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Research paper

Epigenetic and senescence markers indicate an accelerated ageing-like state in women with preeclamptic pregnancies

Sonja Suvakov[a], Ranine Ghamrawi[a], Hajrunisa Cubro[a], Haitao Tu[a,b], Wendy M. White[c], Yvonne S. Butler Tobah[c], Natasa M. Milic[a,d], Joseph P. Grande[e], Julie M. Cunningham[e], Fouad T. Chebib[a], Larissa G.P. Langhi Prata[f], Yi Zhu[f], Tamara Tchkonia[f], James L. Kirkland[f,g], Karl A. Nath[a], Aleksandar Milosavljevic[h], Vesna D. Garovic[a,c,*]

[a] Division of Nephrology and Hypertension, Mayo Clinic, Rochester, MN, USA
[b] Division of Nephrology, The First Affiliated Hospital of Guangzhou University of Chinese Medicine, Guangzhou, Guangdong, China
[c] Department of Obstetrics and Gynaecology, Mayo Clinic, Rochester, MN, USA
[d] Department of Medical Statistics and Informatics, Medical Faculty, University of Belgrade, Serbia
[e] Department of Laboratory Medicine and Pathology, Mayo Clinic, Rochester, MN, USA
[f] Robert and Arlene Kogod Center on Aging, Mayo Clinic, Rochester, MN, USA
[g] Division of General Internal Medicine, Mayo Clinic, Rochester, MN, USA
[h] Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, TX, USA

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ABSTRACT

Background: Preeclampsia is a pregnancy-specific hypertensive disorder characterized by proteinuria and/or multisystem involvement. Disease-specific therapy has yet to be developed due to the lack of understanding of underlying mechanism(s). We postulate that accelerated ageing in general, and particularly cellular senescence, play a role in its pathophysiology.
Methods: We compared women with preeclampsia *vs.* normotensive pregnancies with respect to epigenetic markers of ageing and markers of senescence in tissues/organs affected by preeclampsia (blood, urine, adipose tissue, and kidney).
Findings: We demonstrate that preeclamptic compared to normotensive pregnant women: (i) undergo accelerated epigenetic ageing during pregnancy, as demonstrated by an "epigenetic clock"; (ii) exhibit higher levels/expression of senescence-associated secretory phenotype factors in blood and adipose tissue; (iii) display increased expression of $p16^{INK4A}$ in adipose tissue and renal sections, and (iv) demonstrate decreased levels of urinary α-Klotho (an anti-ageing protein) at the time of delivery. Finally, we provide data indicating that pre-treatment with dasatinib, a senolytic agent, rescues the angiogenic potential of mesenchymal stem cells (MSC) obtained from preeclamptic pregnancies, and promotes angiogenesis, even under pro-inflammatory conditions.
Interpretation: Taken together, our results identify senescence as one of the mechanisms underpinning the pathophysiology of preeclampsia. Therapeutic strategies that target senescent cells may offer novel mechanism-based treatments for preeclampsia.
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* Corresponding author at: Division of Nephrology and Hypertension, Mayo Clinic, Rochester, MN, USA.
E-mail addresses: Suvakov.sonja@mayo.edu (S. Suvakov), ghamrawi.ranine@mayo.edu (R. Ghamrawi), hajrunisa.cubro@louisville.edu (H. Cubro), White.Wendy@mayo.edu (W.M. White), ButlerTobah.Yvonne@mayo.edu (Y.S.B. Tobah), grande@mayo.edu (J.P. Grande), cunningham.julie@mayo.edu (J.M. Cunningham), Chebib.Fouad@mayo.edu (F.T. Chebib), langhi.larissa@mayo.edu (L.G.P.L. Prata), Zhu.Yi@mayo.edu (Y. Zhu), Tchkonia.Tamar@mayo.edu (T. Tchkonia), Kirkland.James@mayo.edu (J.L. Kirkland), Nath.Karl@mayo.edu (K.A. Nath), amilosav@bcm.edu (A. Milosavljevic), garovic.vesna@mayo.edu (V.D. Garovic).

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Research in context

Evidence before this study

Preeclampsia is a pregnancy-specific hypertensive disorder that portends significant maternal and foetal morbidity and mortality. The aetiology and pathogenesis remain elusive, resulting in a failure to develop specific treatment strategies that target the underlying mechanistic processes. Delivery remains the only therapy and commonly results in prematurity.

Added value of this study

We have demonstrated that senescence is implicated in the development of preeclampsia. Pregnant women with preeclampsia undergo an accelerated ageing-like state and demonstrate an increase in senescent burden and upregulation of senescence-associated phenotype (SASP) components.

Implications of all the available evidence

Our study provides proof-of-concept evidence regarding the role of senescence, in general, and particularly MSC senescence, in the development of a systemic pro-inflammatory state and impaired angiogenesis, respectively. This may result in identifying new biomarkers and novel therapeutics using MSC and/or drugs that target fundamental senescence processes.

1. Introduction

Preeclampsia (PE) is a pregnancy-specific hypertensive disorder that affects 3–5% of all pregnancies [1]. Women with PE classically present with new-onset proteinuria (≥300 mg *per* 24 h) and/or severe organ dysfunction, including impaired liver function, acute renal insufficiency, pulmonary oedema, and/or new-onset of cerebral or visual disturbances. Delivery is the only available therapy for PE. Effective targeted therapies are unavailable, primarily due to the lack of understanding of the mechanisms underpinning the pathophysiology of this disorder. Preventive strategies are limited to the use of aspirin in a subset of patients at high risk for PE [2].

Research to date has identified impaired angiogenesis [3] and inflammation as two major mechanisms that contribute to the pathophysiology of PE. Our recent study demonstrated that impaired angiogenesis in PE, at least in part, is due to the impaired angiogenic potential of mesenchymal stem cells (MSC), isolated from adipose tissue during Caesarean (C-)sections from women with PE [4]. Additionally, our data indicate that MSC dysregulation is mechanistically related to cellular senescence, an essentially irreversible cell-cycle arrest mechanism [5]. Under physiological conditions, this mechanism prevents proliferation of cells that have undergone DNA damage and/or oxidative/metabolic stress. Under pathological conditions, senescent cell burden has been associated with metabolic derangements and functional impairment in various organs and tissues mediated by $p16^{INK4A}$ and $p21,^{CIP1}$ as well as release of pro-inflammatory markers [for example, Interleukin (IL)-6, IL-8, monocyte chemoattractant protein 1 (MCP-1), and plasminogen activator inhibitor 1 (PAI-1)], that represent components of the senescence-associated secretory phenotype (SASP) [5]. Notably, senescence has been increasingly associated with age-related dysfunction and multiple chronic diseases. Senescence has been described in PE placentas, which show signs of an aberrant ageing-like state with an increased burden of senescent cells [6]. While several SASP components are elevated in PE compared to normotensive pregnancies, [7] they have not been studied specifically in the context of PE.

Our overall hypothesis in the present study is that an accelerated ageing-like state in general, and especially senescence, play a role in the pathophysiology of PE. To test this hypothesis, we performed a series of experiments comparing women with PE *vs*. normotensive pregnancies with respect to epigenetic markers of ageing and markers of senescence in tissues and organs affected by PE (blood, urine, adipose tissue biopsies, and renal sections). We further posit that clearing the senescent phenotype with senolytic agents will rescue the angiogenic potential of MSC, even under pro-inflammatory conditions. These agents target survival pathways in senescent cells and promote selective apoptosis of senescent cells, while sparing proliferating cells [8]. Our results identify cellular senescence as one of the mechanisms underpinning the pathophysiology of PE. Our study opens new venues for developing mechanism-based therapies for PE, including treatment with senolytic agents.

2. Methods

2.1. Participants

Women with PE and normotensive pregnancies were recruited from the Mayo Clinic Family Birth Centre. The diagnosis of PE was based on the presence of the following criteria: (1) hypertension after 20 weeks of gestation, defined as a blood pressure (BP) ≥ 140/90 mm Hg; proteinuria, defined as ≥300 mg of protein in a 24 h urine specimen; protein/creatinine (Cr) ratio of ≥ 0·3; and/or 1 + (30 mg/L) dipstick urinalysis in the absence of a urinary tract infection. In the absence of proteinuria, the diagnosis of PE was confirmed if one or more of the following laboratory abnormalities were present: thrombocytopenia <100,000/μL, serum Cr > 1.1 mg/dL (or its doubling from baseline), or elevated liver function tests, AST and ALT (>2x upper limits of normal), or the presence of pulmonary oedema, cerebral or visual symptoms. Based on the timing of clinical presentation, women with PE were further grouped as those with early (<34 gestational weeks) versus late PE (≥34 gestational weeks) [9].

Three separate patient cohorts were used for the current studies: (1) A convenient sample from our previous longitudinal prospective study [10] of women with PE (n = 11) and normotensive pregnancies n = 33), for whom buffy coats were collected for epigenetic studies during the first and second trimesters and at delivery, which enabled studies of epigenetic alterations during their pregnancies. In the same cohort, we studied circulating SASP components in women with PE (n = 11) and those with normotensive pregnancies (n = 13) for whom plasma samples at delivery were readily available; (2) A cross-sectional study of women with PE (n = 8) and normotensive (n = 8) pregnancies who were recruited at the time of clinically indicated C-section. Abdominal adipose tissue explants (3–5 g) were obtained, from which adipose-derived MSC were isolated; (3) Pregnant women from whom autopsy renal tissues were available, and who died either from PE and its complications (n = 5) or from accidental causes during otherwise uncomplicated pregnancies (n = 3). Some or all of these participants had participated previously in studies of (i) podocyturia, *i.e*. urinary presence of glomerular epithelial cells (podocytes) as a predictor of PE [10], (ii) assessment of the role of cellular senescence in impaired angiogenesis in PE [4], and (iii) glomerular expression of podocyte-specific proteins in the kidney sections from women with PE [11].

2.2. Ethics

This study was approved by the Mayo Clinic Institutional Review Board (protocol # 2105-05) and all participants provided written informed consent prior to participating.

2.3. Sample processing for epigenetic studies

Blood collection, separation, storage, and DNA extraction were performed in the Biospecimens Accessioning and Processing (BAP) laboratory. Each blood sample was drawn into a 10 ml EDTA “purple

top" tube; the buffy coat (leukocytes) was separated immediately. The buffy coat was aliquoted in 2−ml tubes and frozen at −80 °C until methylation profiles were ready to be conducted. Genomic DNA was extracted using the QIAgen Flexigene Reagent kit on an AutoGen FLEXSTAR DNA instrument using half of each frozen buffy coat (cell number equivalent to 5 ml of whole blood). DNA was then quantified with a Trinean Spectrophotometer, normalized with Qubit methodology, and plated in 1000 ng aliquots.

The extracted DNA was sent to the Genotyping Shared Resource (GSR) Laboratory, where methylation analysis was performed. First, bisulphite modification was performed using the EZ DNA Methylation Kit (Zymo Research, Orange, CA) that, when performed correctly, is a chemical reaction that is >99.5% efficient. In the presence of bisulphite, methylated cytosines are unaffected, while non-methylated cytosines are chemically deaminated to uracil.

Plate maps were generated to determine the random location for each sample on the plate, as well as for the samples that were run in duplicate. All samples were run in a single batch. For the proposed study, we used the 450 K Illumina Human Methylation Assay, which evaluated 450,000 CpG sites (Illumina, San Diego, CA). Bisulphite-converted DNA samples are whole-genome amplified, enzymatically fragmented, and purified. Samples are then hybridized in batches of 8 to the BeadChip, which contains locus-specific DNA oligomers. For each CpG locus interrogated, two bead types are present on the chip: one that corresponds to the methylated (C) state, and the other to the non-methylated (T) state. After allele-specific primer annealing, DNP- and Biotin-labelled dNTPs are used to extend the primer by a single base. The array is then fluorescently stained, scanned, and assessed for fluorescence intensities at the methylated and non-methylated bead sites.

2.3.1. Genome-wide methylation analyzes

The raw fluorescence data from the scanner were processed using GenomeStudio software (or R package, minfi) to yield beta values that were used in statistical analyzes. Beta-values are continuous variables ranging from 0 to 1, which represent the ratio of fluorescence intensity at the methylated bead type over the combined fluorescence intensity of the locus. Rigorous quality control was performed using embedded control probes on the chip. The sample independent controls allow for the evaluation of the quality of the chip processing steps, while the sample-dependent controls allow for the evaluation of the performance across samples. We removed sites with control spot values greater than 4 standard deviations from their mean values. In addition, we have developed and published a normalization scheme to reduce batch and chip effects by linearly regressing the methylated and non-methylated intensity signals onto the set of control probes that are orthogonal (*i.e.* independent) predictors of the control spot distributions [12]. We have also evaluated and adjusted for differences between Type 1 and Type 2 probe biases.

2.3.2. Quality control

The samples were processed and then scanned using Illumina's iScan instrumentation. The raw data were then analyzed using Illumina's Genome Studio software (version 2011.1) with methylation module (version 1.9.0). Quality assessment of the array was conducted using the "Control Dashboard" in the Genome Studio software package, which includes graphical inspection of the 10 types of embedded control probes: staining, extension, hybridization, target removal, bisulphite conversion, G/T mismatch, specificity, non-polymorphic controls, negative controls, and restoration controls.

Overall sample performance was determined by the total number of detected CpGs, the average detection *P* value across all CpG sites, and the distribution of average beta values for all CpGs. Call rates for each CpG site and sample were determined. Methylation sites and samples were excluded if the unreliable call rate (detection *P* value) was greater than 5%. Technical replicate reproducibility was estimated by the Pearson correlation coefficient.

All samples were bisulphite modified, plated, and run concurrently to avoid batch effect. However, different BeadChips, even when processed at the same time, can have variations in assay integrity leading to the "chip" effect. Data were examined using principal components analysis and unsupervised hierarchical clustering.

2.4. Blood, urine, and adipose tissue senescence marker analysis

Plasma SASP components were tested using the Milliplex map Human Adipocyte Magnetic Bead Panel (Cat#HADCYMAG-61K) and the Luminex 200 system for plate reading according to the manufacturer's protocol. These included neuronal growth factor (NGF), IL-6, Leptin, IL-8, hepatocyte growth factor, Adiponectin, MCP-1, TNF-α, Resistin, IL-1β, and PAI-1. The adipose tissue SASP was measured in approximately 100 mg of adipose tissue obtained from each subject. The samples were homogenized in 1 ml of ice-cold homogenization buffer containing 10% glycerol, 150 mM NaCl, 2 mM EDTA, Pierce Protease, and phosphatase inhibitor mini tablets, EDTA-free (Thermo Scientific) in 10 mM Tris-HCl (pH 7.0), with up/down and side-to-side strokes for 20 s at setting No. 6 using a Polytron apparatus with the tubes immersed in ice. The crude homogenate was centrifuged at 3000 × *g* for 15 min at 4 °C, and the fat layer was discarded. The homogenate was centrifuged again at 15,000 × *g* for 20 min at 4 °C. The supernatants were stored in aliquots at −80 °C until all samples were processed. The total protein for each sample was measured using the BCA protein assay and all lysates were then adjusted to the same protein concentration. The lysates were returned to −80 °C until assayed with the Luminex 200 system.

Plasma $p16^{INK4A}$ and $p21^{CIP1}$ were measured using commercial ELISA kits (Human CDKN2A [Sandwich ELISA] ELISA Kit Cat# LS-F22610, Human CDKN1A/WAF1/p21 [Sandwich ELISA] ELISA Kit Cat#LS-F20417). Urinary α-Klotho concentrations were measured using the Alpha Klotho Human Soluble ELISA Assay kit (IBL-America. Inc, Cat#27998). α-Klotho was normalized to urinary creatinine concentration measured using a creatinine assay kit (R&D System, Cat# KGE005). All ELISA studies were performed according to the manufacturers' protocols.

2.5. $p16^{INK4A}$ staining of renal and adipose tissue sections

Histologic sections, 4−5 μ thick, from pregnant women who died from PE (n = 5) and control kidneys (n = 3), were stained using monoclonal mouse antihuman anti-$p16^{INK4A}$ antibody (ab54210, Abcam, Cambridge, UK). Antigen retrieval was performed by steam heating in 0·5 mmol EDTA for 30 min, followed by enzymatic treatment with trypsin for 10 min at 37°C. Commercially available kits (Vectastain ABC kit, Vector Laboratories and Envision Plus HRP kit, DakoCytomation) were used for the blocking, secondary antibody, and amplification steps. Color development was performed using NovaRed (Vector Laboratories) followed by hematoxylin counterstaining. To facilitate consistency among the staining batches, most of the stains were performed on a DakoAutostainer, an automated staining machine. Adipose tissue sections (patients with preeclampsia n = 8, normotensive pregnant women n = 5) were stained in the same manner.

For counting $p16^{INK4A+}$ nuclei, a Motic Slide Scanner (Motic Company, China), with a 40x objective was used to scan adipose and kidney biopsy slides. In the kidney tissue samples, the glomeruli present in each scanned image were identified and numbered. Glomeruli with incomplete sections were excluded from counting. For the counting of $p16^{INK4A+}$ nuclei in the adipose tissue, at least 10 fields were used. Incomplete adipocytes were excluded from the counting. Subsequently, $p16^{INK4A+}$ nuclei were counted in each glomerulus using ImageJ.

Neither the person who captured images for quantification nor the observer counting $p16^{INK4A+}$ cells in the captured images was aware of the identity of the samples.

2.6. Mesenchymal stem cell isolation

MSC were isolated from the adipose tissue from pregnant women during C-section. MSC identification/verification was confirmed as previously reported [4].

2.7. Angiogenesis assay

Angiogenesis was assessed using IncuCyte S3 Live-Cell Analysis. We measured a total network length *per* mm^2 of GFP-expressing human umbilical vein endothelial cells (IncuCyte CytoLight Green HUVEC Cells) seeded in a 96-well plate (Corning Incorporated, USA), as instructed in the manufacturer's kit. The HUVECs were treated for 24 h with 50 ng/ml of TNF-α. To rescue angiogenesis, a total of 4000/well normotensive MSC (NT-MSC) and PE-MSC were transferred and co-cultured with previously seeded HUVECs and human fibroblasts (IncuCyte NHDF Cells). A total of three experimental settings for MSC were explored: (i) control (MSC grown in regular media) (ii) vehicle (MSC grown in 1% DMSO) and (iii) MSC previously treated for 24 h with a senolytic (dasatinib, 1 μM). Dasatinib is a tyrosine kinase inhibitor that specifically targets senescent MSC. We have previously identified 1 μM dasatinib (a senolytic that causes apoptosis in senescent cells only) as the optimal concentration of the drug leading to apoptosis in PE-MSC, but not in NT-MSC [4]. Real-time images were captured every 6 h for 6 days, analyzed using IncuCyte S3 Software (Essen Bioscience), and compared between groups.

2.8. Statistics

Baseline characteristics, epigenetic age, levels of blood, urine, adipose and kidney senescence markers, and angiogenic potential of MSCs from normotensive and PE women are presented as means with standard deviations/standard errors or medians, with 25−75th percentiles and analyzed according to data distribution. Normality of data was assessed by graphical methods and the one-sample Kolmogorov-Smirnov test. Changes in epigenetic ageing during the pregnancy course in normotensive *vs.* PE pregnancies were assessed by repeated measures ANOVA, with the normotensive/PE group as the between subject factors. In addition, we assessed the relative size of the effect of preeclampsia on biological ageing using standardized estimates of effect size according to Cohen's benchmarks, which define *d* as the difference between the means divided by pooled standard deviation (SD) [13]. Baseline characteristics and levels of leptin, IL-8, HGF, adiponectin, MCP-1, TNF- α, resistin and PAI-1 were analyzed using unpaired *t* test under the assumption of equal variance. Levels of NGF, IL-6 and IL-1β, $p16^{INK4A}$ $p21^{CIP1}$ and urinary α-Klotho were analyzed using the Mann-Whitney U test. Renal and adipose tissue $p16^{INK4A+}$ was analyzed using the Mann-Whitney U test. The effects of the pro-inflammatory *milieu* and senolytics on the angiogenic potential of MSC isolated from PE and normotensive pregnancies were examined by repeated measures ANOVA. Analysis was performed using SPSS for Windows (v25.0; IBM SPSS) and GraphPad Prism (v8.0; GraphPad Software). Data are presented according to recently proposed guidelines for basic science data visualization [14]. A power calculation was not performed in advance, as this study is regarded as exploratory due to the lack of published data.

Methodology for Determination of Epigenetic Ageing Horvath's regression model [15] was applied to the DNA methylation dataset to estimate DNA methylation age, or epigenetic age, for women with PE *vs*. normotensive pregnancies. While the 353 marker loci were selected such that they would apply across cell types, the known variation of T-cell composition with ageing was not highly likely to confound our estimates. To rule out any residual confounding, we inferred cell type composition using the epigenomic deconvolution method and reference-based deconvolution methods using the most extensive set of leukocyte reference profiles. This information also helped identify any differences in the ageing of the immune system – as reflected by leukocyte composition – between women with *vs*. without PE.

Table 1
Baseline characteristics of normotensive and preeclamptic women.

Demographics	Normotensive (n = 33)	Preeclamptic (n = 11)	P value
	x ± SD	x ± SD	
Age, years	30·6 ± 4.3	29·6 ± 6.8	NS
BMI, kg/m^2	26·0 ± 6	28·2 ± 8	NS
SBP at delivery, mm Hg	121 ± 15	148 ± 9	<0·001
DBP at delivery, mm Hg	75 ± 13	97 ± 9	<0·001
GA at delivery, weeks	39·3 ± 1.5	38.4 ± 2.0	NS

BMI, body mass index; DBP, diastolic blood pressure; GA, gestational age; SBP, systolic blood pressure; SD, standard deviation.

2.9. Role of funding source

The funders of this study did not have any role in study design, data collection, data analysis, interpretation, or writing of the report.

3. Results

3.1. Epigenetic clock indicates accelerated biological ageing in PE

To examine epigenetic ageing in relation to PE, we profiled blood samples from PE (n = 11) and normotensive (n = 33) women during the first and second trimesters of pregnancy, as well as at the time of delivery, using Illumina 450 k methylation arrays. The baseline characteristics of the normotensive and PE women are presented in Table 1. Notably, women from this longitudinal cohort all developed late-onset PE. We used the profiles to estimate DNA methylation age using the elastic net regression model based on 353 CpG sites, previously proposed as an "epigenetic clock" [15].

Epigenetic ages for women with normotensive pregnancies did not change over the course of their pregnancies (32 ± 6 years for all time points). In contrast, the mean epigenetic ages increased during PE pregnancies: first trimester, 30 ± 8 years; second trimester, 31 ± 9 years; and delivery, 32 ± 9 years. There was a statistically significant difference in epigenetic aging throughout pregnancy in normotensive *vs*. PE pregnancies (P = 0·047, ANOVA for repeated measures with normotensive/PE group as between subject factor) (Fig. 1A). When comparing the estimated DNA methylation (epigenetic) ages to the actual chronological ages, there was an age increase in PE *vs.* normotensive women at delivery, with a 2·4-year difference (Fig. 1B), which corresponds to Cohen's *d* value of 0·51 and a medium effect size [13].

3.2. Senescence burden is increased in blood, urine, and adipose tissue of pregnant women with PE

To examine the physiology of the accelerated ageing-like state observed in PE, we next examined molecular markers of senescence. Circulating indicators of cellular senescence, $p16^{INK4A}$ and $p21^{CIP1}$, were measured in PE and in a subset of normotensive women (n = 13) from the above cohort. Women with PE had significantly higher concentrations of plasma $p16^{INK4A}$ compared to their normotensive counterparts (P = 0·026, Mann-Whitney U test, Fig. 2A). No statistical significance was observed in the $p21^{CIP1}$ levels in women with PE (P = 0·167, Mann-Whitney U test, Fig. 2B). We measured urinary α-Klotho in both PE and normotensive women and observed

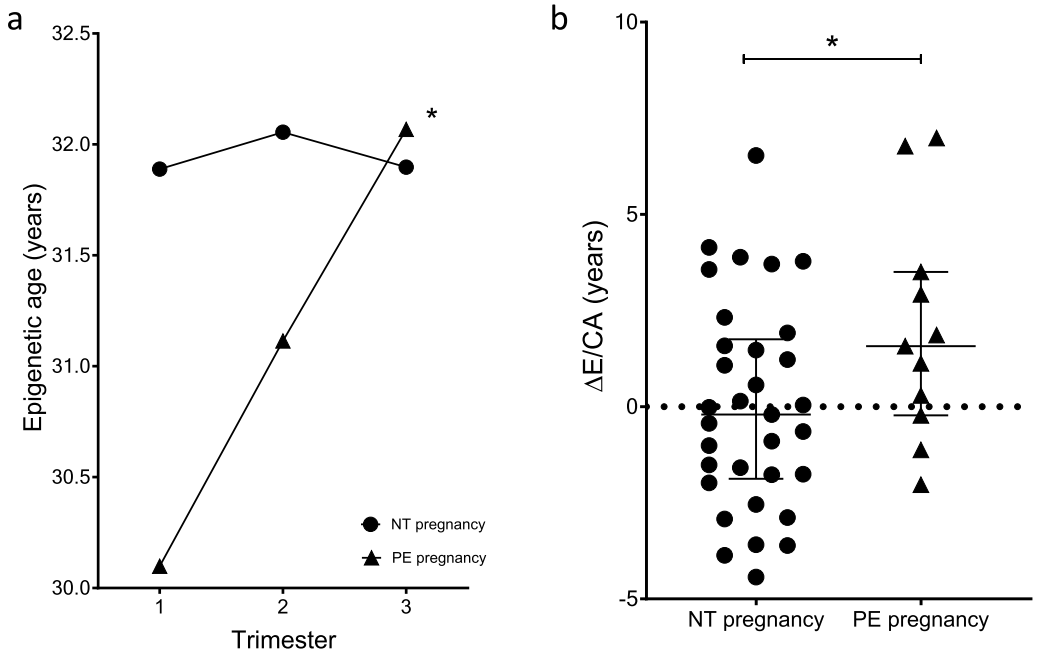

Fig. 1. Epigenetic ageing is accelerated during PE compared to normotensive pregnancies. (a) The epigenetic age of women with PE (n = 11) increased with progression of pregnancy from the first to the third trimester: first trimester, 30 ± 8 years; second trimester, 31 ± 9 years; and delivery, 32 ± 9 years. Epigenetic ages for women with normotensive pregnancies (n = 33) did not change over the course of their pregnancies (32 ± 6 years for all time points). There was a statistically significant difference in epigenetic ages change during the course of pregnancies in PE *vs.* normotensive pregnancies (P = 0·047, ANOVA for repeated measures) (b) When comparing the estimated epigenetic ages to the actual chronological ages (ΔE/CA), there was an age increase in PE *vs.* normotensive women at delivery, with a 2·4-year difference. Horvath's regression model was applied to the DNA methylation dataset to estimate DNA methylation (epigenetic) age, for women with PE and normotensive pregnancies.

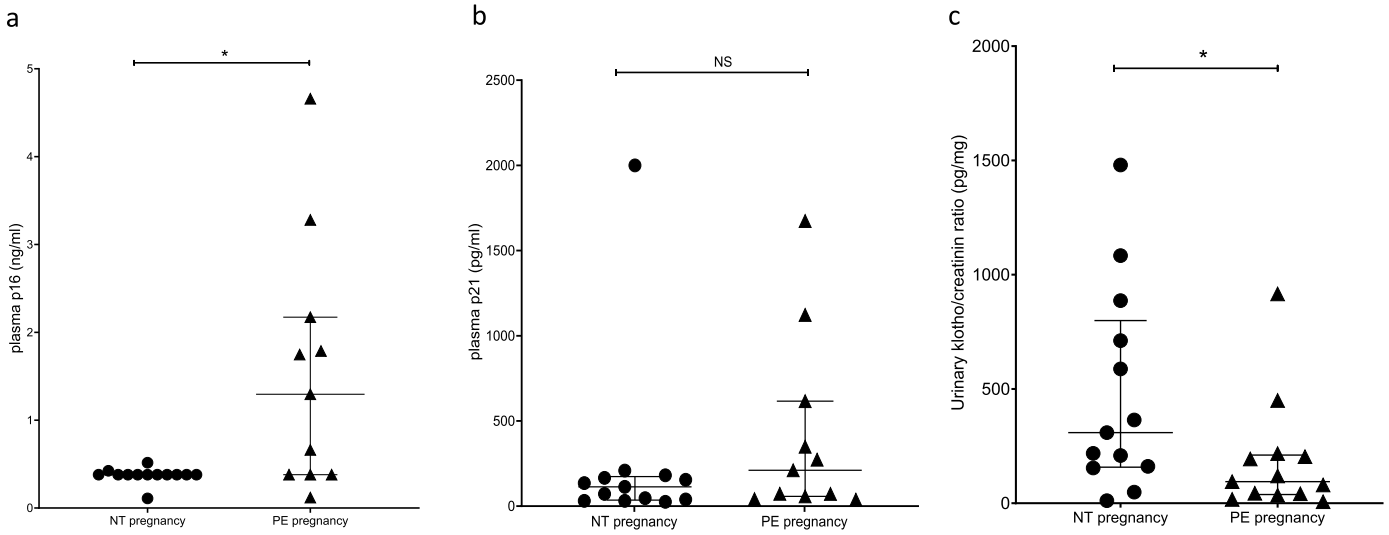

Fig. 2. Higher senescence burden occurs in pregnant women with PE compared to normotensive pregnant women. (a) Women with PE (n = 11) have *higher* plasma $p16^{INK4A}$ concentrations (0·66 [0·06–2·23]) than normotensive women (n = 13) (0·38 [0·38–0·38]), P = 0·026,) (Mann-Whitney U test) (b) Plasma $p21^{CIP1}$ concentrations were not different between the groups (normotensive: 113·5 [35·33–173·50], PE: 210·40 [57·37–616·10]; P = 0·167 (Mann-Whitney U test), NT = 13, PE = 11) (c) Urinary α-Klotho is *lower* in women with PE compared to those with normotensive pregnancies (94·66 [38·36–210·50] *vs.* 308·90 [157·90–799·10], respectively; P = 0·029, Mann-Whitney U test). Dots represent the individual values (mean value of the duplicate); lines represent the median with interquartile range. NT-normotensive, PE-preeclamptic pregnancies; *- $P < 0·05$, NS-not significant.

that women with PE had significantly lower concentrations (P = 0·029, Mann-Whitney U test, Fig. 2C). Other senescence-associated markers for normotensive and PE women at delivery are presented in Table 2. Significant increases in SASP factors were found in PE pregnancies, including neuronal growth factor (NGF) (P = 0·034, Mann-Whitney U test), MCP-1 (P = 0·047, Student's t test), and PAI-1 (P = 0·023, Student's t test).

Senescence markers were measured in adipose tissue explants obtained at delivery (C-section) from normotensive (n = 8) and PE (n = 8) women. Their demographics are presented in Table 3. These women were recruited at the time of C-section, and this cohort included those with both early and late PE (n = 4 and n = 5, respectively). Significant increases in senescence markers in adipose tissue were found in PE pregnancies for the SASP factors, MCP-1 and TNF-α (P = 0·023 and P = 0·015, Student's t test, Fig. 3A and 3B, respectively), indicating increased adipose tissue inflammation. Notably, these two specific senescence markers were also significantly increased in the plasma of PE women (Table 2).

Table 2
Circulating senescence markers from normotensive and preeclamptic women at the time of delivery.

Marker	Normotensive (n = 13) Mean (95%CI)	Preeclamptic (n = 11) Mean (95%CI)	P value
NGF†	0·87 (0·58–0·91)	0·95 (0·81·1·18)	0·034
IL-6†	3·5 (2·7–7·8)	3·9 (3·2–23·5)	0·384
Leptin	6106·31 (3381·09–8831·53)	5883·74 (3264 ·80–8502·67)	0·899
IL-8	4·81 (2·65–6·96)	5·59 (2·75–8·43)	0·626
HGF	512·00 (282·87–741·13)	555·19 (377·38–733·01)	0·754
Adiponectin	57588·85 (28463·21–86714·48)	65900·73 (33038·75–98762·71)	0·680
MCP-1	207·53 (156·30–258·76)	316·95 (206·80–427·09)	0·047
TNF-α	2·06 (1·67–2·45)	2·79 (2·06–3·51)	0·053
Resistin	109215·15 (63344·10–155086·20)	67190·45 (46061·82–88319·09)	0·101
IL-1β†	0·21 (0·05–0·29)	0·21 (0·13–0·87)	0·415
PAI-1	38117·15 (25513·69–50720·62)	58034·73 (45368·29–70701·16)	0·023

† median (25^{-}75th percentile);
NGF, neuronal growth factor; IL-6, Interleukin-6; IL-8, Interleukin-8; HGF, hepatocyte growth factor; MCP-1, monocyte chemoattractant protein 1; TNF-α, tumour necrosis factor alpha; IL-1β, Interleukin-1β; PAI-1, plasminogen-activator inhibitor 1.

Table 3
Baseline characteristics of normotensive and preeclamptic women (Adipose Tissue Analysis).

Demographics	Preeclamptic (n = 9)	Normotensive (n = 8)	P value
	x ± SD	x ± SD	
Age, years	30·3 ± 6·4	31·7 ± 6·1	NS
BMI, kg/m^2	31·7 ± 7·9	30·3 ± 9·3	NS
SBP at delivery, mm Hg	159 ± 19	122 ± 12	<0·001
DBP at delivery, mm Hg	93 ± 15	73 ± 7	<0·001
GA at delivery, weeks	34·7 ± 3·7	38·7 ± 0·7	0·008

BMI, body mass index; DBP, diastolic blood pressure; GA, gestational age; SBP, systolic blood pressure; SD, standard deviation.

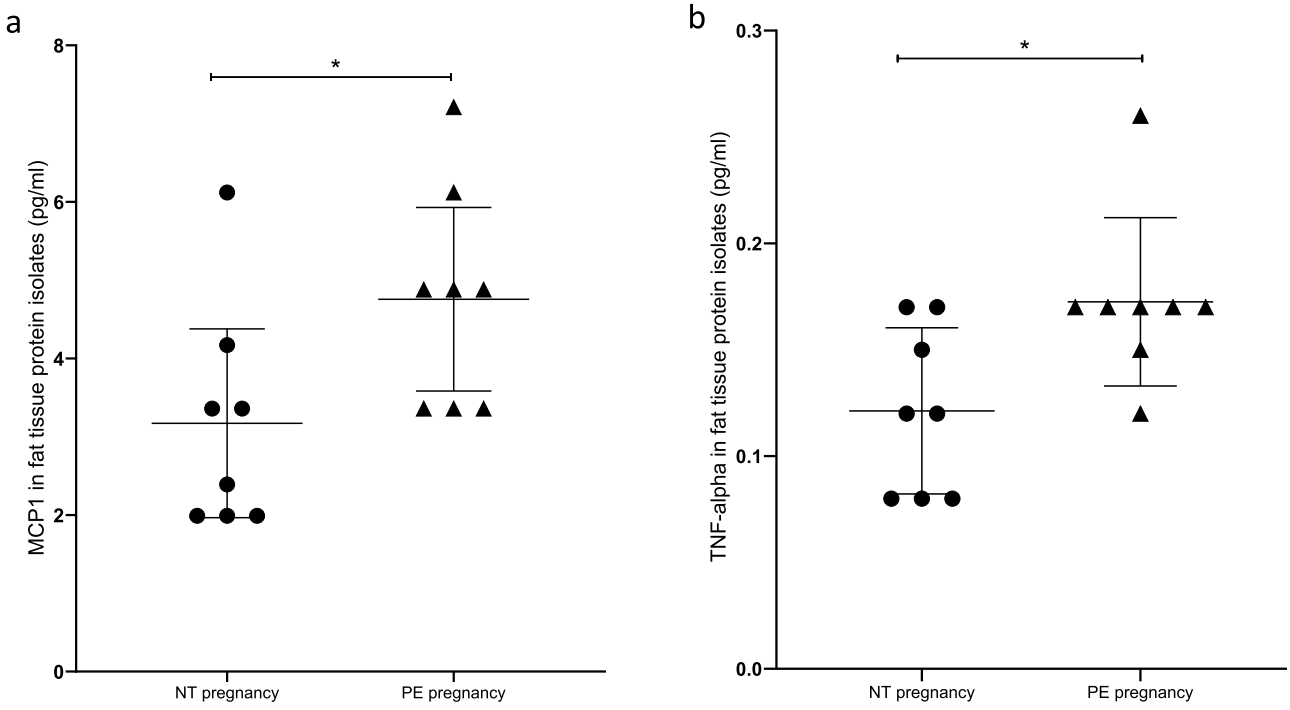

Fig. 3. Senescence-associated secretory phenotype (SASP) components are increased in adipose tissue of pregnant women with PE compared to normotensive pregnant women. (a) After protein isolation from the adipose tissue from PE (n = 8) compared to normotensive (n = 8) pregnancies, SASP component analysis was performed and indicated upregulation of both MCP-1 (4·76 ± 1·40 *vs.* 3·17 ± 1·44, respectively, P = 0·023, Student's t test) and (b) TNF-α (0·17 ± 0·04 *vs.* 0·12 ± 0·03, respectively, P = 0·015, Student's t test). Dots represent the individual values (mean value of the duplicate); lines represent the mean with SD. NT-normotensive, PE-preeclamptic pregnancies *-$p < 0·05$.

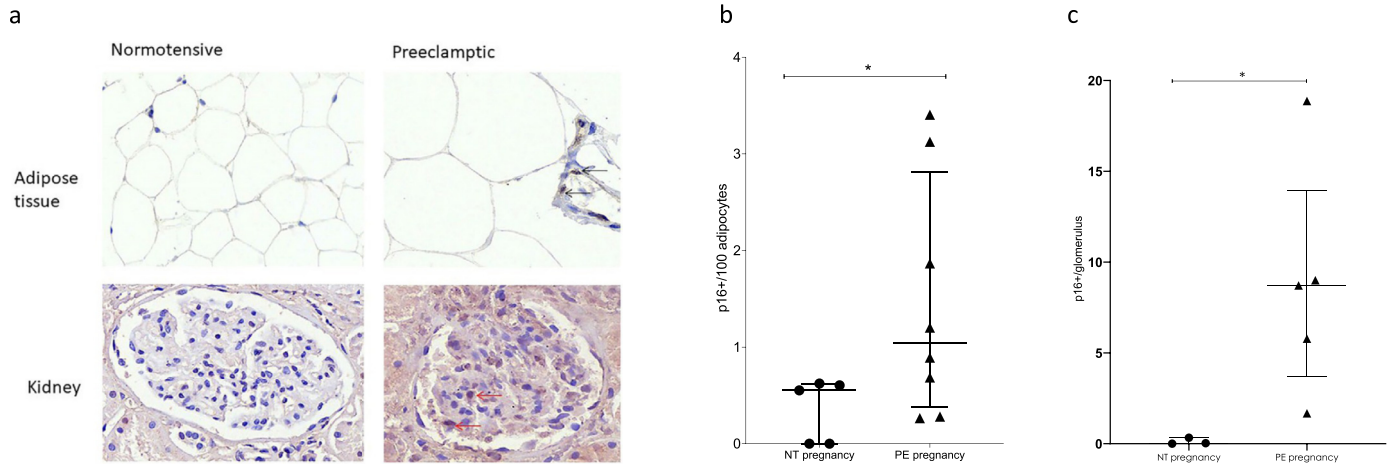

Fig. 4. Pregnant women with PE demonstrate stronger p16^{INK4A} nuclei staining of renal and adipose tissue sections, compared to normotensive pregnant women. (a) Abundant p16^{INK4A} staining in the kidney and adipose tissue sections of PE women, but not in NT pregnancies. (b-c) p16^{INK4A} expression was significantly increased in adipose tissue and renal sections in PE pregnancies compared to normotensive pregnancies: 1·04 [0·38–2·81 *vs.* 0·55 [0·00–0·61], P = 0·043 (Mann-Whitney U test) and 7·39 [3·72–13·93] *vs.* 0·03 [0·00–0·33], P = 0·034, Mann-Whitney U test), respectively. Individual values with median and interquartile ranges are presented in the graph. Red and black arrows indicate positive (brown) staining for p16^{INK4A} in the kidney and adipose tissue, respectively. NT-normotensive, PE-preeclamptic pregnancies, *- $P < 0·05$ (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

3.3. Accelerated senescence affects the kidneys and adipose tissues of pregnant women with PE

We next asked if senescence is reflected in kidney and adipose tissues from women with preeclampsia. We performed p16^{INK4A} staining of kidney and adipose tissue sections, with increased staining of nuclei in both tissue types. (Fig. 4A, arrows). P16^{INK4A+} expression was significantly increased in the women with preeclampsia compared to their normotensive counterparts, both in adipose tissue, P = 0·043 (Mann-Whitney U test) (Fig. 4B) and renal sections, P = 0·034 (Mann-Whitney U test) (Fig. 4C).

3.4. The angiogenic potential of PE-MSCs is impaired by a pro-inflammatory milieu *in the context of a senescent phenotype, and restored by the removal of the senescent phenotype by dasatinib*

We next examined the role of accelerated senescence and the associated pro-inflammatory *milieu* in impaired angiogenesis, a key feature of PE, using human umbilical vein endothelial cells (HUVECs). We monitored the cells under various conditions continuously every 6 h for 6 days and quantified angiogenesis using the total network length (mm/mm^2). To simulate the inflammatory conditions observed in PE, HUVECs were treated for 24 h with the pro-inflammatory factor, TNF-α, at a 50 ng/ml concentration. Endothelial cells treated with TNF-α demonstrated a decrease in total network length development compared to non-treated cells (P = 0·048, ANOVA for repeated measures, Fig. 5A). The angiogenic potential of PE-MSC relative to normotensive (NT)-MSC was determined by co-culturing them with TNF-α-treated endothelial cells. Angiogenesis was significantly improved in the presence of NT-MSC (P = 0·024, ANOVA for repeated measures) compared to PE-MSC (Fig. 5B). After pre-treatment of PE-MSC with dasatinib, the total network length of endothelial cells significantly increased compared to vehicle-treated PE-MSC (P = 0·016, ANOVA for repeated measures) and, indeed, reached the same angiogenic potential as NT-MSC ($P > 0·05$, ANOVA for repeated measures) (Fig. 5C).

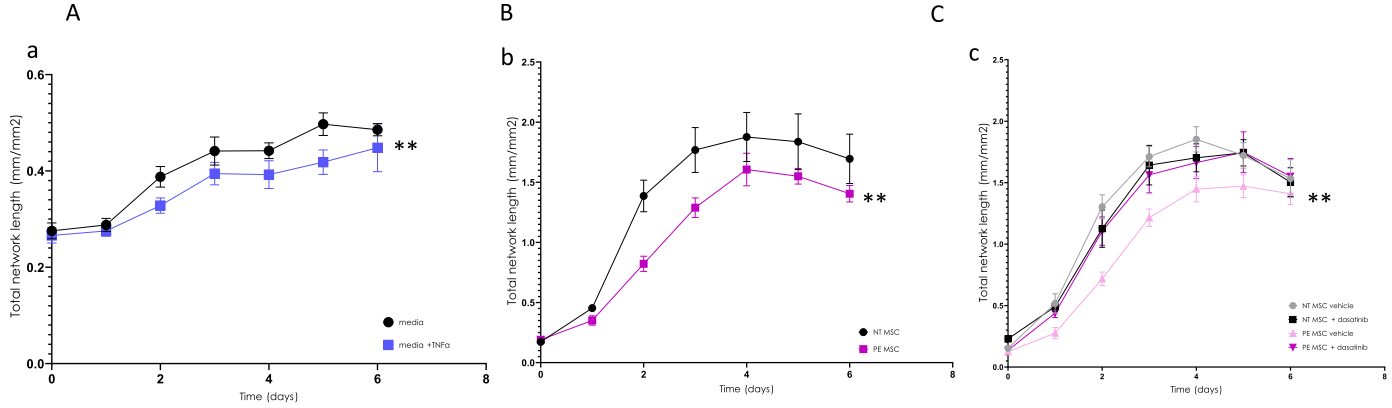

Fig. 5. Under pro-inflammatory conditions, senolytics improve the angiogenic potential of MSC isolated from PE pregnant women. (a) HUVECs pre-treated with the pro-inflammatory factor, TNF-α (50 ng/ml), demonstrate impaired angiogenesis compared to non-treated endothelial cells (P = 0·048, ANOVA for repeated measures); (b) The TNFα-treated endothelial cells were co-cultured with MSC isolated either from PE or NT pregnancies. The PE-MSC group showed a lower angiogenic potential compared to its normotensive counterpart (P = 0·024, ANOVA for repeated measures). (c) After PE-MSC were treated with senolytic agent, dasatinib, angiogenesis improved, reaching the angiogenic potential of NT-MSC ($P > 0·05$, ANOVA for repeated measures). There was a significant difference in total network growth *per* mm^2 in wells incubated with vehicle PE-MSC (treated with 1% DMSO) *vs.* dasatinib-treated PE-MSC (P = 0·016, ANOVA for repeated measures). Endothelial cells co-cultured with vehicle NT-MSC differed significantly from vehicle PE-MSC (P = 0·018, ANOVA for repeated measures). Lines represent total network growth *per* mm^2 measured as an average of sextuplicate with standard error mean. Analyzes were performed on the samples of 3 PE-MSC and 3 NT-MSC. Images were captured every 6 h and the data shown graphically are those obtained at the 24 h time points for each condition. HUVEC-human umbilical vein endothelial cells; MSC- mesenchymal stem cells; NT-normotensive, PE-preeclamptic women, **- $P < 0·05$

4. Discussion

In the current study, we report several new lines of evidence indicating that an accelerated ageing-like state and related cellular senescence contribute to the pathogenesis of PE. First, we demonstrate that PE women undergo accelerated epigenetic ageing during pregnancy, as demonstrated by the application of the "epigenetic clock," while those with normotensive pregnancies show no such change. Second, we show that cellular senescence and SASP indicators are elevated in both blood and adipose tissue sections in PE compared to normotensive pregnancies. Notably, systemic SASP factors can affect multiple organ systems, a hallmark of PE, while SASP factors in adipose tissue may compromise resident MSC, which we have shown previously to be senescent and which exhibit impaired angiogenic potential in PE women [4]. Third, we demonstrate that renal injury - one of the key clinical findings in PE - is associated with cellular senescence, as documented by (i) upregulation of $p16^{INK4A}$ in renal sections from women who died of PE and (ii) downregulation of urinary α-Klotho, a protective anti-ageing protein, [16] at the time of delivery in women with PE compared to those with normotensive pregnancies. Parenthetically, downregulation of α-Klotho is strongly implicated in the pathogenesis of renal injury and has been shown to be reduced in both acute [17] and chronic kidney disease [18]. Fourth, we expand the current understanding of the role of inflammation in PE by demonstrating impaired vascular angiogenesis under pro-inflammatory conditions, and that the angiogenic MSC potential is decreased in TNF-α pre-treated HUVECs. Fifth, we extend our previous findings that pre-treatment with dasatinib, a senolytic agent, rescues the angiogenic potential of MSC obtained from PE pregnancies, even under pro-inflammatory conditions. Taken together, our results identify cellular senescence as one of the mechanisms that drive the pathogenesis of PE.

Horvath [15] showed that age can be estimated accurately based on DNA methylation values across 353 specific CpGs that vary with age. In the diverse tissues examined in Horwath's study, this regression model predicted age with remarkable accuracy for all tissues, including whole blood, which was used in the current study. The "epigenetic clock" showed, not surprisingly, enrichment for genes that control cell survival and death, cellular growth and proliferation, tissue development, and cancer biology. Our prior studies demonstrated the role of methylation in normal and PE pregnancies by (i) demonstrating that maternal leukocyte DNA methylation is associated with maternal adaptations crucial for normal pregnancy;[19] (ii) elucidating that genome-wide methylation profiles in maternal leukocyte DNA at the time of delivery display more methylation in women with PE compared to normotensive pregnancies;[20] and (iii) finding that differential methylation may play a role in the expressions of four genes that have been previously associated with PE [21]. Our current findings further support the role of epigenetic changes in PE by demonstrating a link between PE and acceleration of epigenetic age.

Our scientific premise that an accelerated ageing-like state may play a mechanistic role in the pathophysiology of PE is supported by published studies indicating aberrant ageing and increased senescence in PE placentas [6]. The Holy Grail of PE research is commonly thought to be a substance (or substances) of placental origin with pro-inflammatory and anti-angiogenic effects, which, upon their release into the maternal circulation, lead to systemic disease. We suggest that SASP factors of placental origin may be the missing link between aberrant ageing of the placenta and senescent cell burden and maternal disease. In addition, we have recently shown that, once established, senescence can spread from cell to cell, [22] suggesting that placental senescence may lead to senescence of the remote organs and cells of the mother. We demonstrate that, in keeping with the systemic character and multisystem involvement in PE, accelerated ageing may play a role through upregulation of markers of senescence (increased circulating and abdominal tissue SASP components and upregulation of renal $p16^{INK4A}$), as well as downregulation of urinary α-Klotho, a transmembrane protein, and a known suppressor of age-related phenotypes. However, the sources of elevated circulating SASP, placenta and/or other tissues (such as adipose tissue), remain unclear and will be the topic of our future studies. We extended studies of senescence to MSC, multipotent cells that play critical roles in normal pregnancy through stimulation of angiogenesis, immune tolerance of the semi-allogeneic foetus, and anti-inflammatory effects, [23,24] including downregulation of TNF-α and stimulation of IL-10. We recently demonstrated that the number of senescent cells, as determined by senescence-associated beta-galactosidase staining, was significantly higher in PE-MSC compared to NT-MSC. PE-MSC also had significantly higher expression of the senescence marker, $p16,^{INK4A}$ and SASP-related genes [4]. After treatment with dasatinib, PE-MSC exhibited decreases in senescent cell burden and expressions of senescence markers and SASP components, and improved angiogenic potential. We extend our previous experiments to studies of MSC angiogenic potential under pro-inflammatory conditions (*i.e.* pre-treatment of HUVECs with TNF-α), which are commonly present in PE in the context of systemic inflammation. We now report that, after pre-treatment with senolytics, PE-MSC angiogenic potential improves under pro-inflammatory conditions and is comparable to that of NT-MSC. Given previous studies indicating that the characteristics of MSC residing in different tissues/organs are similar, we conclude that adipose tissue MSC, isolated from the adipose tissue biopsies obtained during C-sections, provide a window into MSC biology in a pregnant patient. Such biopsies are readily obtainable without risk during C-sections and thus provide an invaluable approach in assessing MSC behavior in normal and PE pregnancies.

Pre-intervention testing and pre-conditioning with senolytics may be necessary if MSC function is suboptimal due to cellular senescence. Use of senolytics is contraindicated during pregnancy because the process of cell senescence is critical for embryogenesis, but it can be considered in non-pregnant women with prior histories of PE-related unsuccessful pregnancies. Alternatively, senomorphic agents, such as metformin, [8] which attenuates SASP production, can be safely used during pregnancy. In addition, senolytics can be considered for women with histories of PE, who are at increased risks for post-reproductive multi-morbidity, [25] which, in turn, is considered to be a measure of accelerated ageing, and thus likely associated with an increase in senescent cell burden. Our study has several innovative aspects. We demonstrate that there is an increase in biological age and senescent cell burden at the time of delivery in PE compared to normotensive pregnancies. Additionally, we show that impaired angiogenesis in the context of a pro-inflammatory *milieu* and dysfunctional MSC, which are both present in PE, can be rescued with senolytics. Development of fast and sensitive methods for the detection of increased biological age, epigenetic age acceleration, and senescent cell burden may prove valuable in disease detection preceding symptoms, and in identifying those who may benefit from therapies that target fundamental senescence processes (*i.e.* senolytics), either systemically or when rescuing the angiogenic potential of senescent MSC in preeclampsia.

5. Limitations

The main limitation of our study is the sample size. Although it does not impact the accuracy of the estimation of biological (epigenetic) age, it does influence the determination of statistical significance for the change in biological age during pregnancy (from beginning to end of pregnancy). On the other hand, while differences in some physical factors (such as age and BMI) could have confounding effects on the epigenetic results, it is likely that they have little effect on the assessment of the change in biological age during

pregnancy given the use of longitudinal measurements. Epigenetic ageing was studied in a longitudinal cohort of women who all developed late-onset PE and for whom circulating senescence markers were measured at the time of delivery. Predominance of late PE is not unexpected given our small sample size and the fact that early onset PE accounts for approximately 12% of all PE cases [26]. While we have not tested women with early PE, we postulate that changes in the epigenetic clock and SASP (similar to pro-inflammatory and anti-angiogenic markers) [27] can only be more pronounced in early compared to late PE. This will be the focus of our future studies. In a separate cohort of women who delivered via C-section, we were able to recruit both early and late PE cases for our cross-sectional study of the adipose tissue SASP, but our small sample size did not allow for characterization of senescence markers across the PE clinical spectrum (from early, severe through late, mild). Despite these limitations, our study provides preliminary results and proof-of-concept evidence that set the stage for preclinical studies in animal models that will explore causality and the role of senescence in the pathophysiology of preeclampsia. In addition, we plan prospective clinical studies that will be adequately powered to study markers of senescence across the clinical spectrum of preeclampsia (*i.e.*, early *vs.* late, mild *vs*. severe).

Contributors

SS performed experiments, analyzed data, wrote and reviewed the manuscript. RG, HC, HT, LP, YZ, FTC performed experiments, analyzed data and reviewed the manuscript. WW, YSBT helped with patient recruitment, data analysis, and reviewed the manuscript. NMM performed statistical analysis, verified and analyzed data and wrote the manuscript. TT, JPG, JMC, AM, KN, JLK: study design, analyzed data, and reviewed the manuscript. VDG designed and planned experiments, verified and analyzed data, and wrote and reviewed the manuscript.

Declaration of Competing Interest

YZ, TT, and JLK have a financial interest related to this research. Patents on senolytic drugs are held by Mayo Clinic. This research has been reviewed by the Mayo Clinic Conflict of Interest Review Board and was conducted in compliance with Mayo Clinic Conflict of Interest policies. The other authors have no conflicts of interest.

Data sharing statement

Qualified researchers may request access to raw data and materials by sending a request to the corresponding author. Data will be shared after approval of a proposal, with a signed data access agreement.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ebiom.2021.103536.

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