

# Proteomic analysis reveals angiogenesis-related plasma proteins associated with pre-eclampsia in SLE

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## ABSTRACT

**Objective** Delivery of a small for gestational age (SGA) infant is a common pregnancy complication among women with SLE. Although disease activity and autoantibodies such as anti-Smith and anti-ribonucleoprotein associate with SGA, underlying pathological mechanisms remain unclear and reliable predictors are lacking. To address this, we applied a proteomic approach to identify proteins associated with SGA in SLE.

**Methods** Plasma samples were collected repeatedly during pregnancy, at delivery and from placental intervillous blood in women with SLE (n=83) and healthy controls (n=67) enrolled in the prospective SLE-Placenta study. Postpartum samples (≥6 months) from a subset of women with SLE (n=19) served as non-pregnant controls. Mass spectrometry was performed on a discovery cohort comprising six healthy uncomplicated pregnancies, eight uncomplicated SLE pregnancies and eight SLE pregnancies complicated by SGA (SLE-SGA). Differential protein abundance analysis was performed in R. Candidate proteins were quantified by ELISA in the full cohort.

**Results** Discovery proteomics identified four proteins with increased abundance in SLE-SGA compared with uncomplicated SLE pregnancies: endostatin ( $P_{adj}=0.0003$ ), angiogenin ( $P_{adj}=0.03$ ), insulin-like growth factor-binding protein 5 ( $P_{adj}=0.03$ ) and complement factor H-related protein 5 ( $P_{adj}=0.004$ ). In the full cohort, ELISA quantification did not confirm increased levels of these proteins in SLE-SGA but suggested elevated levels of the angiogenesis-related proteins endostatin and angiogenin in women with SLE who later developed pre-eclampsia. Endostatin levels were consistently higher in SLE compared with controls across all trimesters ( $p\leq 0.0001$ ). Endostatin, but not angiogenin, was enriched in placental blood.

**Conclusion** Our study did not validate the differentially abundant proteins as markers for SLE-SGA but suggested a link between the antiangiogenic and proangiogenic proteins, endostatin and angiogenin, respectively, and pre-eclampsia in SLE. Given the consistent elevation of endostatin throughout pregnancy in SLE compared with controls, its potential effects on placental development in SLE warrant further investigation.

## WHAT IS ALREADY KNOWN ON THIS TOPIC

- ⇒ Women with SLE have a higher risk of adverse pregnancy outcomes than the general population.
- ⇒ Disease activity and specific autoantibodies have been linked to these complications, but the underlying pathophysiological mechanisms remain poorly understood and reliable predictors are lacking.

## WHAT THIS STUDY ADDS

- ⇒ Women with SLE exhibit an imbalance of angiogenesis-related proteins, with the antiangiogenic protein endostatin persistently elevated in blood throughout pregnancy compared with healthy controls.
- ⇒ Elevated endostatin levels in SLE pregnancies are associated with pre-eclampsia, mirroring findings in the general population and suggesting a shared pathophysiological mechanism.
- ⇒ Endostatin is selectively enriched in intervillous blood, implicating the placenta as a likely source of the elevated circulating levels in SLE pregnancy.

## HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

- ⇒ The observed imbalance in angiogenesis-related pathways in SLE pregnancy may help guide future research into the biological role of endostatin and related proteins in placental development and function.

## INTRODUCTION

SLE is a chronic, inflammatory autoimmune disease that predominantly affects women. The onset often occurs during the fertile years and the disease tends to worsen during pregnancy.<sup>1</sup> Pregnancy outcomes among women with SLE have improved over time, likely due to better preconception planning, monitoring and treatment.<sup>2</sup> However, pregnancies in women with SLE still carry increased risks of complications for both mother and fetus,

including pre-eclampsia, preterm birth and delivery of a small for gestational age (SGA) infant.<sup>3,4</sup> Some clinical risk factors have been identified, such as previous lupus nephritis, active lupus prior to conception and chronic hypertension, which are associated with an increased risk of pre-eclampsia and preterm birth in women with SLE.<sup>4</sup> Furthermore, active lupus before conception and the presence of anti-Smith and anti-ribonucleoprotein antibodies are associated with an increased risk of SGA.<sup>4</sup> Still, the pathophysiological mechanisms underlying the increased risk of pregnancy complications in SLE remain poorly understood and current clinical risk factors are insufficient to predict specific adverse pregnancy outcomes.

Giving birth to an SGA infant is a common adverse pregnancy outcome among women with SLE.<sup>3,5,6</sup> We recently reported that SGA is related to circulating interferon alpha levels,<sup>5</sup> a protein with immunostimulatory as well as antiangiogenic effects.<sup>7</sup> In SLE, no studies have systematically screened for circulating proteins associated with SGA. Therefore, we here employed an unbiased proteomic approach in a discovery cohort of pregnant women with SLE, a subset of our longitudinal SLE-Placenta cohort, to identify plasma proteins associated with subsequent SGA births. ELISA-based quantification of candidate proteins was then used for proteomic verification and to investigate their relation to pre-eclampsia and preterm birth in the full SLE-Placenta cohort. We further compared the identified proteins in SLE with healthy pregnancies, irrespective of pregnancy outcome.

## MATERIALS AND METHODS

### Cohort

This study included 83 SLE pregnancies from the prospective Swedish multicentre SLE-Placenta study.<sup>5,8,9</sup> Participants with SLE met the 1997 American College of Rheumatology and/or 2012 Systemic Lupus International Collaborating Clinics classification criteria<sup>10,11</sup> and were recruited from rheumatology clinics in Gothenburg (Sahlgrenska University Hospital, n=27), Stockholm (Karolinska University Hospital, n=38), Uppsala (Uppsala University Hospital, n=3), Linköping (Linköping University Hospital, n=6) and Lund (Skåne University Hospital, n=9). Healthy pregnant women (n=67) were recruited at one antenatal clinic in Gothenburg (Regionhälsan, Gothenburg). Pregnant women with SLE and healthy pregnant controls were enrolled between October 2018 and January 2023. Only live singleton births were included, and seven women with SLE participated in the study twice. Exclusion criteria included the inability to understand study-related patient information and consent forms, the presence of other serious diseases (eg, active malignancy or other rheumatic autoimmune diseases) or treatment with anti-BAFF or anti-CD20 antibodies within 12 months prior to inclusion (online supplemental figure S1). All participants gave their written informed consent.

### Adverse pregnancy outcomes

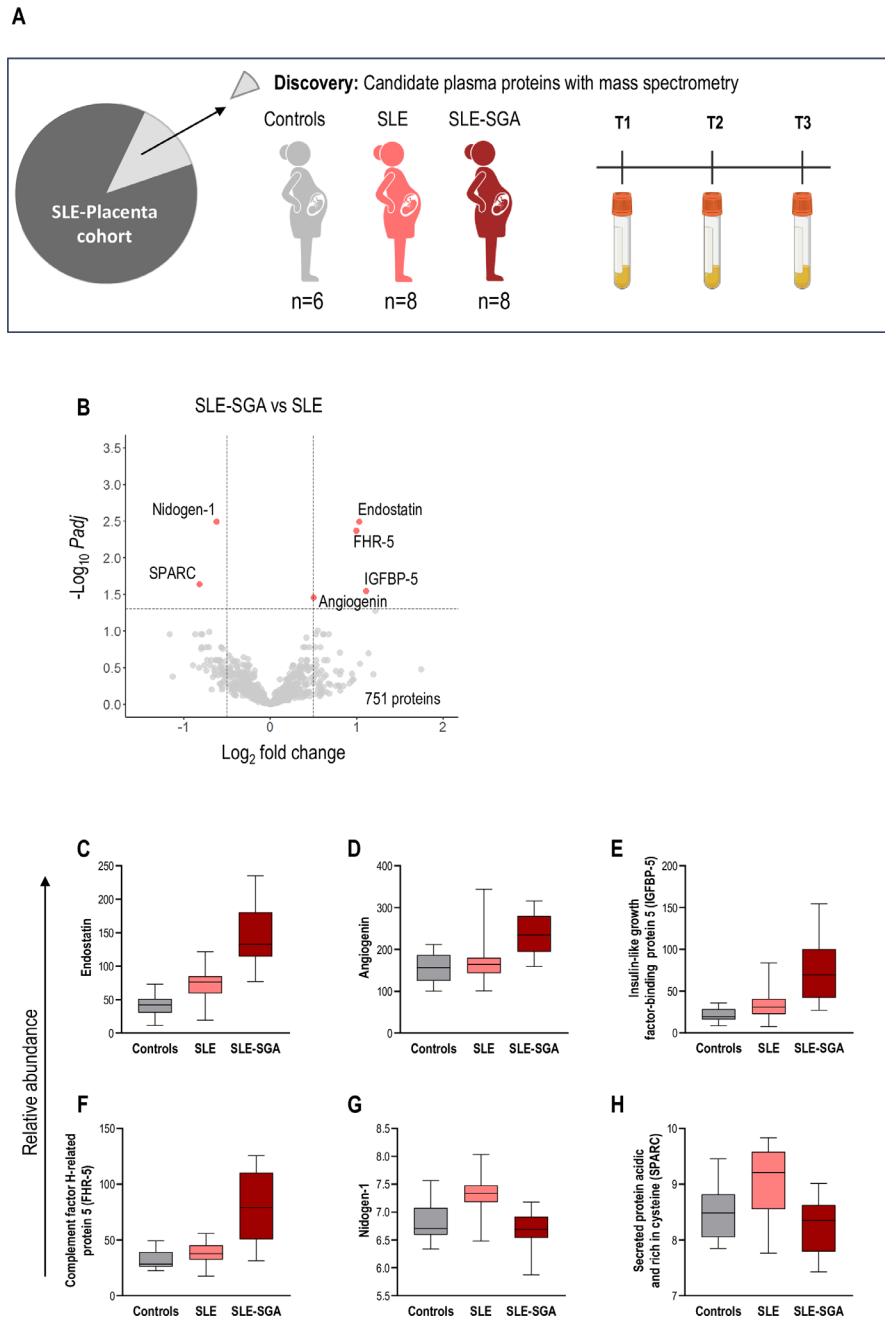
Clinical and obstetric data, including disease duration, medication, gestational age at birth and pregnancy outcome were retrieved from medical records. Disease activity was assessed at least once during pregnancy using the Systemic Lupus Disease Activity Index 2000 (SLEDAI-2K) in accordance with local clinical routines.<sup>12</sup> When multiple assessments were available, the highest SLEDAI-2K score was used. As previously reported for this cohort, SLEDAI-2K values remained stable throughout pregnancy.<sup>8</sup> Autoantibody status during pregnancy was determined as previously described.<sup>8</sup> Adverse pregnancy outcomes included SGA, pre-eclampsia and preterm birth. SGA was defined as birth weight below the 10th percentile of expected according to Marsál and/or Gardosi.<sup>13,14</sup> Pre-eclampsia was defined as a multiorgan disease occurring after 20 weeks of gestation, characterised by hypertension and new-onset clinical symptoms or involvement of one or more organ systems (renal, hepatic, neurological, haematological, circulatory or uteroplacental),<sup>15</sup> and preterm birth as delivery before 37 weeks of gestation. Overlapping outcomes were observed in four women with SLE: one with both SGA and pre-eclampsia, two with SGA and preterm birth and one with pre-eclampsia and preterm birth. In outcome-dependent analyses, data from these individuals were included in both relevant outcome groups.

### Sample collection

Peripheral blood (PB) samples were collected in heparinised tubes from pregnant women with SLE and healthy pregnant women in the first (SLE n=45, controls n=50), second (SLE n=71, controls n=49), third (SLE n=74, controls n=61) trimester and at delivery (SLE n=26, controls n=20). Placentas were collected after birth and maternal-derived intervillous blood (IVB) was collected by manual compression (SLE n=26, controls n=20). For a subset of women with SLE, late postpartum samples were collected at least 6 months after delivery to serve as non-pregnant controls (n=19). Density centrifugation of whole blood was performed to isolate plasma that was kept frozen (-80°C) until further analysis. The number of collected plasma samples is summarised in online supplemental table 1.

### Proteomic analysis of plasma

Based on sample availability, a discovery cohort was selected from the SLE-Placenta cohort for proteomic analysis: SLE pregnancies complicated by SGA (SLE-SGA, n=8), uncomplicated SLE pregnancies (SLE, n=8) and healthy uncomplicated pregnancies (controls, n=6) (figure 1A). In the SLE-SGA group, two delivered preterm, one of whom also developed pre-eclampsia. The proteomic analysis was conducted using mass spectrometry at the Core Facilities, University of Gothenburg, Sweden. The number of plasma samples analysed per trimester is summarised in (online supplemental table 1).



**Figure 1** In the discovery cohort, mass spectrometry-based analysis of combined first-trimester, second-trimester and third-trimester samples identified four candidate proteins associated with SGA births among women with SLE. (A) Illustration of the discovery cohort that was selected as a subset from the full SLE-Placenta study for proteomic analysis. (B) Volcano plot showing differentially abundant proteins among women who gave birth to SGA infants (SLE-SGA, samples  $n=17$ ) compared with uncomplicated SLE pregnancies (SLE, samples  $n=18$ ) in the discovery cohort. Box plots showing the relative abundance of (C) endostatin (uniprot ID: P39060), (D) angiogenin (uniprot ID: P03950) (E) IGFBP-5 (uniprot ID: P24593), (F) FHR-5 (uniprot ID: Q9BXR6), (G) Nidogen-1 (uniprot ID: P14543) and (H) SPARC (uniprot ID: P09486) in uncomplicated healthy pregnancies (controls, samples  $n=14$ ), SLE and SLE-SGA. Differentially abundant proteins were defined as proteins with an adjusted  $p$  value  $<0.05$  and a  $\log_2$  fold change  $>|0.5|$ . (B) Welch's  $t$ -test followed by Benjamini-Hochberg correction for multiple comparison adjustment. FHR-5, complement factor H-related protein 5; IGFBP-5, insulin-like growth factor-binding protein 5; SGA, small for gestational age; SPARC, secreted protein acidic and rich in cysteine.

Sample preparation and liquid chromatography mass spectrometry (LC-MS) analysis: relative quantification was performed to compare protein expression in plasma from the SLE-SGA, SLE and the control groups. A volume of  $5\mu\text{l}$  plasma was diluted with phosphate-buffered

saline (PBS) to a total volume of  $680\mu\text{l}$ , and High Select Depletion Spin Columns and bulk resin (Thermo Scientific) was used to deplete the 14 most abundant plasma proteins (albumin, IgG, IgA, IgM, transferrin, haptoglobin,  $\alpha 1$ -antitrypsin, fibrinogen,  $\alpha 2$ -macroglobulin,

$\alpha$ 1-acid glycoprotein, apolipoprotein A-I, apolipoprotein A-II, complement C3 and transthyretin), using a bead to diluted plasma ratio of 50:20. Proteins (2.1  $\mu$ g) were reduced in 5 mM dithiothreitol (DTT) and 50 mM triethylammonium bicarbonate at 37°C for 30 min and alkylated in 10 mM chloroacetamide at room temperature (RT) for 20 min. Alkylation was quenched by 5 mM DTT and proteins were digested by Lys-C (1:10, Promega) while shaking overnight at 37°C. Trypsin (1:10, Thermo Scientific) was added and incubated for another 3 hours.

Next, LC-MS analysis was performed. In brief, samples were diluted to a final concentration of 25 ng/ $\mu$ L in 0.1% formic acid. Further, 500 ng of the samples were loaded onto Evotips Pure (Evosep) according to the manufacturer's instructions. LC-MS analysis was performed on a timsTOF HT mass spectrometer (Bruker) coupled to an Evosep One LC system (Evosep). The LC system was used running the 30 samples per day method on a Pepsep C18 column (15 cm  $\times$  150  $\mu$ m ID, 1.5  $\mu$ m particle size, Bruker). The timsTOF HT was run in dia-PASEF (Data-independent acquisition Parallel Accumulation Serial Fragmentation) mode using a pyDIA-PASEF long gradient method, covering a mass range of 250–1300 Da. Collision energy was set from 20 to 59 eV along an ion-mobility range of 0.6 to 1.6 Vs/cm<sup>2</sup>.

Raw data was analysed using Spectronaut (V.18.5) using directDIA analysis. The protein database used was Swissprot human (April 2023, 20422 entries). Enzyme was set to trypsin/P allowing one missed cleavage. Fixed Modification was set to Carbamidomethyl (C) and variable modifications were Acetyl (Protein N-term) and Oxidation (M), allowing a maximum of 5 variable modifications. All pulsar search and directDIA settings were kept as default. The proteotypicity filter was set to only protein group specific. The Protein LFQ method was left to automatic, resulting in MaxLFQ being used. Cross-run normalisation was performed choosing the automatically picked local normalisation strategy.

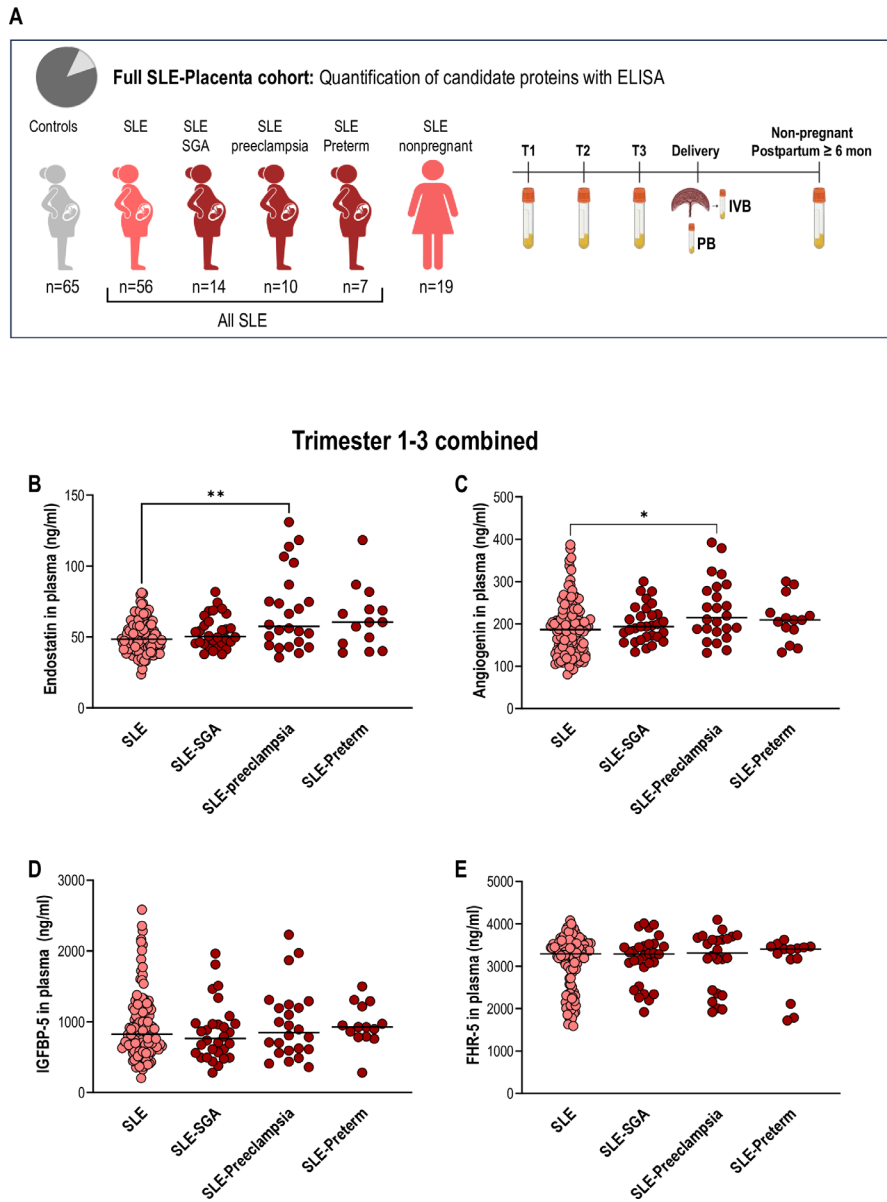
## ELISA

Proteins that showed differential abundance between the SLE-SGA and SLE groups, and differed from the control group in the proteomic analysis, were selected for further exploration and quantification in the full SLE-placenta cohort. In the full cohort, uncomplicated SLE pregnancies (SLE, n=56) were compared with SLE-SGA (n=14), women with SLE and pre-eclampsia (SLE-pre-eclampsia, n=10) or women with SLE and preterm births (SLE-preterm, n=7). In addition, protein levels were measured in plasma from healthy pregnant women (controls, n=67) and from a subgroup of women with SLE in the late postpartum period (SLE-non-pregnant, n=19) (figure 2A). The number of analysed samples at each time point is summarised in online supplemental table 1. Plasma concentrations of endostatin (dilution 1:20), angiogenin (dilution 1:600) and insulin-like growth factor-binding protein 5 (IGFBP-5, dilution 1:300) were

quantified, following the manufacturer's instructions using Human Endostatin DuoSet ELISA (#DY1098, R&D Systems), Human Angiogenin DuoSet ELISA (#DY265, R&D Systems) and Human IGFBP-5 ELISA kit (#CSB-E13263h, CUSABIO). For quantification of complement factor H-related protein 5 (FHR-5, dilution 1:100), a sandwich ELISA was established as detailed below. 96-well half-area microplates (#3690, Corning) were coated with anti-CFHR5 antibody (#MAB3845, R&D Systems) diluted to 1.5  $\mu$ g/mL in PBS and incubated overnight at RT. Plates were washed two times with 0.05% Tween in PBS and then blocked with 1% bovine serum albumin (BSA) (A4503, Sigma-Aldrich) in PBS for 1 hour at RT. After washing plates once with 0.05% tween in PBS, plasma samples and standard Recombinant Human FGF-5 Protein (#3845-F5-050, R&D Systems) were added and incubated for 2 hour at RT. After washing three times, anti-CFHR5 biotinylated antibody (#BAF3845, R&D Systems) (diluted to 0.1  $\mu$ g/mL in 1% BSA in PBS) was added and incubated for 2 hour at RT. Again, plates were washed three times and then incubated with streptavidin-horseradish peroxidase (SA-HRP) (#DY998, R&D Systems) for 20 min at RT. Thereafter, BD OptEIA TMB Substrate (#555214, BD Biosciences) was added and incubated for 13 min at RT. The colour reaction was stopped by adding 1M H<sub>2</sub>SO<sub>4</sub>. Optical density was then assessed at 450 nm. Plasma samples, standard, biotinylated antibody and SA-HRP were diluted in 1% BSA in PBS. For all proteins, standard curves showed good performance, and measured concentrations were within the quantifiable range.

## Statistical analyses

Analysis of proteomic results was performed in R V.4.3.2. Proteins with more than 20% missing values among either SLE or SLE-SGA in the discovery cohort were excluded. The remaining 751 proteins were log<sub>2</sub>-transformed and used for further analyses. Filtering with the inclusion of the control group yielded 740 proteins (online supplemental figure S2). After normality testing, a two-sample Welch's t-test with p values adjusted for multiple comparison using the Benjamini-Hochberg method was chosen to compare protein abundance between SLE-SGA and SLE. Log<sub>2</sub> fold change was calculated as the difference between mean log<sub>2</sub> values of each protein in the two groups. Differentially abundant proteins were defined as proteins with an adjusted p value <0.05 and a log<sub>2</sub> fold change > |0.5|. Additionally, to further explore potential differences that may not reach significance after multiple testing correction, we performed a differentially abundance analysis using an unadjusted significance threshold (p<0.05). Subsequently, Gene Ontology (GO) enrichment analysis was performed separately for upregulated and downregulated proteins using the *clusterProfiler* package in R. Enriched GO terms were identified from the differential abundant



**Figure 2** Quantification of proteomic candidate proteins showed elevated plasma levels of endostatin and angiogenin in women with SLE and pre-eclampsia in the full SLE-Placenta cohort. (A) Illustration of the full SLE-Placenta cohort, including healthy pregnant women (controls) and pregnant women with SLE, that were used for ELISA quantification. Plasma protein concentration of (B) endostatin, (C) angiogenin, (D) IGFBP-5 and (E) FHR-5 in uncomplicated SLE pregnancies (SLE, samples  $n=130$ ) compared with SLE-SGA (samples  $n=30$ ), SLE-Pre-eclampsia (samples  $n=24$ ) or SLE-Preterm (samples  $n=14$ ). One woman was included in both the SLE-SGA and SLE-Pre-eclampsia groups, two were included in both the SLE-SGA and SLE-Preterm groups and one was included in both the SLE-Pre-eclampsia and SLE-Preterm groups.  $*p<0.05$ ,  $**p<0.01$ . (A–B) Kruskal-Wallis followed by Dunn’s multiple comparison test. FHR-5, complement factor H-related protein 5; IGFBP-5, insulin-like growth factor-binding protein 5; IVB, intervillous blood; PB, peripheral blood; SGA, small for gestational age.

proteins based on unadjusted  $p$  values. GO terms with an adjusted  $p$  value ( $p\leq 0.05$ ) and a false discovery rate ( $q\leq 0.2$ ) were considered statistically significant. Kruskal-Wallis followed by Dunn’s multiple comparison test was used to compare protein concentrations quantified by ELISA and for comparison of protein concentrations in PB between the third trimester and delivery, Mann-Whitney U test was used (GraphPad Prism software, La Jolla, California, USA).  $P$  values of  $<0.05$  were considered statistically significant.

## RESULTS

### Proteomic analysis reveals four plasma proteins elevated in SLE pregnancies with SGA

We used a proteomic approach in the discovery cohort to identify proteins associated with subsequent SGA birth in women with SLE. The study design is presented in figure 1A. Demographic and clinical characteristics including maternal age at delivery, body mass index, parity and gestational age at delivery were comparable across all three groups (online supplemental table 2).

Differential abundance analysis was performed on plasma samples from all three trimesters combined (online supplemental table 1). The analysis identified four proteins with significantly higher relative abundance in plasma from SLE-SGA compared with SLE: the antiangiogenic protein endostatin ( $P_{adj}=0.003$ ), the proangiogenic protein angiogenin ( $P_{adj}=0.04$ ), IGFBP5 ( $P_{adj}=0.03$ ) and FHR-5 ( $P_{adj}=0.004$ ) (figure 1B–F). Of these, endostatin also indicated a higher protein abundance in the SLE compared with the controls (figure 1C). These four proteins were selected for further exploration in the full SLE-Placenta cohort. In addition, nidogen-1 ( $P_{adj}=0.003$ ) and secreted protein acidic and rich in cysteine ( $P_{adj}=0.02$ ) were significantly less abundant in SLE-SGA compared with SLE (figure 1B and figure 1G–H). However, as the abundance of these proteins was similar in SLE-SGA and controls, they were not included in further analyses (figure 1G–H). To explore whether proteins of potential biological interest might have been missed due to stringent multiple testing correction, we also performed a differential abundance analysis using unadjusted *p* values. This analysis identified 22 upregulated and 36 downregulated proteins (online supplemental figure S3A). GO enrichment analysis of upregulated and downregulated proteins, respectively, revealed no clear or consistent biological processes, with only a few proteins driving the significant terms (online supplemental figure S3B).

### Endostatin and angiogenin concentrations are associated with pre-eclampsia but not SGA in SLE pregnancies

We pursued the identified candidate proteins that distinguished SLE-SGA from both SLE and controls for further exploration in the full SLE-Placenta cohort (figure 2A). Demographic and clinical characteristics were similar across the groups (table 1). To compare protein concentration between pregnancy outcome groups, we analysed plasma samples from the first, second and third trimester of each woman, which were combined for the analysis (online supplemental table 1). As shown in figure 2B–E, neither endostatin, angiogenin, IGFBP-5 nor FHR-5 levels significantly differed between SLE and SLE-SGA or SLE-Preterm. However, women in the SLE-Pre-eclampsia group presented with significantly higher endostatin and angiogenin levels compared with SLE (figure 2B,C). Nine of the 10 women who developed pre-eclampsia had a late onset (>34 weeks), and in 8 of these cases, the third trimester plasma samples were collected before onset. Separate analysis for the first, second and third trimester for all four proteins is shown in online supplemental figure S4 and S5A,B. In summary, ELISA quantification did not confirm the proteomic results for SGA but suggested elevated levels of the antiangiogenic and proangiogenic proteins endostatin and angiogenin, respectively, in women with SLE and pre-eclampsia.

### Gestational increase of endostatin with consistently elevated levels in SLE pregnancies

To further explore the candidate proteins in SLE pregnancy, we compared their concentrations in plasma from all women with SLE ( $n=83$ ) and controls ( $n=67$ ) in the SLE-Placenta cohort, regardless of pregnancy outcomes. Endostatin levels were consistently higher throughout pregnancy in women with SLE compared with controls (T1 median values (ng/mL): SLE 45.7 vs controls 37.5,  $p\leq 0.0001$ ; T2: SLE 47.7 vs controls 37.9,  $p\leq 0.0001$ ; T3: SLE 55.3 vs controls 46.3,  $p\leq 0.0001$ ). In women with SLE, endostatin levels remained comparable between the non-pregnant state and the first two trimesters, followed by a significant increase in the third trimester (figure 3A). A similar third-trimester increase in endostatin was also found in the controls (figure 3B). However, angiogenin levels were higher in the non-pregnant state compared with the first and second trimesters, followed by an increase in the third trimester, returning to levels comparable to those in non-pregnant women (figure 3C). A third-trimester increase was also observed in the controls (figure 3D). No differences in angiogenin levels were found between women with SLE and controls. In SLE, IGFBP-5 levels slightly decreased between the first and the third trimester and were consistently higher in SLE compared with controls (T1 median values (ng/mL): SLE 950.8 vs controls 658.9,  $p\leq 0.001$ ; T2: SLE 819.2 vs controls 644.5,  $p\leq 0.01$ ; T3: SLE 795.0 vs controls 584.8,  $p\leq 0.01$ ) (online supplemental figure S6A,B). FHR-5 levels were unrelated to pregnancy progression and did not differ between SLE and controls (online supplemental file 6C,D). We next examined whether treatment influenced endostatin and angiogenin levels in SLE pregnancies. Endostatin concentrations were unaffected by prednisone or azathioprine but were slightly higher in women receiving low molecular weight heparin (LMWH) during the second and third trimesters (online supplemental figure S7A–C). Angiogenin levels were unaffected by prednisone, lower in women treated with azathioprine and modestly higher in those receiving LMWH in late pregnancy (online supplemental figure S7D–F). For both proteins, considerable overlap was observed between treated and untreated groups. Hydroxychloroquine and acetylsalicylic acid were not analysed, as most women with SLE received these treatments. To conclude, endostatin levels showed a marked increase in the third trimester compared with the non-pregnant state in SLE. Moreover, concentrations of the antiangiogenic endostatin were consistently higher, independent of treatment, throughout pregnancy in SLE compared with controls.

### Selective enrichment of endostatin in intervillous blood at delivery

At delivery, endostatin levels were markedly elevated in placental maternal-derived IVB compared with PB in

**Table 1** SLE-Placenta cohort: characteristics of pregnant women with SLE and pregnant healthy controls

	Controls (n=67)	SLE (n=83)	SLE non-complicated (n=56)	SLE-SGA (n=14)	SLE-PE (n=10)	SLE-preterm (n=7)
Age at delivery (years)*	33 (18–42)	32 (19–44)	32 (19–42)	33 (27–44)	34 (31–40)	32 (27–36)
BMI, kg/m <sup>2</sup> *	24 (18–37)	23 (18–35)	22 (18–35)	25 (19–32)	25 (19–30)	24 (21–34)
Nulliparous†	43 (64)	50 (60)	34 (61)	8 (57)	6 (60)	2 (29)
Gestational age (weeks)*	40.3 (32.1–42.0)	39.7 (28.1–42.0)	39.9 (37.0–42.0)	39.4 (34.7–41.0)	37.9 (36.7–40.4)	35.7 (28.1–36.9)
ACR criteria ever‡						
Malar rash		31 (38)‡	22 (40)‡	3 (21)	5 (50)	4 (57)
Discoid rash		6 (7)‡	3 (5)‡	1 (7)	1 (10)	2 (29)
Photosensitivity		40 (49)‡	24 (44)‡	7 (50)	6 (60)	4 (57)
Oral ulcers		31 (38)‡	19 (35)‡	7 (50)	5 (50)	2 (29)
Arthritis		68 (83)‡	46 (84)‡	11 (79)	8 (80)	6 (86)
Serositis		18 (22)‡	10 (18)‡	5 (36)	3 (30)	1 (14)
Renal disorder		32 (39)	24 (43)	5 (36)	2 (20)	2 (29)
Neurological disorder		6 (7)	4 (7)	1 (7)	1 (10)	0 (0)
Haematological disorder		53 (64)	32 (57)	9 (64)	9 (90)	6 (86)
Immunological disorder		74 (89)	48 (86)	14 (100)	9 (90)	7 (100)
ANA		82 (99)	55 (98)	14 (100)	10 (100)	7 (100)
Disease duration (years)*		9 (0–26)	8 (0–20)	8 (0–24)	13 (3–26)	14 (0–18)
SLEDAI-2K*		2 (0–18)‡	2 (0–12)‡	2 (0–18)	4 (0–14)	2 (0–18)
ANA fine specificity during pregnancy‡						
Anti-dsDNA		31 (37)	22 (39)	3 (21)	4 (40)	3 (43)
Anti-Sm		12 (14)	7 (13)	2 (14)	1 (10)	2 (29)
Anti-SSA		25 (30)	13 (23)	6 (43)	4 (40)	4 (57)
Anti-SSB		8 (10)	4 (7)	2 (14)	1 (10)	1 (14)
Anti-Sm/RNP		18 (22)	11 (20)	3 (21)	3 (30)	2 (29)
Anti-RNP		11 (13)	7 (13)	3 (21)	1 (10)	1 (14)
Anti-Chromatin		25 (30)	15 (27)	4 (29)	3 (30)	5 (71)
Antiphospholipid antibodies during pregnancy‡						
Anti-CL IgG		6 (7)	6 (11)	0	0	0
Anti-β2GPI IgG		7 (8)	7 (13)	0	0	0
Anti-PS/PT IgG		6 (7)	3 (5)	1 (7)	2 (20)	1 (14)
Medication‡						
HCQ/chloroquine phosphate		77 (93)	52 (93)	12 (86)	10 (100)	6 (86)
Azathioprine		25 (30)	17 (30)	5 (36)	1 (10)	2 (29)
Prednisone		28 (34)	17 (30)	4 (29)	5 (50)	3 (43)
Acetylsalicylic acid		73 (88)	48 (86)	12 (86)	10 (100)	6 (86)
LMWH		19 (23)	14 (25)	1 (7)	4 (40)	1 (14)

One woman was complicated with both SGA and PE and is included in both the SLE-SGA and SLE-PE group. Parts of the data are published previously.<sup>589</sup>

\*Median (range).

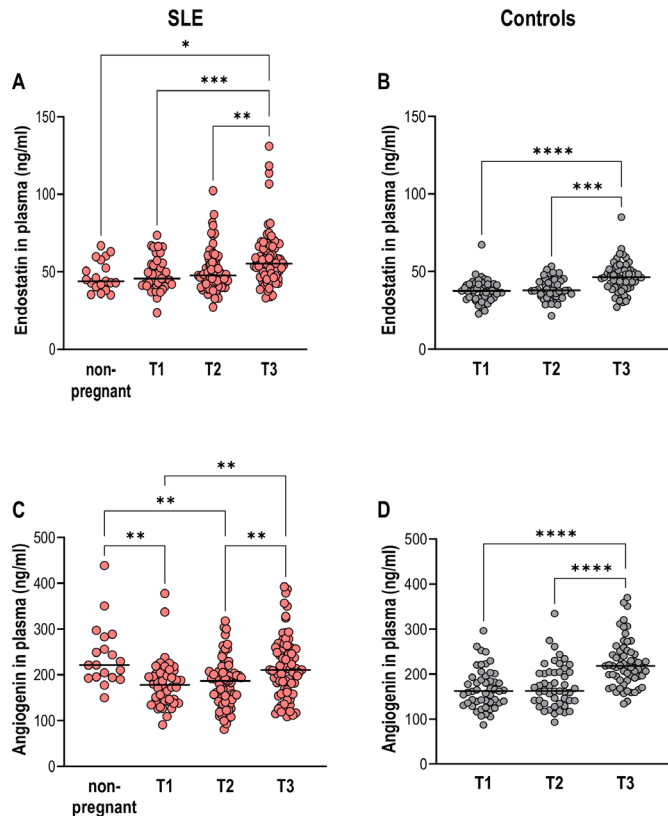
†n (%).

‡Missing data from one woman.

ACR, American College of Rheumatology; anti-dsDNA, anti-double stranded DNA; anti-PS/PT, anti-phosphatidylserine/prothrombin; anti-Sm, anti-Smith; anti-SSA, anti-Sjögren syndrome-related antigen A; anti-SSB, anti-Sjögren syndrome-related antigen B; BMI, body mass index; HCQ, hydroxychloroquine; LMWH, low molecular weight heparin; PE, pre-eclampsia; RNP, ribonucleoprotein; SGA, small for gestational age; SLEDAI 2K, Systemic Lupus Disease Activity Index 2000.

both SLE and controls (figure 4A). In contrast, angiogenin levels were lower in IVB compared with PB in both groups (figure 4B). Likewise, both IGFBP-5 and FHR-5 levels in IVB were lower compared with PB (figure 4C,D). Out of the four proteins, only endostatin concentrations

were higher in PB at delivery compared with in the third trimester (median values (ng/mL) SLE: T3 55.4 vs delivery 83.8,  $p \leq 0.0001$ ; controls: T3 46.2 vs delivery 66.4,  $p \leq 0.0001$ ). To summarise, endostatin was uniquely enriched in IVB compared with PB in both SLE and



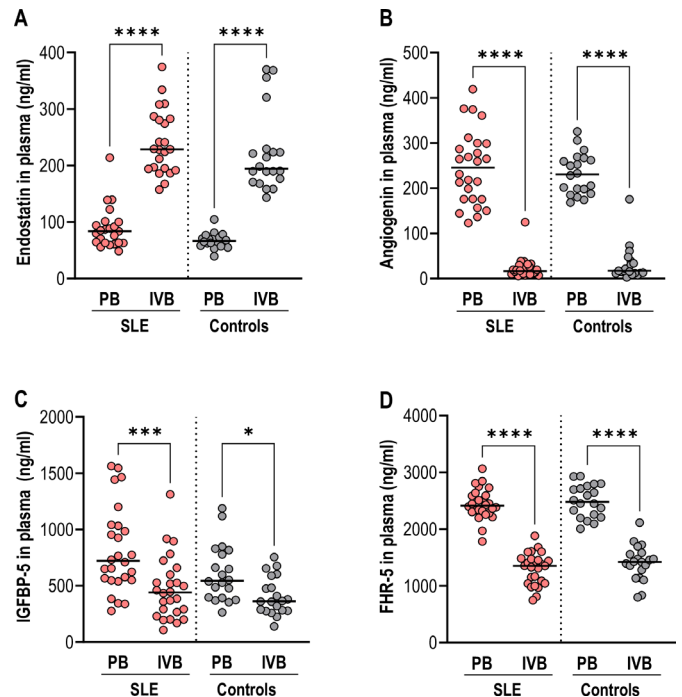
**Figure 3** Quantification of plasma protein levels among all women in the full SLE-Placenta cohort, irrespective of outcome, revealed consistently elevated levels and a gestational increase of endostatin in SLE pregnancies. Endostatin levels in (A) women with SLE in the non-pregnant state ( $n=19$ ) and in the first ( $n=45$ ), second ( $n=71$ ) and third trimesters ( $n=74$ ), as well as in (B) controls in first ( $n=50$ ), second ( $n=49$ ) and third ( $n=61$ ) trimesters. Angiogenin concentrations in (C) women with SLE in the non-pregnant state and in the first, second and third trimesters, as well as in (D) controls in first, second and third trimesters. \* $p<0.05$ , \*\* $p<0.01$ , \*\*\* $p<0.001$  and \*\*\*\*  $p<0.0001$ . (A–D) Kruskal-Wallis followed by Dunn's multiple comparison test.

controls. In contrast, angiogenin, IGFBP-5 and FHR-5 were all lower in IVB, indicating selective placental accumulation of the antiangiogenic protein endostatin at delivery.

## DISCUSSION

Despite improvements in pregnancy outcomes among women with SLE, this population still faces an increased risk of adverse outcomes, including SGA infants. Limited knowledge of the underlying pathophysiological mechanisms has so far hindered the identification of reliable predictors of SGA. Here, we applied an unbiased proteomic approach to a longitudinal pregnancy cohort of women with SLE and healthy controls for the first time, aiming to identify candidate proteins associated with SGA in SLE pregnancy and to provide mechanistic insight.

Using this longitudinal proteomic approach, we identified four proteins in the discovery cohort that were associated with SGA. Although SLE is characterised by



**Figure 4** Endostatin concentrations are higher in maternal IVB compared with PB at delivery. Protein concentrations of (A) endostatin, (B) angiogenin, (C) IGFBP-5 and (D) FHR-5 in PB ( $n=26$ ) and maternal IVB ( $n=26$ ) in all women with SLE and controls, irrespective of outcome, at delivery in full SLE-Placenta cohort. \* $p<0.05$ , \*\* $p<0.01$ , \*\*\* $p<0.001$  and \*\*\*\*  $p<0.0001$ . (A–D) Kruskal-Wallis followed by Dunn's multiple comparison test. FHR-5, complement factor H-related protein 5; IGFBP-5, insulin-like growth factor-binding protein 5; IVB, intervillous blood; PB, peripheral blood.

overactivation in many parts of the immune system, the identified proteins were more related to angiogenesis, specifically endostatin and angiogenin, than to inflammation or immune activation. Endostatin, enzymatically cleaved from the C-terminal region of collagen XVIII, exhibits antiangiogenic properties by inhibiting endothelial cell proliferation and inducing endothelial cell cycle arrest,<sup>16–18</sup> while angiogenin possesses proangiogenic properties and promotes neovascularisation.<sup>19</sup> However, in the full SLE-Placenta cohort, ELISA-based quantification could not confirm the proteomic associations with SGA. Instead, we here for the first time in women with SLE identified that endostatin and angiogenin were associated with pre-eclampsia.

Given that the risk of developing pre-eclampsia is higher in women with SLE compared with those without,<sup>20</sup> and that pre-eclampsia was the second most frequent adverse outcome in our cohort, these findings are particularly relevant. In women without SLE, increased levels of both endostatin and angiogenin have been previously associated with pre-eclampsia.<sup>21–24</sup> Our findings therefore suggest that the dysregulation of these angiogenesis-related proteins is not specific to SLE, but rather reflects a more general mechanism involved in pre-eclampsia development. In non-SLE patients, pre-eclampsia is associated with placental pathologies, including maternal vascular

malperfusion, placental infarctions and decidual vasculopathy.<sup>25–26</sup> These findings underscore the disruption of angiogenesis in pre-eclampsia, but whether the imbalance of angiogenic protein levels drives placental pathology or represents an effect or compensatory response remains to be clarified. Other angiogenic proteins, especially the ratio of soluble fms-like tyrosine kinase-1 (sFlt-1) to placental growth factor (PlGF) (elevated levels of the antiangiogenic sFlt-1 and reduced proangiogenic PlGF) have also been implicated in pre-eclampsia in both patients with SLE and non-SLE, particularly in early-onset cases.<sup>27–29</sup> In our cohort, all but one case of pre-eclampsia was late onset, occurring after gestational week 34, which is also the most common form of pre-eclampsia in women without SLE.<sup>30</sup> Importantly, although the associations between these angiogenesis-related proteins are not strong enough for prediction, our findings provide valuable insights into the pathophysiology of pre-eclampsia and may help clarify why women with SLE are at increased risk of developing this condition. Further studies are warranted to define the specific role of endostatin and angiogenin in late-onset pre-eclampsia and in placental pathology in SLE pregnancies.

Owing to the longitudinal design and the inclusion of non-pregnant women with SLE as well as pregnant healthy controls, we were able to examine the candidate proteins in patients with SLE in relation to pregnancy and to directly compare their levels with healthy pregnancies, independently of outcomes. For the first time, we show that the levels of endostatin were consistently elevated throughout pregnancy in women with SLE compared with healthy women. This contrasts with observations in non-pregnant patients with SLE and healthy controls where endostatin levels did not differ between the groups.<sup>31</sup> Notably, endostatin was the only candidate protein to increase with gestational age in both SLE and healthy women, reaching higher concentrations in late gestation compared with the non-pregnant state in women with SLE. This suggests that antiangiogenic signalling may rise alongside placental development and support the idea that circulating endostatin levels more likely reflect the placental condition rather than contribute to its dysfunction. Indeed, a similar increase has been reported for sFlt-1 during healthy pregnancies.<sup>32</sup> Further, our findings suggest that the growing placenta could be a main source of endostatin. Indeed, endostatin was enriched in placental IVB compared with PB. In accordance, sFlt-1 levels are higher in placental blood from the uterine vein compared with PB in healthy women. After the placenta is removed, sFlt-1 concentration rapidly decreases in circulation.<sup>33</sup>

While our findings are compelling, the lack of confirmation of proteomic candidates by ELISA highlights important methodological considerations. The discrepancy between the proteomic screening and ELISA validation can only be speculated on, but plasma is a complex matrix with high dynamic range and protein heterogeneity, making it inherently challenging to analyse.<sup>34</sup> The

relatively small sample size, together with the inclusion of repeated samples from different trimesters in the discovery cohort, may have introduced variability not related to pregnancy outcome. Moreover, overlapping outcomes could also act as confounding factors, and analytical variation may also have arisen from the extensive sample preparation required for MS.<sup>35</sup> These challenges underscore the difficulty of validating candidate plasma proteins, while our results illustrate the potential of an unbiased proteomic approach to uncover novel pathways relevant to pregnancy complications in SLE. Another limitation was the inability to determine whether the modest treatment-related differences in endostatin or angiogenin levels influenced the observed differences between the SLE–pre-eclampsia and SLE uncomplicated groups due to the small number of SLE pregnancies complicated by pre-eclampsia.

A key strength of our study is the prospectively collected, well-characterised cohort, including both non-pregnant and pregnant women with SLE as well as healthy pregnant controls. The use of ELISA to validate proteomic findings adds robustness to the discovery process. Moreover, the study design provides unique opportunities to examine the dynamics of candidate proteins across gestation and to explore disease-specific effects that may contribute to pregnancy complications in SLE.

In summary, our study identified a link between elevated levels of the antiangiogenic and proangiogenic proteins endostatin and angiogenin with pre-eclampsia in SLE. As endostatin levels were consistently elevated throughout SLE pregnancies compared with healthy controls, its biological role in placental development warrants further investigation.

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