Pediatric RESEARCH

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CLINICAL RESEARCH ARTICLE

Hematopoietic cellular aging is not accelerated during the first 2 years of life in children born preterm

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BACKGROUND: Prematurity in itself and exposure to neonatal intensive care triggers inflammatory processes and oxidative stress, leading to risk for disease later in life. The effects on cellular aging processes are incompletely understood.
METHODS: Relative telomere length (RTL) was measured by qPCR in this longitudinal cohort study with blood samples taken at birth and at 2 years of age from 60 children (16 preterm and 44 term). Viral respiratory infections the first year were evaluated. Epigenetic biological DNA methylation (DNAm) age was predicted based on methylation array data in 23 children (11 preterm and 12 term). RTL change/year and DNAm age change/year was compared in preterm and term during the 2 first years of life.
RESULTS: Preterm infants had longer telomeres than term born at birth and at 2 years of age, but no difference in telomere attrition rate could be detected. Predicted epigenetic DNAm age was younger in preterm infants, but rate of DNAm aging was similar in both groups.
CONCLUSIONS: Despite early exposure to risk factors for accelerated cellular aging, children born preterm exhibited preserved telomeres. Stress during the neonatal intensive care period did not reflect accelerated epigenetic DNAm aging. Early-life aging was not explained by preterm birth.

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IMPACT:

- Preterm birth is associated with elevated disease risk later in life.
- Preterm children often suffer from inflammation early in life.
- Stress-related telomere erosion during neonatal intensive care has been proposed.
- Inflammation-accelerated biological aging in preterm is unknown.
- We find no accelerated aging due to prematurity or infections during the first 2 years of life.

INTRODUCTION

Preterm birth inevitably involves varying degrees of immaturity in organ structure, function, and defense systems. The neonatal period following preterm birth exposes the newborn infant to oxidative stress, simply because the extra-uterine environment is comparably rich in oxygen and the antioxidant response is still incomplete. In addition, neonatal intensive care often includes treatments and risks, such as mechanical ventilation, oxygen therapy, and systemic infections that may trigger inflammatory responses and further aggravate the oxidative stress.

Inflammation and oxidative stress are major causative factors in several of the most severe neonatal complications,[1] including bronchopulmonary dysplasia (BPD), retinopathy of prematurity (ROP), and necrotizing enterocolitis (NEC). Long-term consequences are less well characterized. Today, most preterm infants survive and growing up after preterm birth carries the risk of increased susceptibility for later diseases, such as cardiovascular dysfunction[2] and chronic lung disease,[3] and possibly a shorter life expectancy.[4] The short- and long-term effects of preterm birth on cellular aging processes are largely unknown.

Telomere length, a marker of cellular replicative history, is negatively influenced by infections, inflammation, and oxidative stress,[5,6] and there is an aging process due to oxidative stress starting already in the perinatal period in life.[7,8] Diseases such as chronic lung disease, cardiovascular disease, diabetes mellitus, and Alzheimer's disease are oxidative stress related and have been associated with shorter leukocyte telomeres.[9] Telomere attrition can be slowed down by activation of the enzyme telomerase, adding telomeric repeats to the telomere ends, and telomerase activity is highly regulated and not expressed in most somatic cells.[10]

Children have a faster telomere attrition rate compared to adults, shortening with 1000 base pair per year (bp/year) up to 4 years of age compared to 30 bp/year in adults.[11] In preterm infants, an even faster telomere attrition rate has been reported, with a shortening of 230 bp/week.[12,13] A faster telomere attrition rate for males compared to females have also been observed.[14] The faster telomere attrition rate measured in blood cells during childhood may be explained by the active cell expansion and renewal of the hematopoietic precursors.[13] In addition, the white

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blood cell composition changes during life, especially in infancy,[15] which may influence cellular aging measurements made on whole blood. One challenge is to achieve longitudinal studies in newborn infants, especially if born preterm, due to the need of repeated blood sampling over a short period of time in infants already at risk of being compromised and with a low circulating total blood volume. A study of preterm baboons showed rapid telomere attrition rate during the first year of life and measured in parallel the turnover of hematopoietic stem cell types and found longer telomere length in T cells compared to granulocytes and B cells at birth. This difference disappeared at 2 years of age. They concluded that there was a biphasic pattern of stem cell turnover with an initially rapid cell division that declined during the second year of life and similarly a rapid telomere attrition during the first year of life that subsequently slowed down.[16]

Infants born preterm are reported to have longer telomeres at birth than those born term;[12,17,18] however, being born smaller than expected for the gestational age (small for gestational age (SGA)) was not associated with shorter telomere length at birth.[19] Data on the development of telomere length over time in newborn infants, telomere attrition, is scarce, especially for preterm infants. Cross-sectional measurements in adolescents show similar telomere length for preterm born and healthy term born children, with longer telomeres in girls irrespective of prematurity and a negative correlation to lung function, that could not be explained by perinatal events.[3] We have previously reported a study of 10-year-old preterm born children with a history of BPD compared to a group of term born children with asthma showing that the strongest predictors for short telomeres at age 10 years were impaired lung function and male sex, rather than prematurity alone.[20]

Epigenetic modifications are dynamic cellular responses to the environment that can silence or activate genes without altering the DNA sequence. DNA methylation (DNAm) is one epigenetic mechanism where a methyl group is added to a carbon atom on a cytosine followed by a guanine (called CpG sites). DNAm is not only an important regulator of cell function and development and for maintaining chromosome stability but can also function as a marker of cellular aging (epigenetic DNAm age).[21] Understanding the role of hypermethylated or hypomethylated genomic regions in disease development and progression in relation to prematurity is a rapidly growing field of research, mainly in relation to stressful exposures during neonatal intensive care, and was recently reviewed by Casavant et al.[22]

It is important to further elucidate telomere biology in relation to preterm birth as it may provide both prognostic markers and effect measures for future disease. We hypothesize that telomere attrition rate would be accelerated in children born preterm and that this would be reflected in faster biological aging. Therefore, the objective of this cohort study was to longitudinally investigate leukocyte cellular aging measured by telomere length and attrition rate and estimate epigenetic biological DNAm age during the first 2 years of life in relation to preterm birth, perinatal factors, and postnatal viral infections.

METHODS

Study protocol

The study was based on a subgroup of preterm born children ($n = 16$) and term born controls ($n = 44$) with longitudinal blood samples identified from a larger longitudinal case–control study of lung function development during the first 2 years of life (the LUFT study, a follow-up study of lung function in preterm and term born infants and the impact of viral infections during the first year of life). Eligible for inclusion in the LUFT study were infants born preterm (before gestational week 36^{+6}) during January 2009 to March 2011 and admitted to one neonatal unit at Karolinska University Hospital, Stockholm. Exclusion criteria were children with major malformations or neurological impairment preventing later lung function testing. The control group were healthy children born at term (gestational week 37^{+0}–41^{+6}) on the same day as the index child and matched for sex and maternal smoking. The protocol included blood sampling at two time points: cord blood at birth and peripheral blood at follow-up at 2 years of age. Respiratory infections during the first year of life were monitored. Parents kept a diary and were instructed to report to the hospital for clinical evaluation when their child showed symptoms of infection. Nasopharyngeal sampling for viral infections (adenovirus, influenza A and B, parainfluenza 1, 2, and 3, respiratory syncytial virus, metapneumovirus, coronavirus (Oc 43, 229 E, NL 63, HKU1), enterovirus, rhinovirus, mycoplasma pneumoniae, and chlamydia pneumoniae) was performed at every infection episode and analyzed by routine PCR methods at Karolinska University Hospital Laboratory. Episodes of wheeze and hospital admissions for bronchiolitis were reported.

Inclusion criteria for the telomere study were available longitudinal samples, i.e., blood samples from both time points, birth and follow-up, which resulted in 60 infants of the 197 infants with any available blood sample included in the LUFT study. Analyses performed were (1) telomere length measurements in all samples at birth and at follow-up, (2) telomere attrition rate in all cases, and (3) DNAm analysis in a smaller selected group, Fig. 1. For the selection of the smaller group to analyze epigenetic DNAm age ($n = 23$), we initially included matched pairs (10 preterm and term individuals). Owing to difficulties in getting longitudinal samples and enough amount of extracted DNA, we included an additional 13 individuals, chosen by the investigators to balance the groups for sex, parity, quarter of birth, mode of delivery, and chronological age at follow-up.

Perinatal information and neonatal morbidity data were retrieved from medical records. Perinatal data included obstetric and maternal characteristics (premature rupture of membrane, antenatal steroids, mode of delivery, breech position, parity, small for gestational age, gestational age at birth, maternal age, and smoking), neonatal morbidity (days of oxygen treatment, mechanical ventilation, days on continuous positive pressure support, surfactant treatment, patent ductus arteriosus requiring

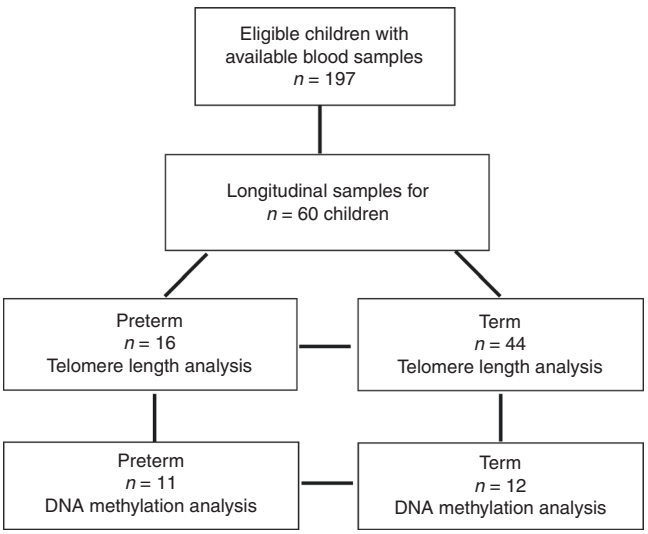

Fig. 1 The study cohort. The analyzed subgroups of children originate from a larger study cohort of lung function development during the first 2 years of life including preterm born children and matched term born children at Karolinska University Hospital during a 26-month period, from 2009 and 2011. Children with longitudinal blood samples available from both birth and 2 years of age were eligible for inclusion in the telomere and cellular aging analyses.

treatment, NEC, and intraventricular hemorrhage), and neonatal outcome (grading of BPD, ROP, and postnatal growth). Anthropometric data included postnatal growth measurements using weight and length at birth and at the follow-up to calculate body mass index (BMI) and age-specific *z*-scores according to World Health Organization.[23]

No sample size calculations were performed because of the predefined study cohort. Lung function data are not reported here.

Ethics

Parental written informed consent was obtained. The Regional Ethical Review Board in Stockholm granted approval for the LUFT study (Dnr 2008/1896-31/4, Dnr 2009/625-32, Dnr 2010/1747-32, and Dnr 2011/402-32) and the add-on protocol for telomere analyses and DNAm (Dnr 2018/780-32 2018-05-21).

Measurements of telomere length

Whole blood (2–3 ml) was collected in a vial containing ethylenediaminetetra acetic acid (EDTA) by puncture of the umbilical cord at birth and by venous puncture at follow-up and frozen at −80 °C within 1 h. Genomic DNA was extracted manually according to the manufacturer's instructions with Puregene DNA Purification kits (Genta Systems, Inc., now known as Qiagen, Gaithersburg, MD, USA) based on the salt precipitation method, and after the lysis of red and white blood cells, the process was finished automatically using the Autopure LS instrument (Qiagen, MD, USA). DNA was dissolved in 200 µl of 10 mM Tris buffer containing 1 mM EDTA, pH 7.0–8.0. DNA yield and purity were determined spectrophotometrically using the NanoDrop (Thermo Fisher Scientific Inc., DE, USA).

Relative telomere length (RTL) was determined by quantitative PCR according to the method described by Cawthon et al.,[24] with minor modifications. Briefly, each DNA sample was analyzed in triplicate wells in separate Telomere (TEL) and single copy gene (hemoglobin subunit beta (HBB) Gene ID: 3043) reactions on the ABI7900HT instrument (Applied Biosystems) at two separate time points. Each reaction contained: 17.5 ng DNA, 1× PCR buffer II (Applied Biosystems), 150 nM Rox (Molecular Probes), 0.2× SYBR Green (Roche Diagnostics GmbH), 1% dimethyl sulfoxide, 0.2 mM of each dNTP, 1.25 U AmpliTaq Gold DNA Polymerase (Applied Biosystems), 1.7 mM MgCl/2.5 mM dithiothreitol (DTT; TEL reaction) or 2.5 mM MgCl/5 mM DTT (HBB reaction), and TEL/HBB primers (TEL 1b, 100 nM: CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT and TEL 2b, 900 nM: GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT, or HBB3, 400 nM: TGTGCTGGCCCATCACTTTG and HBB4, 400 nM: ACCAGCCACCACTTTCTGATAGG). The cycling conditions were 95 °C for 10 min and 25 cycles at 95 °C for 15 s and 54 °C for 1 min (TEL) or 35 cycles at 95 °C for 15 s and 56 °C for 1 min (HBB). TEL/HBB (*T/S*) values were calculated by the $2^{-\Delta Ct}$ method, where $\Delta Ct = Ct_{TEL} - Ct_{HBB}$. The RTL value for each sample was generated by dividing the sample's *T/S* value with the *T/S* value of a reference cell line DNA (CCRF-CEM) included in all runs. A standard curve generated by the reference cell line DNA was also included in each run to monitor PCR efficiency. The mean inter-assay coefficient of variation for RTL measurements in our laboratory is between 4% and 8%.

Epigenetically estimated biological DNAm age

High-density arrays covering 485577 CpG sites (HumMeth450K, Illumina, San Diego, USA) were used for genome-wide methylation analysis. Briefly, 500 ng DNA bisulfite converted by the EZ Methylation Gold Kit (Zymo Research, Irvine, USA) and arrays were operated according to the manufacturer's instruction. To each array, 200 ng of bisulfite-converted DNA was applied, and the arrays were scanned with a HiScan array reader (Illumina). The fluorescence intensities were extracted using the Methylation module (1.9.0) in the Genome Studio software (V2011.1). The quality of each individual array was evaluated with built-in controls.

Methylation data were pre-processed and normalized by the BMIQ method as previously described[25] using R (v2.15.0). The methylation levels (i.e., the β value) of each CpG site ranges from 0, corresponding to completely unmethylated DNA, to 1, representing fully methylated DNA.

The epigenetic DNAm age was calculated based on 353 CpG sites "epigenetic clock" prediction model described by Horvath.[21] Chronological age, uncorrected postnatal days of life, was used because this is the time the infants have been exposed to the environment that might affect cellular aging for factors we can control for in this study. Delta age was determined by subtracting the chronological age from the estimated epigenetic DNAm age at each measuring point. DNAm aging rate/year, or epigenetic aging, correspond to (DNAm age at follow-up − DNAm age at baseline)/(chronological age at follow-up). In our study, baseline was birth and therefore represents age 0 years.

Statistical analyses

Differences between preterm and term children were analyzed with *t* tests for numeric variables and Fisher's exact tests for the categorical variables. The normality of the distribution of the numeric variables was tested with Shapiro–Wilk's tests. We fail to reject normality distribution in all numeric variables, except for RTL at birth which showed a *p* value of 0.04. After adjusting for the multiple comparisons performed, however, this was not significant. Changes in mean RTL were evaluated with univariable linear regressions. We considered the following dependent variables in separate models: maternal age, premature rupture of membrane, parity, mode of delivery, birth weight, BMI, sex, postnatal viral infections, and neonatal lung morbidity in the preterm group. RTL was the dependent variable in all models. Figures 2a and 3a show the scatterplot of the data and the interpolating linear regression lines in preterm and term children. Figures 2b and 3b show the boxplots of cellular aging rates. Statistical significance was set at 0.05. The analyses were performed using Stata version 13 (StataCorp, College Station, TX, USA) and SPSS version 24 (IBM corporation, Armonk, NY, USA).

RESULTS

Clinical characteristics

In this longitudinal cohort study, we included 60 children, 16 infants born preterm (mean gestational age of 31^{+6} weeks and birth weight 1894 ± 339 g) and 44 infants born term (40^{+0} weeks and birth weight 3517 ± 449 g), Table 1. No infants were born SGA. Ethnicity, maternal age, and smoking habits did not differ between groups; noteworthy is that only two mothers in the cohort were smokers (Supplementary Table S1). Perinatal characteristics are described in Table 1 and Supplementary Table S1. At the follow-up time point, preterm infants were significantly older (chronological age) than term born children with a mean postnatal age of 25.6 months (range 22–32) compared to 22.6 months in term infants (range 18–28), Table 1B.

Growth

Preterm born infants were significantly smaller at birth with an expected difference in weight and BMI *z*-score, which was no longer seen at 2 years of age. The growth velocity was significantly higher in preterm born infants compared to term born (100 ± 10 g/week versus 90 ± 10 g/week, $p < 0.001$), reflecting an expected postnatal catch-up growth, Table 1B.

Neonatal morbidity

Neonatal morbidity was low in this rather mature preterm cohort. Only 1 out of 16 preterm infants had mild BPD defined as a need for oxygen treatment at 28 days of life, but none required any

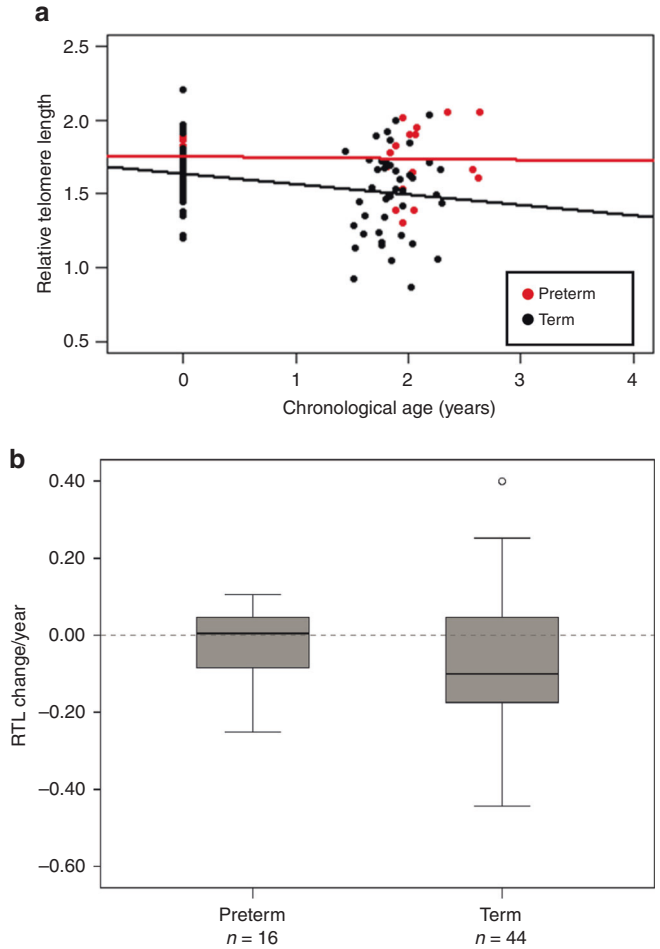

Fig. 2 Telomere length in preterm and term born infants. **a** Relative telomere length (RTL) at birth and at 2 years age. **b** RTL change/year during the first 2 years of life. $p = 0.145$ (t test).

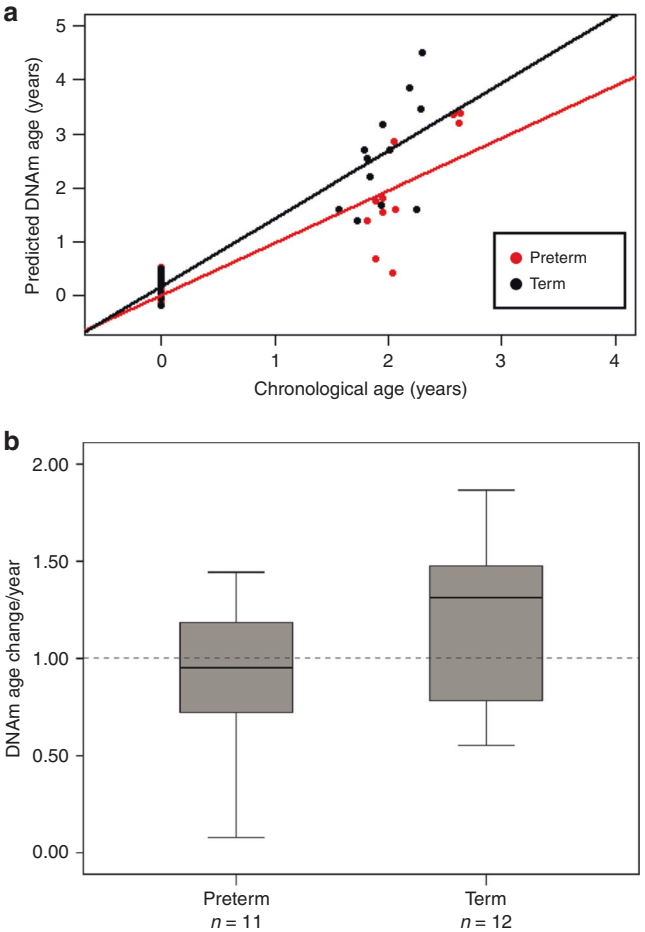

Fig. 3 Epigenetic aging in preterm and term born infants. **a** Predicted biological epigenetic DNAm age at birth and at 2 years of age. **b** Predicted DNAm age change/year during the first 2 years of life. $p = 0.075$ (t test).

oxygen or ventilatory support at 36 weeks of gestational age. Continuous positive airway pressure was needed for a mean of 4 days (range 0–27), and none required mechanical ventilation. Oxygen was given to 6 infants (38%) for, on average, 4 days (maximum 28 days), and surfactant treatment was given to 3 infants (19%). No infant had intraventricular hemorrhage grade 3–4 or needed surgery for NEC, patent ductus arteriosus, or ROP (Supplementary Table S1).

Telomere length

Preterm infants had significantly longer RTL compared to term infants both at birth (1.76 ± 0.1 versus 1.64 ± 0.2, $p < 0.05$) and at 2 years of age (1.73 ± 0.2 versus 1.50 ± 0.3, $p < 0.01$), Table 2 and Fig. 2a. There was no sex effect within each group (preterm or term) comparing females versus males. When comparing groups, preterm females had significantly longer telomeres than term females at birth ($p < 0.05$). At 2 years of age, preterm male sex had significantly longer telomeres than term males ($p < 0.01$), Table 2. RTL did not correlate to perinatal factors, such as maternal age, premature rupture of membrane, parity, mode of delivery, birth weight, or sex (data not shown).

Telomere attrition rate

Telomere attrition rate per year ((RTL at follow-up − RTL at partus)/chronological age at follow-up in years) did not significantly differ between preterm and term born infants during the first 2 years of life as shown in Fig. 2b. We calculated telomere attrition per year (telomere attrition/postnatal age) and compared by group level and sex, Table 2. Children with male sex in the term group had accelerated telomere attrition per year compared to preterm born male infants (0.01 ± 0.1 in preterm versus -0.12 ± 0.2 in term, $p < 0.01$), a difference that was not seen with female sex. Telomere attrition rate did not correlate to any perinatal factors or any neonatal respiratory morbidity (data not shown).

Epigenetic DNAm age

Preterm children were generally younger in predicted DNAm age at birth and at 2 years of age but only statistically significant when comparing delta age (-0.13 ± 0.8 years for preterm infants compared to 0.65 ± 0.9 years in term, $p < 0.03$) at 2 years of life, shown in Fig. 3a and Table 3. Predicted DNAm age did not correlate with sex or neonatal respiratory morbidity. Epigenetic DNAm aging showed a tendency to be slower in the preterm group (0.87 ± 0.4 years versus 1.2 ± 0.4 years, $p = 0.075$) but did not reach statistical significance, Fig. 3b. We found no correlation between epigenetic aging and telomere attrition rate in preterm and term born infants during the first 2 years in life, Fig. 4.

Cellular aging and infections

The exposure of respiratory viral infections during the first year of life was similar for children in both groups (94% in preterm versus 86% in term born infants). Preterm born infants required more often hospital admissions and had more bronchiolitis compared to

Table 1. Characteristics, perinatal data, and respiratory viral infections during the 2 first years of life in 60 infants.

	Preterm, $n = 16$	Term, $n = 44$
(A) Birth		
Gestational age, weeks^{+days} (range)	31^{+6} (27^{+6}–33^{+5})[a]	40^{+0} (37^{+6}–42^{+3})[a]
Birth weight, g	1894 ± 339[a]	3517 ± 449[a]
BMI *z*-score	−2.7 ± 1.2[a]	0.3 ± 1.0[a]
Male gender, *n* (%)	9 (56)	25 (57)
Maternal age, years	34 ± 4	32 ± 5
Premature rupture of membrane, *n* (%)	3 (19)	1 (2)
Cesarean section, *n* (%)	7 (44)	8 (18)
(B) Follow-up at 2 years of age		
Chronological age, months	25.6 ± 3[a]	22.6 ± 3[a]
Weight, kg	13.0 ± 1	12.4 ± 1
BMI *z*-score	1.0 ± 1	0.6 ± 1
Postnatal growth per week, kg	0.10 ± 0.01[b]	0.09 ± 0.01[b]
Experienced one/several infections during the first year of life, *n* (%)	15 (94)	38 (86)
Bronchiolitis/admittance to hospital, *n* (%)	6 (38)[b]	2 (5)[b]

Mean values ± SD.
BMI body mass index.
[a]Significance level $p < 0.01$.
[b]Significance level $p < 0.05$.

Table 2. Relative telomere length (RTL) and telomere attrition rate in longitudinal samples from birth and follow-up at 2 years of age in preterm and term born infants.

	Preterm, $n = 16$	Term, $n = 44$
RTL at partus		
All	1.76 ± 0.1[a]	1.64 ± 0.2[a]
Male	1.74 ± 0.1	1.66 ± 0.2
Female	1.80 ± 0.1[a]	1.61 ± 0.2[a]
RTL at 2 years of age		
All	1.73 ± 0.2[b]	1.50 ± 0.3[b]
Male	1.76 ± 0.2[b]	1.45 ± 0.3[b]
Female	1.70 ± 0.3	1.57 ± 0.3
RTL attrition rate/year[c]		
All	−0.02 ± 0.1	−0.08 ± 0.2
Male	0.01 ± 0.06[b]	−0.12 ± 0.2[b]
Female	−0.05 ± 0.14	−0.02 ± 0.15

Mean values ± SD.
[a]Significance level $p < 0.05$.
[b]Significance level $p < 0.01$.
[c]RTL attrition rate = (RTL at follow-up − RTL at birth)/(chronological age at follow-up in years).

the control group (38% versus 5%, $p < 0.01$), Table 1B and Supplementary Table S2. Since the burden of viral infection was high in both the preterm and term groups, we are not able to analyze cellular aging in relation to infections.

RTL was longer (1.6 ± 0.3 versus 1.3 ± 0.3, $p < 0.05$) and telomere attrition rate slower (−0.05 ± 0.2 versus −0.17 ± 0.2, $p = 0.06$), in the group of infants exposed to viral infections during the first year of life (Supplementary Table S2). Correlations between epigenetic DNAm age and viral infections could not be analyzed as the majority of children had one or more infections during the first year of life (Supplementary Table S2).

Table 3. Predicted epigenetic biological DNAm age[a] at birth and at 2 years of age in preterm and term born infants.

	Preterm, $n = 11$	Term, $n = 12$
Predicted DNAm age at partus (years)	0.08 ± 0.2	0.23 ± 0.2
Predicted DNAm age at 2 years of age (years)	2.0 ± 1.0	2.6 ± 1.0
Delta DNAm age at partus	0.25 ± 0.2	0.23 ± 0.2
Delta DNAm age at 2 years of age	−0.13 ± 0.8[b]	0.65 ± 0.9[b]
Epigenetic DNAm aging[c]/year	0.87 ± 0.4	1.20 ± 0.4

Mean ± SD.
[a]According to Horvath.
[b]Significance level $p < 0.05$.
[c]Epigenetic DNAm aging/year = (predicted DNAm age at follow-up − predicted DNAm age at birth)/(chronological age at follow-up in years).

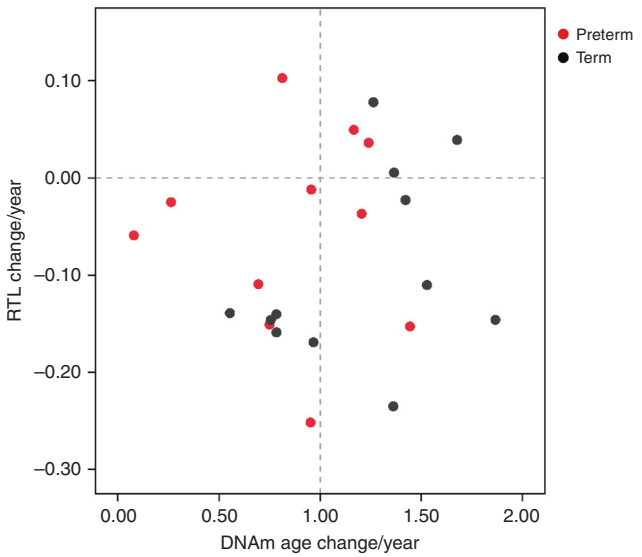

Fig. 4 Correlation telomere attrition and epigenetic aging in preterm and term born infants. RTL change/year versus predicted DNAm age change/year during the first 2 years of life.

DISCUSSION

This is the first study to evaluate hematopoietic biological aging early in life in a longitudinal cohort using both telomere length and predicted epigenetic aging. Contrary to our hypothesis, we were not able to detect a faster telomere attrition rate in children following preterm birth compared to those born at term. Preterm children were generally younger in predicted epigenetic DNAm age and term born infants showed a tendency to an accelerated epigenetic DNAm aging at 2 years of age. The results are reassuring and suggest that preterm infants, despite susceptibility to oxidative stress damage and exposure to more severe viral infections during the first year of life, do not exhibit accelerated cellular aging.

Interestingly, we found that exposure of viral infection during the first year of life resulted in significantly longer telomeres at 2 years of age and a trend toward slower telomere attrition rate (Supplementary Table S2). Respiratory infections during the first year were common in both preterm and term born children (overall 88%), although more severe in the preterm group with more bronchiolitis or hospital admissions. The hypothesis that infections will increase oxidative stress and thereby result in faster telomere attrition could not be confirmed in our cohort. As previously reported, preterm children in our study had longer

telomeres compared to term born children[17,26] both at birth and at 2 years of age. The longer telomeres at 2 years of age in children with infections found in our study could not be explained by preterm having more viral infections during the first year of life since preterm and term born children were exposed to infections to the same extent (Supplementary Table S2).

Another reason explaining preserved telomeres could be that we measured telomere length in a different subset of white blood cells, in which the cell composition had been changed after an infection. Telomere length is known to be longer in naive T lymphocytes compared to granulocytes[27] but not in memory T and B cells.[28] Rufer et al. showed that telomere attrition rate was faster in lymphocytes compared to granulocytes in 500 individuals between 0 and 90 years of age suggesting that a rapid hematopoietic turnover in early childhood results in increased telomere attrition and then a shift with aging from naive to memory T cells[29] and thereby a slower attrition rate. In a recent publication by Olin et al. we showed that preterm infants have higher levels of CD4+ T cells and lower levels of neutrophils compared to term born infants at birth but these differences normalize early in life.[30] We still do not know exactly how cell composition develops and affect telomere length and within what time frame from a viral infection a change in telomere length could be detected. Therefore, we cannot be sure if this will reflect how viral infections during the first year of life will affect telomere length at 2 years of age that we measured in our cohort.

Provenzi et al. suggested stress-related telomere erosion in children needing neonatal intensive care.[18] Our cohort of sixteen preterm infants was rather mature and had little neonatal morbidity. Standard of care for extremely preterm born infants in Sweden include management for pain relief, parental involvement with both skin-to-skin care and supporting parental–infant bonding from the moment of birth as well as having the parents stay at the ward together with their infants around the clock, from admission to discharge from the hospital. Breastfeeding is also promoted and all infants born preterm in our cohort received bank milk if mother's own milk was not available. More than half of the infants born term were exclusively breastfed at 4 months of chronological age. Wojcicki et al. showed in 4-year-old children that exclusive breastfeeding at 1 month of age was correlated to longer telomeres.[31] In our study, breast milk was highly available for both term and preterm born infants early in life and do not explain the longer telomeres we found in preterm infants. Wojcicki et al. also measured change in telomere length from birth to 2.4 years of age with a yearly change of -0.08 ± 0.6,[32] which correspond to the same telomere attrition rate for term infants that we found in our study. Despite spending 5–10 weeks after birth at the hospital and being exposed to more severe respiratory tract infections during the first year of life, preterm infants in our cohort had longer telomeres than term born infants and cellular aging was not accelerated. The low neonatal morbidity and very close parental involvement might elude some of that stress-related telomere erosion described by Provenzi et al. in infants in need of neonatal intensive care.

Many other types of telomere erosion might be in play with oxidative stress, infections, and inflammation that together with the cell turnover affects telomere attrition rate; however, these might not be as important to cellular aging measured as DNAm.[33] In our study, we could not find any strong correlation between telomere length attrition and biological aging measured by epigenetics (Fig. 4), which may imply that there are different processes involved in the cellular aging process.

Telomerase activity can also affect telomere length, and we know from Svenson et al. that telomere length is a dynamic feature later on in life,[34] although exactly how this is regulated is still not known.[28] Telomerase counteracts telomere attrition, but Ahmed et al.[35] proposed an interesting mechanistic thought that the catalytic subunit of human telomerase, TERT, also protects the mitochondria from oxidative stress-induced damage in a telomere-independent way, later verified by Haendeler et al.[36] This underlines the importance of studying telomere biology in newborn infants and account for the aspects of both telomere erosion and telomere elongation. In our study, we are limited to longitudinal telomere lengths and thereby telomere attrition but can only speculate on possible explanations for not seeing that increased telomere attrition rate that we expected due to prematurity.

Epigenetic DNAm age was younger in preterm born compared to term born infants at 2 years of age. The biological age of an individual can be estimated by DNAm analysis and we used the 353 CpG sites "epigenetic clock" prediction model described by Horvath to determine the biological epigenetic (DNAm) age of the blood samples.[21] The predicted age model has an error of 3.9 years, and our significant difference lies within that margin. Despite that, we think the evidence in our study suggests that preterm infants do not have accelerated cellular aging. We used two methods to measure cellular aging based on telomere attrition and biological aging measured by epigenetics and we found no evidence for faster hematopoietic cellular aging in preterm born infants compared to term during the first 2 years of life.

Neonatal DNAm profiles are not explained by genetic factors but alter in response to interactions with the environment[37] and can play a role in the regulation of leukocyte function.[38] In a study by de Doede et al.,[15] both cell composition and DNAm were analyzed in cord blood of five term and five preterm infants showing that granulocytes and T cells are abundant but with great inter-individual variability and differences in DNAm changes might not be associated with prematurity but differences in cell composition. However, in that study all infants were born by cesarean section. Schlinzig et al. described methylation variation due to the mode of delivery where children born with cesarean section showed increased DNAm, but that decreased within a week.[39] We have no knowledge of the cell composition at birth or at 2 years of age in our cohort and can therefore not evaluate differences in cell composition due to the exposure of viral infections the first year after birth or the impact of that exposure and prematurity on the DNAm profiles.

In our study, the samples at birth come from cord blood in contrast to venous blood samples at 2 years of age. We know from Olin et al. that cell composition in cord blood does not correlate with postnatal cell composition due to drastic changes induced at birth,[30] and how much the different sources of blood affect DNAm profiles and whether it will affect DNAm age is unknown. Wang et al.[40] reported the same level of inter-individual variability in DNAm for both sex in a longitudinal study of 105 infants with paired blood samples from the umbilical cord and a venous blood sample within the first 2 years of life. For the DNAm analysis of 23 preterm and term infants in our study, we aimed for performing paired analysis with one preterm and one term born matched for day of birth, sex, and maternal smoking, but due to difficulties in achieving true matched pairs, we were only able to do analysis on a group level and due to small sample size sex effects could not be studied.

One major limitation of our study is the low sample size with longitudinal blood samples. It was difficult recruiting preterm infants at birth, as infants are born round the clock, often preventing consent from parents in a stressful situation. Owing to the low sample size, our analyses of telomere length and DNAm age were based on differences on a group level comparing preterm with term born children. We also had a rather mature and healthy group of preterm infants and could therefore not draw any conclusions of neonatal morbidity and neonatal outcomes.

In this longitudinal cohort study, we used two different measurements to evaluate cellular aging the first 2 years in life in relation to prematurity and exposure of respiratory viral infections: telomere attrition rate using measurements of RTL at birth and at 2 years of age and epigenetic aging using predicted biological DNAm age. We could not establish any clear sex effects, but term born male infants had an accelerated telomere

shortening during the first 2 years of life irrespectively of infections, maternal age, and growth.

To conclude, despite early exposure to risk factors for accelerated aging, children born preterm exhibited preserved telomeres and did not show accelerated epigenetic DNAm aging. The stress of neonatal intensive care and the added stress following exposure to respiratory viral infections during the first year of life did not lead to accelerated biological aging. Cellular aging is faster early in life compared to later in childhood or in adulthood, and we still do not fully understand why. With a better comprehension of how postnatal exposures affect newborn infants, particularly if compromised by being born preterm, the neonatal period in life can serve as a "window of opportunity" to affect causes that lead to accelerated telomere attrition and epigenetic changes with the potential to prevent disease development later in life.

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AUTHOR CONTRIBUTIONS

Conceptualization and writing—original draft, E.H., K.B., S.D.; data acquisition, analysis, and/or interpretation, writing—review and editing, and final approval: all authors.

ADDITIONAL INFORMATION

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