; range 7.3-34.3) (**Table 2**). There was a positive association between chronological age and DNA methylation age (Pearson's r=0.32, p-value = 0.0001, **Figure 1**). The correlation was moderate compared with the one reported in the original Horvath's study (r >0.90 in most of the tissues and cell types)[8] due to the much narrower chronological age range of our sample (7-17 months). Several studies with restricted sample age ranges reported analogous correlations between the estimated Horvath's epigenetic age and chronological age.[17,19,21-23] The median absolute difference (median error) between DNA methylation age and chronological age was 6.8 months (mean 6.9; SD 3.9).

Using the weighting metric described in Methods, the twenty top ranked CpG sites, i.e. the strongest CpG predictors from the Horvath's epigenetic clock in our study, contributed collectively to the 25.9% of the total weight metric. Among these, eleven CpGs overlapped with the twenty most influential CpG sites in the newborn saliva study by Phang et al,[41] adding to 14.9% of the total weight in our study and 21.0% in the study by Phang et al. The eleven overlapping CpG in studies on newborn/infant saliva map to the following genes: NHLRC1, CSNK1D, PPP1R14A, FZD9, FXN, RASSF4, BCMO1, SCGN, PAWR, RXRA and DPP8. The complete list of CpG sites, with their rankings and functional characteristics are shown in **Supplementary Table S2**.

In the NINFEA saliva samples, the PedBE clock had weaker correlation with chronological age compared with Horvath's epigenetic clock (Pearson's r=0.24 for PedBE clock, r=0.32 for Horvath's epigenetic clock), and showed slightly higher saliva epigenetic age compared with Horvath's epigenetic age method (**Supplementary Table S1**, **Supplementary Figure S1**).

Parental socioeconomic characteristics, pregnancy outcomes and offspring saliva epigenetic AA

The associations of parental socioeconomic characteristics and pregnancy outcomes with the offspring epigenetic AA are shown in **Table 3** and **Figure 2**. Overall, there was a suggestion of an association between the indicators of low SEP and epigenetic AA, but the evidence for each indicator was weak, also due to large confidence intervals and low SEP-associated heterogeneity in the study sample. In particular, infants born to unemployed mothers or mothers with low or medium educational level had on average 1 month higher epigenetic age compared with infants of mothers with high education and employment (coefficient 0.78 months, 95% confidence intervals (CI): -0.79, 2.34 for maternal low/medium educational level, and coefficient 0.96, 95%CI: -1.81, 3.73 for maternal unemployment). Similar estimates were also found for a low total household income (1st and 2nd quintile compared with the highest three quintiles 0.74, 95%CI: -0.95, 2.43) and large family size (>4 members compared

with 3-4 members 0.86; 95% CI: -2.02, 3.75). The estimate observed for paternal unemployment status (2.82 months; 95% CI: -2.23, 7.88) should be interpreted with caution as in our sample only three children had fathers who were unemployed (**Table 1**).

In our study, there was no evidence for associations of gestational age, birthweight or delivery by caesarean section with infant saliva epigenetic AA estimated using Horvath's epigenetic clock (**Table 3**, **Figure 2**).

Similarly, we found no association of parental SEP or pregnancy outcomes with infant PedBE AA (data not shown).

Discussion

Using saliva samples from 139 infants of the NINFEA birth cohort study we examined epigenetic DNA methylation age, its correlation with chronological age and the associations of parental socioeconomic characteristics and pregnancy outcomes with epigenetic age acceleration/deceleration. We found a moderate association between epigenetic and chronological age in the first 1.5 years of life, and only an indication of an accelerated epigenetic aging in infants from lower socio-economic backgrounds. This study did not identify associations of gestational age, birthweight or delivery by caesarean section with the rate of early life epigenetic AA.

The sample analysed in our study had a quite narrow age range (7-17 months, SD 2.2 months), so the correlation between the estimated epigenetic and chronological age was moderate (Pearson's r= 0.32), with the mean difference between epigenetic and chronological age of approximately six months. This is, however, expected and in line with other studies that analysed samples with low standard deviations in chronological age.[8,17,19,21-23]

Most of the previous studies focused on the effects and causes of accelerated/decelerated epigenetic age in adult populations,[42] and some studies reported that a difference between

DNA methylation age and chronological age may already occur at birth as a consequence of unfavourable prenatal environment.[16,18,19] To our knowledge no studies looked at these changes in infancy. We showed that Horvath's epigenetic clock predicts the chronological age in the first months of life, with a similar performance of studies based on saliva samples collected at later ages,[22,43] and can be used to explore epigenetic AA in infancy.

Previous findings on socio-economic status and epigenetic ageing are conflicting. An accelerated epigenetic ageing has been found in adults with early life or current socio-economic disadvantage,[27,28] but not all studies confirmed these associations,[17,44] and no such epigenetic signatures were observed at birth or in childhood.[16,20,45] We used several maternal, paternal and household SEP measures, as well as the predicted equivalised total disposable household income, which captures yet another dimension of SEP, namely the material property in the country-specific context. Our findings support previous findings of no strong association between parental SEP and the rate of offspring epigenetic ageing in the first years of life. Nevertheless, the direction of the association observed in our and other studies based on children may indicate that the changes observed in adults may initiate early in life and that these small effects may then accumulate as a result of life-long deprivation. It should be, however, acknowledged that despite the existing contrast between medium and high socioeconomic level in our sample, few children came from very low socioeconomic background, and therefore, the effect of severe early childhood deprivation might not have been captured in this study.

The lack of association between gestational age and epigenetic AA is consistent with previous studies that assessed epigenetic AA either at birth from cord blood or later in childhood from peripheral blood samples, where there was nearly no association between Horvath's epigenetic AA and gestational age.[18,20] Horvath's DNA methylation age was designed to estimate chronological age and its consistent weak association with gestational age could

reflect different postnatal age-specific changes unrelated to prenatal development. In fact, several tissue-specific gestational age predictors have been developed using DNA methylation from umbilical cord blood or blood spot samples,[46,47] but only few CpG sites from these predictors overlap with the Horvath's epigenetic age predictor. Despite these differences, gestational epigenetic age acceleration and deceleration have also been associated with some of the maternal characteristics and pregnancy and birth outcomes.[16,18] In particular, the directions of the associations were mixed for birthweight, where a negative association with gestational AA was observed in a Finish study,[18] while a positive association was reported in a UK study.[16] Inconsistent findings on birthweight were also found using the Horvath's method in another UK study based on the same cohort, where birthweight was not associated with epigenetic AA at birth, while the association was in the opposite direction in the analyses carried out in childhood compared to those conducted in adolescence.[20] Similarly, we found no association between birthweight and saliva epigenetic age acceleration in infancy.

In contrast with two previous studies,[16,20] we did not find an accelerated epigenetic age in children born by caesarean section. It should be, however, noted that in both previous studies there was only a marginal positive association with caesarean delivery at birth, and that it disappeared later in childhood.

Epigenetic AA can be seen as a proxy of adverse environmental exposures and a mechanism through which these exposures influence later disease risk, or as a consequence of adverse health outcomes. In fact, it has been associated both with prenatal and early postnatal environmental adversity and with later health outcomes, such as high BMI, asthma and mortality.[11-14, 16-28] Despite mainly weak associations, epigenetic AA has been linked with multiple exposures and outcomes, which makes complicated the understanding of what is actually measured with DNA methylation clock. It could be seen as an overall epigenetic shifting in response to adverse events, not exclusive of any specific insult, but an intrinsic

response of an organism to unfavourable environment in general. If so, it is not unexpected that the associations reported so far with single adverse factors operating early in life are relatively small in magnitude and often inconsistent. However, even small-magnitude effect sizes are important and could have functional relevance for later childhood and adulthood health.

The lack of associations with parental SEP and pregnancy outcomes in our study may be due to several factors. First, most of the previous studies that found associations with these two groups of exposures estimated DNA methylation age using cord or peripheral blood samples. It is well-established that epigenetic mechanisms are cell type specific, so it might be well possible that pregnancy outcomes and SEP-related changes in epigenetic AA are not present in all tissues and cell types. Second, the potential epigenetics marks of prenatal exposures that are present at birth may be reversible (or, conversely, might further accumulate) postnatally. Due to the extensive growth and development in the first months of life, it is expected that the dynamic of epigenetic changes is particularly rapid in infancy, and thus the epigenetic marks of prenatal exposures might have already disappeared by the first year of life. Third, some of the characteristics analysed in our study, e.g. paternal unemployment, small family size and large for gestational age, had less than ten exposed children available for the analyses. The sample size was thus relatively small, and our results should be seen in the overall context of the available and the future literature rather than a single ultimate study with high statistical power.

Conclusions

We have provided evidence that, using the Horvath's method, DNA methylation age can be fairly accurately predicted from saliva samples already in the first months of life. This is important as saliva represents one of the most easily accessible non-invasive biological samples for DNA methylation analyses in large population studies, especially at young ages.

Horvath's epigenetic clock indicates only a suggestion of an accelerated epigenetic aging in infants from lower socio-economic backgrounds, and no evidence of association between epigenetic AA and pregnancy outcomes, including gestational age, birthweight and mode of delivery.

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Conflicts of Interest

None

Ethical Standards

The authors assert that all procedures contributing to this work comply with the ethical standards and with the Helsinki Declaration of 1975, as revised in 2008. The NINFEA study protocol and subsequent amendments have been approved by the Ethical Committee of the San Giovanni Battista Hospital—CTO/CRF/Maria Adelaide Hospital, Turin, Italy (approval N.0048362). All women gave their informed consent at recruitment and when donating saliva samples.

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Table 1. Characteristics of the study population (N=139)

Maternal characteristic	N (%) or Mean
Maternal age (years)	34.5 (4.0)
Maternal parity	
Nulliparous	90 (66.7)
≥1 previous pregnancies	45 (33.3)
Missing	4
Maternal pre-pregnancy BMI (kg/m2)	
Underweight	11 (8.0)
Normal	107 (77.5)
Overweight/Obese	17 (14.5)
Missing	1
Smoking during pregnancy	
No	136 (97.8)
Yes	3 (2.2)
Parental socio-economic characteristics	
Maternal educational level	
High	100 (72.5)
Medium	36 (26.1)
Low	2 (1.4)
Missing	1
Maternal employment status	
Employed	125 (90.6)
Unemployed	13 (9.1)
Missing	1
Paternal educational level	
High	64 (46.4)
Medium	54 (39.1)
Low	20 (14.5)
Missing	1
Paternal unemployment status	
Employed	136 (97.8)
Unemployed	3 (2.2)
Predicted equivalised total disposable household income	
Euros	1768.6 (303.86)
The NINFEA cohort quintiles	
1st quintile	9 (7.1)
2nd quintile	29 (22.8)
3rd quintile	24 (18.9)
4th quintile	32 (25.2)
5th quintile	33 (26.0)
Missing	12
Family size (including the index child)	
2 members	4 (2.9)
3 members	83 (59.7)
4 members	41 (29.5)
≥ 5 members	11 (7.9)

Pregnancy outcomes		
Gestational age at delivery (weeks)		39.5 (1.5)
Birthweight (grams)		3241 (450.1)
Size for gestational age		
	Small for gestational age	22 (15.8)
	Appropriate for gestational age	110 (79.2)
	Large for gestational age	7 (5.0)
Mode of delivery		
	Vaginal delivery	88 (63.3)
	Caesarean section	51 (36.7)
Matching variables		
Case (child wheezing 6-18 months)		
	No	68 (48.9)
	Yes	71 (51.1)
Child sex		
	Females	60 (43.2)
	Males	79 (56.8)
Season of saliva sampling		
	Spring or summer	75 (54.0)
	Autumn or winter	64 (46.0)
Age at saliva sampling (months)		10.8 (2.2)

Table 2. Chronological age and predicted Horvath's DNA methylation age in the NINFEA sample

Epigenetic and chronological age measures	Mean (SD)	Range	
Chronological age (months)	10.8 (2.2)	7.0, 17.0	
DNA methylation age (months)	17.5 (4.4)	7.3, 34.3	
Difference between epigenetic and chronological age			
Difference (months)	6.7 (4.3)	-4.1, 19.2	
Absolute difference (months)	6.9 (3.9)	0.0, 19.2	
Median absolute difference (median error; months)	6.8	/	
Epigenetic age acceleration	**Coef.**	**s.e.**	**R^2**
Epigenetic age ~ chronological age	0.64	0.16	0.09
Residuals (months)			
Mean; SD; Range	0.0	4.2	-10.5, 14.1
Epigenetic age ~ chronological age + chip + cellular	0.75	0.17	0.20

SD –Standard deviation
Coef. – Linear regression coefficient
s.e. – Standard error
R^2 – Coefficient of determination

Table 3. Associations of familial socio-economic status and pregnancy outcomes with saliva epigenetic AA in infants

Familial socio-economic characteristics	Epigenetic age acceleration (months)[a]	
	Coef.[b] (95% CI)	**Adjusted coef.[c] (95% CI)**
Maternal age at delivery (per 5 years)	0.20 (-0.68, 1.08)	0.17 (-0.82; 1.17)[d]
Maternal educational level		
High	*Reference*	*Reference*
Medium / Low	0.60 (-0.92, 2.12)	0.78 (-0.79, 2.34)
Maternal employment status		
Employed	*Reference*	*Reference*
Unemployed	0.84 (-1.66, 3.35)	0.96 (-1.81, 3.73)
Paternal educational level		
High	*Reference*	*Reference*
Medium / Low	0.06 (-1.33, 1.45)	0.11 (-1.31, 1.53)
Paternal employment status		
Employed	*Reference*	*Reference*
Unemployed	2.76 (-2.22, 7.73)	2.82 (-2.23, 7.88)
Predicted equivalised total disposable household income (quintiles of Euros)		
≥3rd quintile	*Reference*	*Reference*
1st and 2nd quintile	0.81 (-0.85, 2.47)	0.74 (-0.95, 2.43)[e]
Family size (including the index child)		
2 members	-0.43 (-4.54, 3.67)	-0.34 (-4.57, 3.89)
3-4 members	*Reference*	*Reference*
≥ 5 members	1.20 (-1.34, 3.74)	0.86 (-2.02, 3.75)
Pregnancy outcomes	**Coef.[b] (95% CI)**	**Adjusted coef.[f] (95% CI)**
Gestational age at delivery (weeks)	0.21 (-0.26, 0.69)	0.06 (-0.52, 0.64)[g]
Birthweight (per 100 grams)	0.11 (-0.06, 0.27)	0.05 (-0.16, 0.26)[g]
Size for gestational age		
Small	0.22 (-1.67, 2.10)	0.63 (-1.35, 2.60)
Appropriate	*Reference*	*Reference*
Large	1.77 (-1.75, 5.29)	0.80 (-3.09, 4.69)
Mode of delivery		
Vaginal delivery	*Reference*	*Reference*
Caesarean section	0.01 (-1.46, 1.49)	0.14 (-1.36, 1.65)

Coef. – Regression coefficient; CI – Confidence Intervals

[a] Unstandardized residual regressing DNA methylation age on chronological age

[b] Adjusted for child sex, technical batch (chip), estimated saliva cell count types, and child wheezing.

[c] Adjusted as [b] and additionally adjusted for maternal age and parity.

[d] Adjusted as [b] and additionally adjusted for maternal education, occupation, parity, and pre-pregnancy body mass index (BMI)

[e] Adjusted as [b] and additionally adjusted for maternal parity.

[f] Adjusted as [b] and additionally adjusted for maternal age, parity, maternal education and maternal BMI.

[g] Gestational age and birthweight were mutually adjusted.

Figure 1 A scatterplot with a smoothed regression line and 95% confidence intervals showing associations between saliva DNA methylation age and chronological age in infants from the NINFEA birth cohort

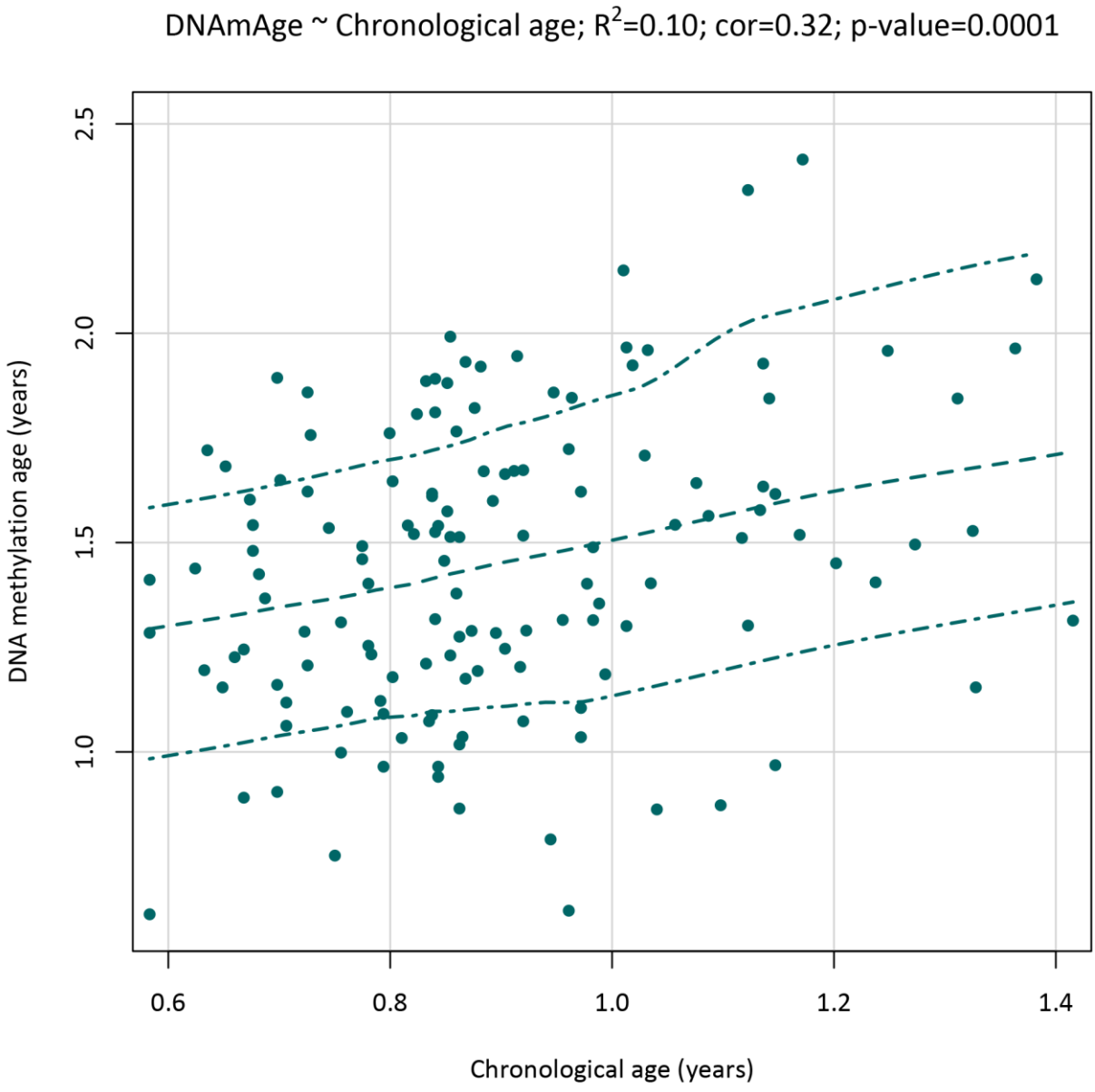
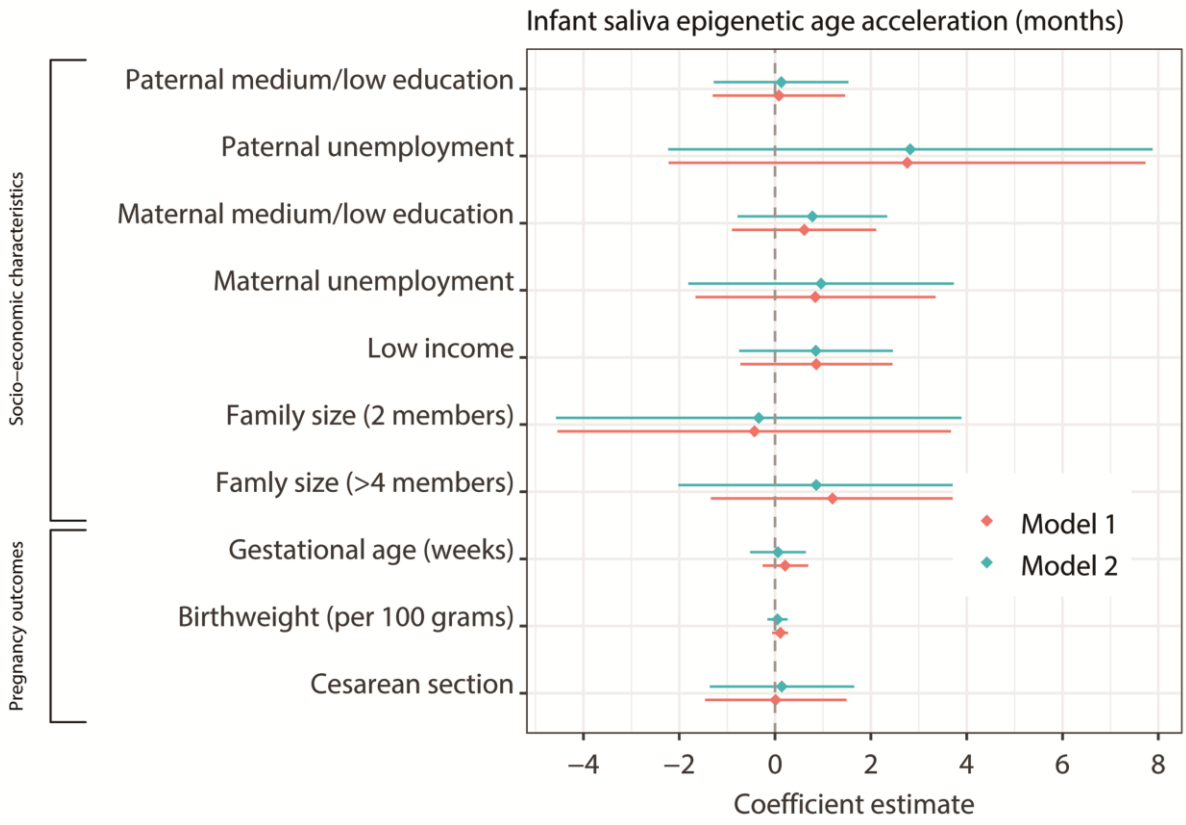


Figure 2 Coefficient estimates with 95% confidence intervals for the association of parental and familial socioeconomic characteristics and pregnancy outcomes with infant saliva epigenetic age acceleration (months). Reference groups: parental high education (University or higher), parental employment, high income (ranked to ≥3rd quintile), 3-4 family members in the household, vaginal delivery. *Model 1* adjustment (red): child's sex, technical batch (chip), estimated saliva cell count types, and child wheezing as a selection factor; *Model 2* adjustment (blue): as *Model 1* and additional adjustment for maternal age and parity in analyses of socioeconomic characteristics, and for maternal age, parity, maternal education and maternal pre-pregnancy body mass index (BMI) in analyses of pregnancy outcomes. Gestational age and birthweight were mutually adjusted in *Model 2*.



Determination of saliva epigenetic age in infancy,
and its association with parental socioeconomic characteristics
and pregnancy outcomes

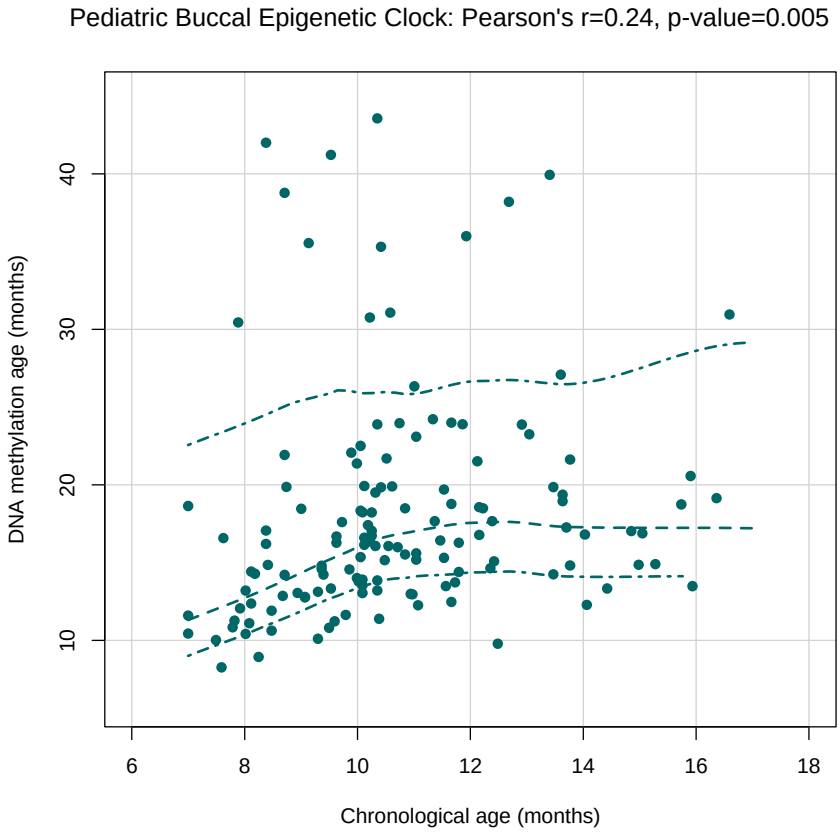
Supplementary Material

Supplementary Table S1. Chronological age and the predicted pediatric buccal epigenetic age (PedBE clock) in the NINFEA sample (N=139; two samples with more than 4SD in PedBE clock were removed)

Epigenetic and chronological age measures	Mean (SD)	Range	
Chronological age (months)	10.8 (2.2)	7.0, 17.0	
Pediatric buccal epigenetic age (months)	18.3 (7.6)	8.3, 47.2	
Correlation between epigenetic and chronological age	Pearson r	p-value	
Pediatric buccal epigenetic age (months)	0.24	0.005	
Difference between epigenetic and chronological age	Mean	Range	
Pediatric buccal epigenetic age (months)			
Difference (months)	7.5 (7.4)	-2.7, 33.6	
Absolute difference (months)	7.6 (7.2)	0.1, 33.6	
Regression epigenetic age ~ chronological age	Coef.	s.e.	R^2
Pediatric buccal epigenetic age (months)			
Epigenetic age ~ chronological age	0.82	0.29	0.05
Epigenetic age ~ chronological age + chip	1.00	0.32	0.04
Epigenetic age ~ chronological age + chip + cell type 1 + cell	0.58	0.11	0.89

SD – Standard deviation
Pearson r – Pearson correlation coefficient
Coef. – Regression Coefficient
s.e. – Standard error
R^2 – Coefficient of determination

Supplementary Figure S1. Scatterplot with smoothed regression line and 95% confidence intervals showing association of chronological age with Pediatric Buccal Epigenetic Clock estimated in infant saliva samples in the NINFEA birth cohort.



Supplementary Figure S2. Scatterplot with smoothed regression line and 95% confidence intervals showing association of Pediatric Buccal Epigenetic Clock estimated in infant saliva samples with the two saliva cell type proportions estimated using Houseman's reference-free method.