Dysregulated non-coding telomerase RNA component and associated exonuclease XRN1 in leucocytes from women developing preeclampsia—possible link to enhanced senescence

Tove Lekva1,2,*, Marie Cecilie Paasche Roland3,4, Mette E. Estensen4, Errol R. Norwitz6, Tamara Tilburgs7,8, Tore Henriksen2,5, Jens Bollerslev5,9, Kjersti R. Normann5,9, Per Magnus10, Ole Kristoffer Olstad11, Pål Aukrust1,5,12,13,14 & Thor Ueland1,5,13,14

Senescence in placenta/fetal membranes is a normal phenomenon linked to term parturition. However, excessive senescence which may be induced by telomere attrition, has been associated with preeclampsia (PE). We hypothesized that the telomerase complex in peripheral blood mononuclear cells (PBMC) and circulating telomere associated senescence markers would be dysregulated in women with PE. We measured long non-coding (nc) RNA telomerase RNA component (TERC) and RNAs involved in the maturation of TERC in PBMC, and the expression of TERC and 5'-3' Exoribonuclease 1 (XRN1) in extracellular vesicles at 22–24 weeks, 36–38 weeks and, 5-year follow-up in controls and PE. We also measured telomere length at 22–24 weeks and 5-year follow-up. The circulating senescence markers cathelicidin antimicrobial peptide (CAMP), β-galactosidase, stathmin 1 (STMN1) and chitotriosidase/CHIT1 were measured at 14–16, 22–24, 36–38 weeks and at 5-year follow-up in the STORK study and before delivery and 6 months post-partum in the ACUTE PE study. We found decreased expression of TERC in PBMC early in pregnant women who subsequently developed PE. XRN1 involved in the maturation of TERC was also reduced in pregnancy and 5-year follow-up. Further, we found that the senescence markers CAMP and β-galactosidase were increased in PE pregnancies, and CAMP remained higher at 5-year follow-up. β-galactosidase was associated with atherogenic lipid ratios during pregnancy and at 5-year follow-up, in PE particularly. This study suggests a potential involvement of dysfunctional telomerase biology in the pathophysiology of PE, which is not restricted to the placenta.

Pregnancy is a state of oxidative stress as the higher metabolic demand of the growing fetus results in increased reactive oxygen species production1,2. Oxidative stress is implicated in the pathophysiology of many reproductive

---

1Research Institute of Internal Medicine, Oslo University Hospital, Rikshospitalet, Oslo, Norway. 2Department of Obstetrics, Oslo University Hospital, Rikshospitalet, Oslo, Norway. 3National Research Center for Women's Health, Oslo University Hospital, Oslo, Norway. 4Department of Cardiology, Oslo University Hospital, Rikshospitalet, Oslo, Norway. 5Faculty of Medicine, University of Oslo, Oslo, Norway. 6Newton-Wellesley Hospital, Boston, MA, USA. 7Division of Immunobiology, Center of Inflammation and Tolerance, Cincinnati, OH, USA. 8Department of Pediatrics, University of Cincinnati College of Medicine, Cincinnati, OH, USA. 9Section of Specialized Endocrinology, Department of Endocrinology, Oslo University Hospital, Rikshospitalet, Oslo, Norway. 10Centre for Fertility and Health, Norwegian Institute of Public Health, Oslo, Norway. 11The Blood Cell Research Group, Department of Medical Biochemistry, Oslo University Hospital, Ullevål, Oslo, Norway. 12Section of Clinical Immunology and Infectious Diseases, Oslo University Hospital, Rikshospitalet, Oslo, Norway. 13K.G. Jebsen Inflammatory Research Center, University of Oslo, Oslo, Norway. 14K. G. Jebsen Thrombosis Research and Expertise Center, University of Tromsø, Tromsø, Norway. *email: tove.lekva@ous-research.no
complications including infertility, miscarriage and pre-eclampsia (PE), but the mechanisms of these complications are not fully elucidated. Cellular senescence, a condition in which a cell no longer has the ability to proliferate, can be activated by intrinsic and extrinsic factors like oxidative stress, inflammation, DNA damage and epigenetic stress. The increased senescence may be induced by telomere attrition (shortening), but also by long uncapped and dysfunctional telomeres, which have been associated with pregnancy complications, including PE.

Telomere length is regulated by the enzyme telomerase that adds telomeric repeats to the ends of the chromosomes. Telomerase is a ribonucleoprotein complex with a catalytic core composed of telomerase reverse transcriptase (TERT) and the non-coding RNA (ncRNA) TERC. Regulation of telomerase activity occurs through the control of TERT transcription and through a post-transcriptional maturation process of the 3' end of TERC, which determines circulating levels of mature TERC and mature telomerase complex. TERC transcripts undergo a multistep process of maturation that includes cycles of adenylation and de-adenylation, which together control steady state circulating levels of TERC. These mechanisms are crucial for the regulation of telomerase levels, and subsequent telomere length, and are disrupted in many disease states. Shorter telomeres and decreased expression of TERT has been reported in trophoblasts from PE patients, and may reflect a process of accelerated telomere shortening during pregnancy due to increased stress including oxidative stress. A progressive physiological senescence and aging of decidual cells and placental membranes may be important for the onset of labor at term, whereas premature aging related to telomere pathology may lead to PE.

In age-related diseases, telomere shortening is accelerated and has become the proxy parameter for overall poor health. TERC−/− mice with critically short telomeres develop excessive inflammation, oxidative stress, endothelial dysfunction, and hypertension, all features that are typical in PE. Furthermore, shorter leukocyte telomere length is associated with a long-term risk of aging and cardiovascular disease (CVD) and circulating senescence markers of human aging and several diseases associated with telomere shortening have been identified. A meta-analysis of 22 studies with >6.4 million women including 258,000 women with preeclampsia adjusting for confounders identified a fourfold increase in future incident heart failure and a twofold increased risk in coronary heart disease, stroke and death due to CVD. The risk of future CVD is highest associated with early-onset PE.

In a pilot array from 22 to 24 weeks’ gestation in peripheral blood mononuclear cells (PBMC), including lymphocytes, monocytes, natural killer cells and dendritic cells, from women who subsequently developed PE and normotensive controls, we found lower ncRNA TERC in the PE women, suggesting dysfunctional telomerase biology. To further explore these novel data we measured 1) levels of ncRNA TERC and other mRNAs involved in the maturation of TERC in PBMC and extracellular vesicles at different time points during pregnancy, in term placenta, and at 5-year follow-up comparing PE and controls; 2) telomere length during pregnancy and at 5-year follow-up in PE and controls; 3) levels of the senescence markers cathelicidin antimicrobial peptide (CAMP), β-galactosidase, stathmin 1 (STMN1), and chitotriosidase/CHIT1 in two independent cohorts at different time-points during pregnancy and at follow-up comparing PE and controls; and 4) evaluated if these senescence markers were associated with future risk of CVD as evaluated by atherogenic lipid ratios and arterial stiffness 5 years post-partum in women with PE and controls.

Methods

The STORK study is a prospective longitudinal cohort study in which 1031 low-risk women of Scandinavian heritage with singleton pregnancies were followed throughout pregnancy and who gave birth at Oslo University Hospital Rikshospitalet between 2002 and 2008. Exclusion criteria included pre-gestational diabetes and any severe chronic diseases (lung, cardiac, gastrointestinal or renal). Each pregnant woman had four study-related antenatal visits at 14–16, 22–24, 30–32, and 36–38 weeks. The follow-up study was performed 5-years after the index delivery in three hundred women. In the current study women with gestational diabetes mellitus were excluded and we included only normotensive controls that were included in the follow-up study (215 controls, 38 PE in pregnancy and 10 PE at follow-up). In the ACUTE PE study there were 34 PE and 61 control subjects. Blood samples were collected at 25–38 weeks and at 6 months postpartum. The cohorts are presented in Fig. 1, Supplemental File. Measurements of brachial arterial systolic and diastolic blood pressure (BP) were made with an automated oscillometric technique (Dinamap ProCare 300-Monitor, Criticon, GE Medical Systems). Systolic and diastolic BP were assessed as the mean of three recordings. Written informed consent was obtained from all study participants. All clinical investigations were conducted in accordance with the principles enshrined in the Declaration of Helsinki. The study was approved by the Regional Committee for Medical Research Ethics of Southern Norway in Oslo, Norway.

**Preeclampsia.** PE was diagnosed by new-onset BP ≥ 140/90 mmHg and significant proteinuria (urinary total protein/creatinine ratio >30 or +1 on urine dipstick). In the STORK study, all cases (n = 38) were diagnosed after 34 weeks’ gestation (late-onset PE). The ACUTE PE study included cases diagnosed after 34 weeks (late-onset PE [n = 23]) and (early-onset PE [n = 11]).

**Collection, storage and RNA extraction of placental biopsies.** As previously reported, placental biopsies were collected after vaginal or cesarean delivery. Blocks of 2–4 cm were taken from the placental parenchyma, briefly washed in phosphate buffer saline, snap frozen in liquid nitrogen, and stored at −80 °C until RNA isolation. Half of each biopsy was homogenized in TRIzol reagent (Invitrogen, Life Technologies) on ice with a tissue grinder (Sigma Aldrich, St. Louis, MO). Total RNA was extracted using TRizol reagent (Invitrogen, Life Technologies) and purified with RNeasy microkit columns (Qiagen, Netherlands). Purity and concentration of isolated total RNA was measured using Nanodrop ND-1000 Spectrophotometer (Thermo Fisher Scientific Inc.,
USA) and RNA integrity number (RIN) was estimated using Agilent 2100 Bioanalyzer (Agilent Technologies, USA). Placental biopsies available for this study include 13 PE and 107 controls.

**Collection, storage and RNA extraction of PBMC.** In the STORK study, PBMC was isolated from venous blood using BD Vacutainer CPT Tubes (BD, NJ) at weeks 22–24, 36–38, and at 5-year follow-up and stored at −70 °C until extraction. RNA was extracted using Magnapure Isolation Kit and instrument (Roche Life Science, Penzberg, Germany) at weeks 22–24 and with Magmax isolation kit and instrument (Applied Biosystems, Carlsbad, CA) at weeks 36–38 and at follow-up, due to change in instruments at the laboratory over the years, as previously published. RNA available from PBMC in this cohort included 189 controls/32 PE at 22–24 weeks, 194 controls/24 PE at 36–38 weeks and 213 controls/10 PE at 5-year follow-up.

**Microarray and data analysis.** 100 ng of total RNA from PBMC from 4 controls and 5 PE at week 22–24 was subjected to GeneChip HT One-Cycle cDNA Synthesis Kit and GeneChip HT IVT Labeling Kit, following the manufacturer’s protocol for whole genome expression analysis (Affymetrix, Santa Clara, CA). Labeled and fragmented single stranded cDNAs were hybridized to the GeneChip Human Gene 1.0 ST Arrays (28,869 transcripts) (Affymetrix). The arrays were washed and stained using FS-450 fluidics station (Affymetrix). Signal intensities were detected by Hewlett Packard Gene Array Scanner 3000 7G (Hewlett Packard, Palo Alto, CA). The scanned images were processed using Affymetrix GeneChip Command Console (AGCC). The CEL files were imported into Partek Genomics Suite software (Partek, Inc. MO). Robust microarray analysis (RMA) was applied for normalization. Differentially expressed genes between groups were identified using one-way ANOVA analysis. Cluster analysis were generated in Partek Genomics Suite. Further bioinformatics analysis was conducted on the significant genes to identify functional significance by means of Ingenuity Pathways Analysis (Ingenuity Systems, Redwood City, CA).

**Collection, storage and RNA extraction of extracellular vesicles.** From the STORK study, 350 µl plasma from venous blood using BD Vacutainer CPT Tubes (BD, NJ) at weeks 22–24, 36–38, and at postpartum follow-up was used to isolate RNA from extracellular vesicles. The previous unthawed plasma stored at −70 °C until extraction was thawed at room temperature centrifuged at 3000 g for 5 min and 300 µl was used further in the exoRNeasy plasma kit protocol (Qiagen). The spike-in control C. elegans miR-39 miRNA was added to the lysate after adding the Qiazol. We used 35 controls/35 PE at 22–24 weeks, 35 controls/27 PE at 36–38 weeks, and 35 controls/9 PE at 5 years follow-up for this analysis.

**Quantitative real-time polymerase chain reaction.** Reverse transcription was performed using a High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) of RNA from PBMC and miScript II RT Kit Qiagen of RNA from extracellular vesicles. mRNA quantification was performed using SYBR Green PCR Fast Mix (Quantabio, Beverly, MA) for PBMC and miScript SYBR Green PCR Kit Qiagen for extracellular vesicles using the standard curve method on an ABI Prism 7900 (Applied Biosystems). Primers for TERC RT² lncRNA qPCR Assay (LPH26581A) and Ce_miR-39_1 miScript Primer Assay was bought from Qiagen (Netherlands). Sequence specific intron spanning oligonucleotide primers were designed using the Primer Express software version 2.0 (Applied Biosystems) (Table 1, Supplemental File). Transcript expression levels were nor-
malized to β-actin and GAPDH and expressed as relative mRNA levels in PBMC and geometric mean of β-actin, GAPDH, and miR-39 in extracellular vesicles.

**Biochemical analysis.** Peripheral venous blood was drawn in the morning between 07:30 and 08:30 AM after an overnight fast, into tubes with citrate additives in the STORK study and EDTA additives in the ACUTE PE study, centrifuged for 25 min at 3000 g at 4 °C, separated, and stored at −80 °C until analyzed. Fasting plasma levels of CAMP and STMN1 were measured in duplicate by enzyme immuno-assay with antibodies obtained from Mybiosource (San Diego, CA). β-galactosidase and chitotriosidase/CHIT1 were measured in duplicate by fluorescence. Briefly, 20 µl of citrate plasma and standards rhGLB1 (R&D Systems, Minneapolis, MN) diluted in 50 mM sodium citrate (pH 3.5) and 20 ul of 1.2 mM substrate (4-methylumbelliferyl-β-D-galactopyranoside (Sigma-Aldrich) was loaded into 384-well black plates. The plates were incubated in 37 °C for 1 h and the reaction stopped by 40 µl of 0.17 M glycine-carbonate buffer (pH 9.8) and the plates read by the fluorescent plate reader. The β-galactosidase and chitotriosidase/CHIT1 activity was measured by excitation and emission wavelengths of 355 and 460 respectively. All 5 samples from one person were analyzed on the same plate. The variation (CV) for these assays in our hands were 17% for CAMP, 19% for STMN1, 7% for β-galactosidase, and 17% for chitotriosidase/CHIT1.

**Telomere length.** DNA was extracted from the PBMC isolated from venous blood at weeks 22–24 (controls n = 186, PE n = 31) using Magnapure Isolation Kit and instrument (Roche Life Science) and using a salting out procedure of EDTA blood collected at the 5-year follow-up visit in the STORK study (controls n = 206, PE n = 10). Equal amounts of DNA (2 ng/µL) were used to measure telomere length by PCR using telomere-specific primers relatively quantified to the single-copy gene RPLP0 (Table 1, Supplemental File). All samples were run in duplicates. Quantification was performed using SYBR Green PCR Fast Mix (QuantaBio, Beverly, MA) on an ABI Prism 7900 (Applied Biosystems).

**Statistical analysis.** Statistical analyses were conducted using SPSS for Windows, version 21.0 (Chicago, IL). Data are expressed as mean ± SD when normally distributed and median (25th, 75th percentile) when skewed. Comparison between women with PE and controls was performed using t-test or Mann–Whitney U depending on distribution, and Chi-square test for categorical variables. Associations were evaluated by Pearson correla-

| STORK study            | Controls | PE     | p-value   |
|------------------------|----------|--------|-----------|
| N                      | 215      | 38     |           |
| Age (years)            | 32 ± 4   | 30 ± 4 | 0.001     |
| Gestational age at delivery (week) | 40 ± 1   | 39 ± 3 | <0.001    |
| Multiparous (%)        | 110 (47) | 11 (29) | 0.035     |
| BMI (kg/m²) 14–16 week | 23.5 (21.3, 25.4) | 27.4 (23.1, 29.9) | <0.001 |
| BMI (kg/m²) 22–24 week | 24.8 (22.5, 26.8) | 29.0 (23.8, 32.1) | <0.001 |
| BMI (kg/m²) 30–32 week | 26.1 (23.7, 28.3) | 31.2 (26.0, 33.3) | <0.001 |
| BMI (kg/m²) 36–38 week | 27.2 (24.8, 29.5) | 32.2 (27.3, 34.8) | <0.001 |
| BMI (kg/m²) 5 years FU | 22.6 (20.8, 24.5) | 23.0 (20.1, 25.8) | 0.800     |

| ACUTE PE study         | Controls | PE     | p-value   |
|------------------------|----------|--------|-----------|
| N                      | 61       | 34     |           |
| Age (years)            | 32 ± 5   | 32 ± 6 | 0.151     |
| Gestational age (weeks) | 36 ± 0   | 35 ± 4 | <0.001    |
| Multiparous (%)        | 27 (42%) | 13 (33%) | 0.10     |
| BMI (kg/m²) 5 years FU | 27.3 (24.6, 28.9) | 30.1 (25.8, 34.2) | 0.001 |
| MAP (mmHg) 5 months FU | 22.8 (21.2, 25.2) | 25.3 (22.1, 30.1) | 0.010 |

| Table 1. Maternal characteristics in controls and preeclamptic (PE) pregnancies. Data given as mean ± SD when normal distributed and median (25th, 75th) when skewed distributed. BMI body mass index, MAP mean arterial pressure, FU follow-up. a N = 10 PE. bAt blood sampling.
We performed a mixed model analysis adjusting for age in the longitudinal PBMC, plasma and extracellular vesicles analysis. Two-tailed $p$-values < 0.05 were considered significant.

Results

Table 1 shows the characteristic of the STORK study cohort and of the ACUTE PE study. In both populations, women with PE had significantly higher mean arterial pressure (MAP) and body mass index (BMI) during pregnancy as well as lower gestational age at delivery, while the women with PE were younger in the STORK study.

**TERC** and mRNAs involved in the maturation of **TERC** from PBMC. The pilot array showed **TERC** as one of the top ten down-regulated transcripts (fold change $-1.8$) from the ingenuity report in PBMC from women with PE at 22–24 weeks (Table 2, Supplemental File). We then assessed **TERC** RNA in PBMC at 22–24 weeks and found a decreased expression in women with PE (Fig. 1A). We next investigated the temporal course and follow-up levels of **TERC** and some of the mRNAs involved in the maturation of **TERC** ($\text{DCP2}, \text{XRN1}, \text{NCBP1}, \text{PABPN1}, \text{MTREX/MTR4}$) (Fig. 1B). First, **TERC** increased from 22 to 24 weeks to term with similar levels at 5-year follow-up with no differences between groups at the two latter time-points (Fig. 1C). **XRN1** was markedly decreased at weeks 22–24, 36–38 and at 5-year follow-up in the women with PE (Fig. 1C). Further, **XRN1** and **TERC** were positively correlated at weeks 22–24 ($r = 0.20$, $p = 0.003$) with a stronger association in the PE group (Fig. 1D, $r = 0.59$, $p = 0.001$). Whereas **NCBP1** significantly increased, $\text{DCP2}$ decreased during pregnancy (Fig. 1E). All other RNAs measured ($\text{PABPN1}, \text{MTREX/MTR4}$) were low during pregnancy and increased at the 5-year follow-up visit, except **TERC** which had similar levels at weeks 36–38.

**TERC** and **XRN1** expression were not differentially regulated between control and PE term placenta samples. Although **TERC** and **XRN1** expression in PBMC was regulated and correlated in PE as compared with controls, this was not seen in term placenta (Fig. 2, Supplemental file). However, we did find an association between **TERC** levels in the term placenta and **TERC** in PBMC at week 36–38 ($r = 0.21$, $p = 0.024$) (data not shown).

| 14–16 | 36–38 | 5 years FU |
|-------|-------|------------|
| Controls | PE | Controls | PE | Controls | PE |
| $r$  | ($p$-value) | $r$  | ($p$-value) | $r$  | ($p$-value) | $r$  | ($p$-value) |
| $\beta$-galactosidase | | | | | | | |
| TG/HDL ratio | 0.19 (0.006) | 0.24 (0.148) | 0.21 (0.002) | 0.13 (0.488) | 0.06 (0.356) | 0.82 (0.004) |
| LDL/HDL ratio | 0.15 (0.025) | 0.40 (0.013) | 0.16 (0.018) | 0.50 (0.006) | −0.12 (0.070) | 0.59 (0.073) |

Table 2. Associations between $\beta$-galactosidase, CAMP and lipids. Significant associations in bold.
Telomere length were not differentially regulated between control and PE during pregnancy or at 5 years follow-up. We found no difference in the telomere length at 22–24 weeks or at 5-year follow-up comparing controls and women with PE (Fig. 1F). We also found an association between XRN1 and telomere length ($r = 0.15, p = 0.024$) at 5-year follow-up in the whole cohort and in the PE group ($r = 0.83, p = 0.005$) (data not shown).

**TERC expression in extracellular vesicles from plasma was decreased in PE at 5 years postpartum.** PBMC TERC levels increased during pregnancy and we wanted to explore its expression in extracellular vesicles, possibly reflecting feto-maternal crosstalk mechanisms. We found no increased expression of TERC and XRN1 in circulating extracellular vesicles during pregnancy. However, TERC was significantly decreased in PE patients at 5-year follow-up (Fig. 2A). We found no difference in XRN1 expression between controls and PE, but a significant correlation between XRN1 in PBMC and XRN1 in extracellular vesicles was found in controls at 22–24 weeks and at 5-year follow-up (Fig. 2B).

The circulating senescence markers CAMP and β-galactosidase were increased in PE. TERC expression and telomere length is related to senescence. As such we next investigated levels of circulating senescence markers CAMP, β-galactosidase, STMN1 and chitotriosidase/CHIT1 in the STORK and ACUTE PE study (Fig. 3). In the STORK study, we found β-galactosidase increased at 22–24 and 36–38 weeks, and CAMP increased at 30–32, 36–38 and at 5 years follow-up in women with PE compared to controls, after adjusting for age. CAMP and β-galactosidase increased significantly from 14 to 38 weeks during pregnancy in the whole cohort, while decreasing again at 5-year follow-up. Investigating the same markers in the ACUTE PE study we found CAMP increased at 6 months both in early- and late-onset PE, and β-galactosidase increased in late-onset PE during pregnancy compared to controls. We found no significant difference in the STMN1 and chitinase markers between controls and women with PE after adjusting for age or any significant changes during pregnancy or compared to follow-up. Thus, the senescence markers CAMP and β-galactosidase seems to be increased during PE pregnancies.

**CAMP and β-galactosidase were associated with atherogenic lipid ratios during pregnancy.** We then investigated whether CAMP and β-galactosidase were associated with CV risk as reflected by the atherogenic lipid ratios TG/HDL-C and LDL/HDL-C. In the control group in the STORK study, the TG/HDL-C ratio and LDL/HDL-C ratio were associated with β-galactosidase at week 14–16 and 36–38 (Table 2). In the PE group, the LDL/HDL-C ratio correlated with β-galactosidase during pregnancy and the TG/HDL-C ratio during postpartum follow-up. We found few associations between these markers at 5-year follow-up, although this may be due to smaller numbers of PE patients. We did not find any association between the senescence markers and arterial stiffness at 5-year follow-up in the STORK study or systemic arterial properties in the ACUTE PE study (data not shown).

**Few associations between BMI, telomere length, mRNAs involved in the maturation of TERC and senescence markers.** Evaluated within PE and controls separately we observed no correlation between BMI and telomere length during pregnancy or at follow-up. We observed no correlations between BMI and TERC or XRN1 RNA levels or with CAMP and β-galactosidase at any time-point during pregnancy or at follow-up with the exception of a correlation between BMI and β-galactosidase at week 14–16 ($r = 0.19, p = 0.007$) and at week 36–38 ($r = 0.14, p = 0.044$) in controls, but not in PE.
Discussion
In the present study we found decreased expression of TERC in PBMC in pregnant women with PE. XRN1 involved in the maturation of TERC was also low during pregnancy and at 5-year follow-up in patients with PE. Further, we found that the senescence markers CAMP and β-galactosidase were significantly elevated during pregnancy in women with PE, and CAMP remained elevated at 5-year follow-up. β-galactosidase was positively associated with atherogenic lipid ratios during pregnancy and at 5-year follow-up in women with PE.

Telomerase activity and cell senescence are altered during pregnancy and delivery since the placenta/fetal membranes are unique to this context, and there is evidence that the timing of these mechanisms may be temporally related to the timing of parturition1,2,23. There is also data to suggest that telomere-related cellular aging may be associated with PE, with premature activation of this pathway leading to placental villous telomere shortening, telomere aggregates, and trophoblast dysfunction in PE placenta5-24,26. Although we demonstrate changes in maternal leukocyte (PBMC) expression of TERC and senescence markers in PE, we were unable to detect any differences in telomere length in leukocytes or components related to telomere maturation between PE and controls in biopsies taken from placental parenchyma. Similar results were reported by previous studies comparing telomere length in circulating leukocytes from PE and normal pregnancies27,28 as well as leukocytes isolated from cord blood29. Furthermore, the similar maternal telomere length was not due to differences in age between the groups as we adjusted for age in the comparison and leukocytes were isolated at the same gestational age. As suggested by others, the lack of telomere shortening in blood leukocytes in PE suggests that these processes are probably restricted primarily to the placenta30. However, we found dysfunction in the markers of the telomerase complex in maternal leukocytes and propose that these processes are not restricted only to the placenta. For components related to telomere maturation between PE and controls in biopsies taken from placental parenchyma, it should be noted that the tissues examined were collected at delivery—often delivery at term—rather than at an earlier gestational age, and that specific cell lines within the placenta/fetal membranes were not separately examined. As the placenta secretes a range of biologically active substances, hormones, cells and extracellular vesicles into the maternal circulation, many of which may be taken up and affect cellular function within the maternal vasculature30, we speculate that the marked increase in TERC during pregnancy could reflect enhanced feto-maternal trafficking of extracellular vesicles. To this end, we did detect TERC in extracellular vesicles, but with a different temporal pattern which did not correlate with PBMC levels. However, RNA levels of TERC at 5-year follow-up were similar to term levels arguing against accumulation of placental derived TERC in PBMC during pregnancy.

A major finding in our study was the decreased levels of TERC and the RNA exonuclease XRN1 as early as 22–24 weeks and at 5-year follow-up in PE patients, suggesting a possible underlying predisposition in such women that is unmasked during pregnancy, further supported by the strong positive correlation between TERC and senescence markers in PE, we were unable to detect any differences in telomere length in leukocytes or components related to telomere maturation between PE and controls in biopsies taken from placental parenchyma. Similar results were reported by previous studies comparing telomere length in circulating leukocytes from PE and normal pregnancies27,28 as well as leukocytes isolated from cord blood29. Furthermore, the similar maternal telomere length was not due to differences in age between the groups as we adjusted for age in the comparison and leukocytes were isolated at the same gestational age. As suggested by others, the lack of telomere shortening in blood leukocytes in PE suggests that these processes are probably restricted primarily to the placenta30. However, we found dysfunction in the markers of the telomerase complex in maternal leukocytes and propose that these processes are not restricted only to the placenta. For components related to telomere maturation between PE and controls in biopsies taken from placental parenchyma, it should be noted that the tissues examined were collected at delivery—often delivery at term—rather than at an earlier gestational age, and that specific cell lines within the placenta/fetal membranes were not separately examined. As the placenta secretes a range of biologically active substances, hormones, cells and extracellular vesicles into the maternal circulation, many of which may be taken up and affect cellular function within the maternal vasculature30, we speculate that the marked increase in TERC during pregnancy could reflect enhanced feto-maternal trafficking of extracellular vesicles. To this end, we did detect TERC in extracellular vesicles, but with a different temporal pattern which did not correlate with PBMC levels. However, RNA levels of TERC at 5-year follow-up were similar to term levels arguing against accumulation of placental derived TERC in PBMC during pregnancy.

The secreted proteins, CAMP, STMN1, β-galactosidase and chitinase, were previously identified as senescence markers in telomere-dysfunctional mice (G4mTerc−/−)12. The increase in CAMP and β-galactosidase during pregnancy may reflect the progressive natural physiological senescence and aging of decidual cells and placenta/fetal membranes important for the onset of labor. During pregnancy, placental extravillous trophoblasts invade the pregnant decidua, losing their replicative potential and entering a senescent state characterized by high β-galactosidase activity. Moreover, in support of increased CAMP and β-galactosidase activity in PE pregnancies, prior studies have shown increase staining in trophoblast collected from women with both early- and late-onset PE25,26. Differences were less pronounced in the ACUTE PE study compared with the STORK study, which may be explained by the shorter gestational age, especially in regards to early-onset PE. While it is tempting to speculate that the increased circulating CAMP and β-galactosidase in PE women may be a consequence of the telomerase dysfunction, we were unable to detect any correlation with the decreased TERC and XRN1 levels in PBMC. However, whereas cellular senescence suggests decreased proliferation, the cells that are present have a strongly pro-inflammatory phenotype. In relation to this, the release of CAMP is essential in innate immunity, with the ability to modulate both local innate and adaptive immune responses35–39. Thus, enhanced levels could reflect persistent inflammation and high amounts of ncRNA in the circulation of PE women.

Senescent cells often show a global change in their metabolism, including enhanced glycogen and lipid storage, induction of fatty acid synthesis, and increased secretion of inflammatory molecules. The increase in CAMP at 5-year follow-up in the PE women is especially interesting for investigating well-recognized long-term adverse events after PE40, although we observed no association with atherogenic lipid ratios. However, we found that circulating β-galactosidase activity, another marker of cellular senescence, correlated with TG/HDL and LDL/HDL ratios during pregnancy, with a stronger association in PE. This is consistent with prior studies that have shown an association between PE and dyslipidemia41. However, as β-galactosidase activity was normalized in PE at 5-year follow-up, the impact of this finding is unknown. Although we found no strong correlation between BMI and senescence markers or telomere length, there are reports that show accumulation of senescent cells in obesity42,43 and senescence is linked to CVD44,45. Our PE women had higher BMI during pregnancy and we cannot exclude that some effect of premature senescence in PE on CV risk could be mediated through interaction with adipose tissue mass.

A limitation of the study is the low numbers of PE women followed-up after 5-year in the STORK cohort and the different number of samples available in the experiments. Also, evaluation of specific cell lines within the placenta/fetal membranes could reveal regional differences in telomere homeostasis. Several factors including...
serum starvation, confluent culture, H$_2$O$_2$ treatment, age of cells and pH may change β-galactosidase activity\(^4\). However, our experiment on plasma samples were controlled for age and performed under controlled conditions with the identical reagents with similar pH at the same day. Still, our clinical cohorts were well described, with temporal samples during pregnancy and at 5-year follow-up.

In the present study, we found low levels of the long ncRNA TERC in PBMC coupled with low circulating XRNI levels in women with PE compared with normotensive controls. This may reflect dysfunctional telomerase activity in PE women. Although we did not show a significant overall decrease in telomere length in PBMC in women with PE, it is possible that such changes may occur later or may be localized to uterine tissues and the placenta/fetal membranes. The telomere-associated senescence markers CAMP and β-galactosidase were increased in PE during pregnancy and CAMP remained elevated at 6 months and 5 years after pregnancy.

Received: 14 July 2021; Accepted: 21 September 2021
Published online: 05 October 2021

References

1. Kohlrusch, F. B. & Keefe, D. L. Telomere erosion as a placental clock: From placental pathologies to adverse pregnancy outcomes. *Placenta* 97, 101–107 (2020).
2. Poletti, N. & da Silva, M. G. Telomere-related disorders in fetal membranes associated with birth and adverse pregnancy outcomes. *Front. Physiol.* 11, 561771 (2020).
3. Vicentelli, S. & Passos, J. F. Telomeres and cell senescence—Size matters not. *ElBioMedicine* 21, 14–20 (2017).
4. Roake, C. M. & Artandi, S. E. Regulation of human telomerase in homeostasis and disease. *Nat. Rev. Mol. Cell Biol.* 21, 384–397 (2020).
5. Jiang, H.
6. Sultana, Z., Maiti, K., Dedman, L. & Smith, R. Is there a role for placental senescence in the genesis of obstetric complications and long-term health in adulthood. *Front. Psychiatr.* 8, 208 (2017).
7. Hoffmann, J. et al. Telomerase as a therapeutic target in cardiovascular disease. *Arterioscler. Thromb. Vasc. Biol.* 41, 1047–1061 (2021).
8. Ross, M. G. & Gorospe, M. Noncoding RNAs controlling telomere homeostasis in senescence and aging. *Trends Mol. Med.* 26, 422–433 (2020).
9. Jiang, H. et al. Proteins induced by telomere dysfunction and DNA damage represent biomarkers of human aging and disease. *Proc. Natl. Acad. Sci. U.S.A.* 105, 11299–11304 (2008).
10. Wu, P. et al. Preeclampsia and future cardiovascular health: A systematic review and meta-analysis. *Circ. Cardiovasc. Qual. Outcomes* 10, e003497 (2017).
11. Dall’Asta, A. et al. Cardiovascular events following pregnancy complicated by preeclampsia with emphasis on comparison between early- and late-onset forms: Systematic review and meta-analysis. *Ultrasound Obstet. Gynecol.* 57, 698–709 (2021).
12. Roland, M. C. et al. Fetal growth versus birthweight: The role of placenta versus other determinants. *PLoS ONE* 7, e39324 (2012).
13. Lekva, T. et al. beta-cell dysfunction in women with previous gestational diabetes is associated with visceral adipose tissue distribution. *Endocr. J.* 67, 63–70 (2015).
14. Estensen, M. E. et al. Elevated inflammatory markers in preeclamptic pregnancies, but no relation to systemic arterial stiffness. *Pregnancy Hypertens.* 5, 325–329 (2015).
15. Lekva, T. et al. Gene expression in term placenta is regulated more by spinal or epidural anesthesia than by late-onset preeclampsia or gestational diabetes mellitus. *Sci. Rep.* 6, 29715 (2016).
16. Lekva, T. et al. CXC chemokine ligand 16 is increased in gestational diabetes mellitus and preeclampsia and associated with lipoproteins in gestational diabetes mellitus at 5 years follow-up. *Diabetes Vasc. Dis. Res.* 14, 525–533 (2017).
17. Enderle, D. et al. Characterization of RNA from exosomes and other extracellular vesicles isolated by a novel spin column-based method. *PLoS ONE* 10, e0136135 (2015).
18. Miller, S. A., Dykes, D. D. & Polesky, H. F. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucl. Acids Res.* 16, 1215 (1988).
19. Lekva, T. et al. Aortic stiffness and cardiovascular risk in women with previous gestational diabetes mellitus. *PLoS ONE* 10, e0136892 (2015).
20. Chen, J. C., Chu, T. C., Huang, S. C., Chow, S. N. & Hsieh, C. Y. Telomerase activity in gestational trophoblastic disease and placental tissue from early and late human pregnancies. *Hum. Reprod.* 17, 463–468 (2002).
21. Sukenik-Haley, R. et al. Telomere aggregate formation in placenta specimens of pregnancies complicated with pre-eclampsia. *Cancer Genet. Cytogenet.* 195, 27–30 (2009).
22. Faraldukus-Gershshnel, S. et al. Telomere homeostasis and senescence markers are differently expressed in placentas from pregnancies with early- versus late-onset preeclampsia. *Reprod. Sci.* 26, 1203–1209 (2019).
23. Cox, L. S. & Redman, C. The role of cellular senescence in aging of the placenta. *Placenta* 52, 139–145 (2017).
24. Harville, E. W., Williams, M. A., Qui, C. F., Mejia, J. & Risques, R. A. Telomere length, pre-eclampsia, and gestational diabetes. *BMJ Res. Notes* 3, 113 (2010).
25. Xu, J. et al. Reduced fetal telomere length in gestational diabetes. *PLoS ONE* 9, e86161 (2014).
26. Sukenik-Haley, R. et al. Telomere homeostasis in trophoblasts and in cord blood cells from pregnancies complicated with preeclampsia. *Am. J. Obstet. Gynecol.* 214, 203.e1–203.e7 (2016).
27. Apflik, J. D., Myers, J. E., Timms, K. & Westwood, M. Tracking placental development in health and disease. *Nat. Rev. Endocrinol.* 16, 479–494 (2020).
28. Wolin, S. L. & Maquat, L. E. Cellular RNA surveillance in health and disease. *Science* 366, 822–827 (2019).
29. Labno, A., Tomecki, R. & Dziembowski, A. Cytoplasmic RNA decay pathways—Enzymes and mechanisms. *Biochim. Biophys. Acta* 1863, 3125–3147 (2016).
30. Cesena, D. et al. Regulation of telomere metabolism by the RNA processing protein Xrn1. *Nucl. Acids Res.* 45, 3860–3874 (2017).
31. Minns, D. et al. The neutrophil antimicrobial peptide cathelicidin promotes Th17 differentiation. *Nat. Commun.* 12, 1285 (2021).
36. Kahlenberg, J. M. & Kaplan, M. J. Little peptide, big effects: The role of LL-37 in inflammation and autoimmune disease. *J. Immunol.* **191**, 4895–4901 (2013).
37. Majewski, K., Agier, J., Kozlowska, E. & Brzezinska-Blaszczyk, E. Serum level of cathelicidin LL-37 in patients with active tuberculosis and other infectious diseases. *J. Biol. Regul. Homeost. Agents* **31**, 731–736 (2017).
38. Yang, Y. M., Guo, Y. F., Zhang, H. S. & Sun, T. Y. Antimicrobial peptide LL-37 circulating levels in chronic obstructive pulmonary disease patients with high risk of frequent exacerbations. *J. Thorac. Dis.* **7**, 740–745 (2015).
39. Tran, D. H. *et al.* Circulating cathelicidin levels correlate with mucosal disease activity in ulcerative colitis, risk of intestinal stricture in Crohn's disease, and clinical prognosis in inflammatory bowel disease. *BMC Gastroenterol.* **17**, 63 (2017).
40. Pittara, T., Vryides, A., Lannios, D. & Giannakou, K. Pre-eclampsia and long-term health outcomes for mother and infant: An umbrella review. *BJOG* **128**, 1421–1430 (2021).
41. Spracklen, C. N., Smith, C. J., Safilas, A. F., Robinson, J. G. & Ryckman, K. K. Maternal hyperlipidemia and the risk of preeclampsia: A meta-analysis. *Am. J. Epidemiol.* **180**, 346–358 (2014).
42. Muezzinler, A., Zaineddin, A. K. & Brenner, H. Body mass index and leukocyte telomere length in adults: A systematic review and meta-analysis. *Obes. Rev.* **15**, 192–201 (2014).
43. Xu, M. *et al.* Targeting senescent cells enhances adipogenesis and metabolic function in old age. *Elife* **4**, e12997 (2015).
44. Childs, B. G. *et al.* Senescent intimal foam cells are deleterious at all stages of atherosclerosis. *Science* **354**, 472–477 (2016).
45. Roos, C. M. *et al.* Chronic senolytic treatment alleviates established vasoconstrictor dysfunction in aged or atherosclerotic mice. *Aging Cell* **15**, 973–977 (2016).
46. Yang, N. C. & Hu, M. L. The limitations and validities of senescence associated-beta-galactosidase activity as an aging marker for human foreskin fibroblast Hs68 cells. *Exp. Gerontol.* **40**, 813–819 (2005).

**Author contributions**

T.L., M.C.P.R., M.E.E., T.U. conceptualized and designed the study. T.L., M.C.P.R., M.E.E., and K.R.N collected data and biospecimens. T.L., M.C.P.R., K.R.N. and T.U. processed biospecimens. O.K.O. performed bioinformatics. T.L. and T.U. analyzed, interpreted data and drafted the manuscript. All authors interpreted data, revised and approved the final version of the manuscript.

**Funding**

This work was supported by grants from The Norwegian Health Association (TL).

**Competing interests**

The authors declare no competing interests.

**Additional information**

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-021-99140-z.

**Correspondence** and requests for materials should be addressed to T.L.

**Reprints and permissions information** is available at www.nature.com/reprints.

**Publisher’s note** Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2021, corrected publication 2021