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1. The association between circulating SIGLEC6 and preeclampsia: observational studies of seven cohorts.

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1.1. Abstract

No abstract available.

1.2. Background

Preeclampsia is a serious complication of pregnancy.

1.3. Methods

We did an observational study using seven tissue bank/cohorts to examine the association between circulating SIGLEC6 and preeclampsia. We included samples from participants with preterm disease (delivering 34 weeks gestation in Australia), examined whether levels altered with clinical disease severity (samples collected in South Africa) and whether there were alterations preceding disease onset using samples collected at 15- and 20-weeks gestation in New Zealand, samples collected between 26 and 34 weeks in the UK and samples collected at 28 or 36 weeks gestation in Australia. Circulating SIGLEC6, sFlt-1, and PlGF were measured via ELISA or a electrochemiluminescence immunoassay platform.

1.4. Findings

SIGLEC6 was elevated 9.5-fold (23,397 pg/ml, IQR 16701-32,267) in preterm preeclampsia (34 weeks gestation), compared to normotensive pregnancies (2441 pg/ml, IQR 871.9-6547; $p = 6.3 \times 10^{-9}$). SIGLEC6 levels correlated with disease severity: compared to preeclampsia without severe features, SIGLEC6 was raised 1.5-2.5-fold with eclampsia, or preeclampsia with life-threatening complications. There was a stepwise increase in SIGLEC6 with increasing numbers of maternal complications, accentuated when expressed as a SIGLEC6/PlGF ratio (10.7-fold rise with ≥ 3 maternal complications, versus no complications). Circulating SIGLEC6 concentrations were significantly increased among those later diagnosed with preeclampsia in samples collected at 36 weeks (n = 1032; Australia), 26-34 weeks (n = 235; UK), 28 (n = 283; Australia), and 20 weeks gestation (n = 1945; New Zealand).

1.5. Interpretation

SIGLEC6 is elevated with preeclampsia and levels correlate with disease severity.

1.6. Funding

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1.7. Evidence before this study

Currently there are no biomarkers used clinically that can rule-in those at risk of later developing preeclampsia. A ratio of a sFlt-1/PlGF is being used clinically in some settings to rule out later disease. Thorough characterisation of new biomarkers for preeclampsia may improve outcomes. SIGLEC6 is a protein that has been identified as significantly increased in placentas from pregnancies complicated by preeclampsia. It has also been identified in proteomic screens as elevated in circulation.

1.8. Added value of this study

We singularly focused on characterising SIGLEC6 and its association with preeclampsia using seven international cohorts spanning those with established preterm disease, to prediction cohorts and examining its association with increasing disease severity. We report a strong association between elevated circulating SIGLEC6 and preeclampsia, both in those with established disease and preceding disease onset. We also demonstrate an association with increasing preeclampsia disease severity.

1.9. Implications of all the available evidence

Our data, combined with prior findings cement SIGLEC6 as a protein strongly dysregulated in preeclampsia. While its sensitivity for predicting preeclampsia falls short of it being useful as a clinical tool, its biological role in disease pathogenesis and association with increasing disease severity warrants further investigation.

1.10. Introduction

Preeclampsia is a major complication of pregnancy that threatens the lives of mothers and babies. 1 Finding biomarkers with exceptionally strong associations with preeclampsia is important for two reasons. 2 First, they may be useful as screening tests to improve clinical outcomes. 3 Second, molecules strongly linked with disease may play a role in the pathogenesis. Teasing out their molecular pathways and role in disease evolution may reveal them to be therapeutic targets.

SIGLECs are sialic acid recognising members of the immunoglobulin family and SIGLEC6 is a receptor for the hormone Leptin. In silico analysis reveals SIGLEC6 to be highly expressed in placenta in a human-specific manner 5 , 6 and recent work suggests that it may be a signalling molecule in human trophoblast. 7 SIGLEC6 binds sialoglycans and leptin and its mRNA and protein are measurable in syncytiotrophoblast derived extracellular vesicles. 8 , 9

Prior evidence has raised the possibility that SIGLEC6 has potential as a biomarker for preeclampsia. It has previously been reported as highly expressed in preeclamptic placentas. 10 , 11 , 12 , 13 In proteomic screens of blood samples from longitudinal studies, SIGLEC6 has appeared among a list of analytes significantly increased in the circulation in women likely to develop preeclampsia. 14 , 15 , 16 The association was especially strong with early onset preeclampsia. 14 , 15 , 16 However, until this report, no one has singularly focussed on the diagnostic potential of SIGLEC6 a priori , nor measured it in multiple large groups of pregnant women.

Here, we report a strong association between elevated circulating SIGLEC6 and preeclampsia across numerous sample sets from multiple countries. We first measured circulating plasma SIGLEC6 and placental expression in pregnancies complicated by preterm preeclampsia (34 weeks gestation) and gestational age matched controls from Australia. We next correlated circulating levels with the degree of disease severity in plasma samples from women with preeclampsia in South Africa, where many developed severe, life-threatening complications. Using sample sets from Australia (28- and 36-weeks gestation), United Kingdom (26-32 weeks gestation), and New Zealand (15 and 20 weeks gestation), we examined whether circulating levels measured via ELISA can predict preeclampsia months before clinical diagnosis. Last, we described how SIGLEC6 levels change across normal pregnancy in an Australian set of samples collected longitudinally across the same pregnancy. Across many of these sample sets, we also measured soluble fms-like tyrosine kinase 1 (sFlt-1) and placental growth factor (PlGF) to compare their diagnostic or predictive performance with SIGLEC6.

1.11. Methods

1.12. Identification of SIGLEC6 as dysregulated in preeclampsia

SIGLEC6 was originally discovered as increased preceding preeclampsia diagnosis as part of our biomarker screening pipeline. This is a rolling screening pipeline. We initially identified molecules in silico (using two publicly available repositories; BioGPS and Protein Atlas) as highly expressed in placenta relative to other human tissues, and/or located within the syncytiotrophoblast. 2 , 4 This identified a list of many potential biomarkers. We went through this list and identified proteins where research grade ELISAs were available (To date we have tested 567; see Fig. 1). ELISA plates were then purchased to test for the presence of the potential biomarkers in plasma from pregnant women, in batches of 10-20 proteins at a time. This involved testing standard curve and sample dilution. To conserve plasma, we preference assays that require a dilution of 1:2 or greater (meaning we do not screen potential proteins that need plasma that is not diluted).

Of the assays that performed well, we proceeded to order new plates, and did a pre-screen for their potential to predict preeclampsia in a case cohort, selected from 1000 samples. These were samples that were collected in Melbourne, Australia at 36 weeks gestation.

Given each ELISA was run independently of the next (e.g., no multi-plexing and each assay had its own separate standard curve), and we validate candidate biomarkers in independent sample sets, adjustment for multiple testing was not done. As described in the statistical section, case cohort data was tested for normality before either a students t test or Mann-Whitney U test was performed to determine significant changes between the cases and controls. Importantly, the discovery samples were independent of the samples sets in which validation was then tested at the population level. The total number of proteins we have screened through our pipeline to date, and the relevant outcomes are shown in Fig. 1 . SIGLEC6 was identified early on as one of the proteins that was most significantly dysregulated with preeclampsia (small p value when comparing cases to controls, see Figure S1) and was thus selected for further validation.

1.13. Biobanks and cohorts used to measure SIGLEC6 concentrations in plasma

For all studies, preeclampsia was defined according to the guidelines published by the International Society for the Study of Hypertension in Pregnancy (ISSHP). 25 All participants gave written informed consent prior to sample collection. All participants in the seven studies provided written informed consent and permission for their samples to be used for biomarker research, including the measurement of novel proteins.

1.14. Preterm preeclampsia-case control study from Melbourne, Australia

A case control set of plasma samples were collected at the Mercy Hospital for Women: 41 with preeclampsia who birthed at 37 weeks). Clinical details are shown in Table S1 .

1.15. Preeclampsia and varying disease severity-tissue bank from Cape Town, South Africa

Plasma samples were obtained from women with preeclampsia and prospectively recruited to three large studies at Tygerberg Hospital, Cape Town, South Africa from 2016. The first was the Preeclampsia Intervention with Esomeprazole (PIE) trial that randomised 120 women diagnosed with preterm preeclampsia (26 + 0-31 + 6 weeks gestation) to 40 mg esomeprazole daily, or placebo (trial registration number PACTR201504000771349). 18 , 26 The second was the Preeclampsia Intervention 2 (PI2) trial that randomised 180 women with preterm preeclampsia to 3 g of metformin daily, or placebo (trial registration number PACTR201608001752102). 19 , 27 The third study was the Preeclampsia Obstetric Adverse Effects (PROVE) biobank study that collected samples from women with preeclampsia at the same site that ran the PIE and PI2 trials. 17 , 18 , 19

319 samples from all three studies were combined and stratified according to the degree of clinical severity. 111 women had preeclampsia without severe features (defined as hypertension, proteinuria and no other maternal organs involved). 28 The remaining 208 had preeclampsia and the following clinical features of severe disease: i) 36 had eclampsia; ii) 14 had pulmonary oedema; iii) 23 developed any of haemolysis, elevated liver enzymes, low platelet count (HELLP) syndrome, disseminated intravascular coagulation (DIC) or severe renal involvement (creatinine ≥ 120 μM/L); and iv) 135 had severe hypertension (systolic blood pressure ≥ 160 or diastolic blood pressure ≥ 110 mmHg). For those delivering with preeclampsia without severe features, 73 delivered at 34 weeks gestation, 37 delivered between 34 and 36 + 6 weeks gestation and 1 delivered at term gestations. For those delivering with eclampsia, 20 delivered at 34 weeks gestation, 10 delivered between 34 and 36 + 6 weeks gestation, and 6 delivered at term gestations. For those delivering with pulmonary oedema, 11 delivered at 34 weeks gestation, 2 delivered between 34 and 36 + 6 weeks gestation, and 1 delivered at term gestations. For those delivering with very severe organ injury (HELLP, disseminated intravascular coagulation or severe renal impairment), all 23 delivered at 34 weeks gestation. For those delivering with severe hypertension 98 delivered at 34 weeks gestation, 33 delivered between 34 and 36 + 6 weeks gestation and 4 delivered at term gestations. Participant characteristics are shown in Table 2 . Table 2 Maternal characteristics for the samples from South Africa obtained from women with preeclampsia. Preeclampsia without severe features n = 111 Eclampsia n = 36 Pulmonary oedema n = 14 Other (HELLP, DIC, renal) n = 23 Severe hypertension n = 135 p = Age (years) , median (IQR) 28 (23-34) 21.5 (17-24.5) 28.5 (23-34) 25 (24-27) 30 (24-34) 0.001 Booking body mass index (kg/m 2) , median (IQR) 30.6 (25.6-3...

In a further analysis, women were also categorised by the number of maternal complications present, either none, 1, 2 or ≥3.

1.16. Preeclampsia prediction in the general population-cohorts from Melbourne, Australia and Auckland, New Zealand

To examine the association between circulating SIGLEC6 at 15-, 20-, 28-, and 36-weeks gestation and the development of preeclampsia (i.e., prediction), we examined plasma

samples collected from unselected pregnancies in Melbourne and also unselected women in a cohort from Auckland.

The Fetal Longitudinal Assessment of Growth (FLAG) study was a large prospective collection of plasma samples at the Mercy Hospital for Women in Melbourne Australia. Over 2000 samples were obtained at both 28 (27 +0 -29 +0 days) and 36 (35 +0 -37 +0) weeks gestation. At the time of their oral glucose tolerance test, women were screened for eligibility and invited to participate. English-speaking women aged over 18 years, with a singleton pregnancy and normal mid-trimester fetal morphology examination were eligible. Whole blood was collected in a 9 ml ethylenediaminetetraacetic acid (EDTA) tube and plasma was stored at -80 °C until analysis. This study was approved by the Mercy Health Research Ethics Committee (Ethics Approval Number R14/12) and written informed consent was obtained from all participants.

At 36 weeks gestation we measured SIGLEC6 in a cohort of 992 participants, consisting of n = 41 women who later developed preeclampsia and n = 951 controls (Table S6). At 28 weeks gestation, we selected a case cohort from the entire 2000 FLAG samples, consisting of n = 93 from women who later developed preeclampsia (‘cases’) and 190 randomly selected participants who did not develop preeclampsia, deemed the ‘cohort’ (Table S9).

The Screening for Pregnancy Endpoints (SCOPE) study recruited healthy nulliparous women with singleton pregnancies in Auckland New Zealand. Participants who were healthy nulliparous women with singleton pregnancies were recruited to the SCOPE study between 2004 and 2007 in Auckland, New Zealand. Exclusion criteria included 1) an elevated risk of preeclampsia, small for gestational age or spontaneous preterm birth due to underlying medical conditions (known chronic hypertension, and/or pre-existing diabetes, renal disease, systemic lupus erythematosus, or anti-phospholipid syndrome), previous cervical knife cone biopsy, ≥3 terminations or ≥3 miscarriages or current ruptured membranes; 2) known major fetal anomaly or abnormal karyotype or 3) interventions (such as low dose aspirin) that might modify pregnancy outcome. Women were recruited at 15 ± 1 weeks gestation. For this study SIGLEC6 was measured in samples collected at 15 ± 1 weeks gestation (n = 1923 controls, n = 84 who later developed preeclampsia) and in those collected at 20 ± 1 weeks gestation (n = 1863 controls and n = 82 who later developed preeclampsia). Preeclampsia was defined as gestational hypertension or postpartum hypertension in association with proteinuria (24 urinary protein ≥ 300 mg, or spot urine protein: creatinine ratio ≥ 30 mg/mmol, or urine dipstick protein ≥ 2+) or any multi-system complication of preeclampsia. Participant characteristics are shown in Tables S14 and S15 .

1.17. Preeclampsia prediction in a high-risk population-nested case control samples from Manchester, United Kingdom

Plasma was also obtained from participants attending the Manchester Antenatal Vascular Service (The MAViS clinic) in the United Kingdom. The clinic provides care for high-risk pregnancies. Women recruited to the MAViS clinic are known to have elevated risks of preeclampsia, small for gestational age infants, or fetal growth restriction.

The inclusion criteria were: i) chronic hypertension BP ≥ 140/90 at ≤20 weeks; ii) chronic hypertension requiring antihypertensive treatment at ≤20 weeks; iii) pre-gestational diabetes with evidence of vascular complications (hypertension, nephropathy); iv) history of ischaemic heart disease; and v) previous early onset preeclampsia. We examined a nested case control study of 235 participants with a plasma sample obtained between 24 and 32 weeks (177 controls, 33 who later developed preeclampsia, and 25 who developed preeclampsia and were carrying a small for gestational age infant (birthweight 10th centile). The 235 participants were selected from an overall collection of 518 participants based on the gestation at sampling and whether they developed a complication related to placental disease or had an uncomplicated pregnancy outcome. The clinical characteristics are shown in Table S12 . As participants attending the MAViS clinic have underlying vascular disease and were sampled across a range of gestations, we performed multivariate linear regression. For modelling, the natural logarithm of SIGLEC6 was used as the dependent variable, with disorder status, chronic hypertension, renal hypertension, and gestational age at sampling (in days) as the independent variables. To determine if SIGLEC6 was significantly elevated in preeclampsia cases, adjusting for hypertensive status and gestation at sampling, the fitted regression coefficients were then transformed to represent fold-change in mean SIGLEC6 levels with respect to controls.

1.18. Longitudinal changes in circulating SIGLEC6 across pregnancy and postpartum-samples from Sydney, Australia

To assess longitudinal changes in SIGLEC6 across gestation and postpartum, we measured SIGLEC6 in 75 women recruited to the Microbiome Understanding in Maternity Study (MUMS). Samples were collected at 13 + 0 (first trimester), 20 + 0-24 + 6 (2nd trimester), 32 + 0-36 + 6 (3rd trimester). Participant characteristics are shown in Table S20 .

1.19. Non-pregnant hypertensive and normotensive women-samples from Melbourne, Australia

To assess whether SIGLEC6 is expressed and altered in non-pregnant women with normal and high blood pressure (BP), samples were obtained from women participating in the VicGut (approval 415/16 from the Alfred Hospital, Australian New Zealand Clinical Trials Registry, ACTRN12620000958987) 24 and pHibre (approval 23336 from the Monash University Human Research Ethics Committee, ACTRN12620000284965) studies. Hypertension was defined as elevated 24-h ambulatory monitoring of BP of 130 mmHg systolic BP and/or 80 mmgHg diastolic BP measured with an ambulatory BP monitoring device (VicGut and; pHibre: Mobil-O-Graph), all recruited in Melbourne, Australia. Plasma samples were obtained from 12 hypertensive and 15 normotensive women. Participant characteristics are shown in Table S21 .

1.20. Ethics

For all studies, participants who provided samples gave written informed consent. For the collection of plasma and placentas from participants delivering at 34 weeks gestation, approval was given by the Mercy Human Research Ethics Committee, R11/34. For the samples from South Africa that formed the study assessing increasing disease severity, approval was obtained from the Health Research Ethics Committees of Stellenbosch University (M14/09/038 for PIE, M16/09/37 for PI2, and N17/05/048 for PROVE). For the SCOPENZ study, ethical approval was obtained from the University of Auckland ethics committee (AKX/02/00/364-23 April 2003). For samples obtained from the MAViS clinic (UK), approval was given by the NRES Committee Northwest 11/NW/0426. Samples collected at 28 and 36 weeks gestation were collected after approval by the Mercy Health Research Ethics Committee (Ethics Approval Number R14/12). Samples for the across gestation study (Sydney, Australia) were collected after ethics approval from the South Eastern Sydney Local Health District Research Ethics Committee (17/293) (HREC/17/POWH/605). Samples utilised from the VicGut and pHibre study were approved for use in this study by the Monash University Human Research Ethics Committee (#39204).

1.21. Measuring circulating SIGLEC6, sFlt-1, and placental growth factor (PlGF)

SIGLEC6 was measured in human plasma samples or placental lysates using the human SIGLEC6-/CD327 DuoSet ELISA (RD Systems). This assay shows no cross-reactivity with SIGLEC-1, SIGLEC-2/Fc Chimera, SIGLEC-3/Fc Chimera, SIGLEC-5/Fc-Chimera, SIGLEC-7/Fc Chimera, SIGLEC-9/Fc Chimera, SIGLEC-10/Fc Chimera, SIGLEC-11/Fc-Chimera, with batches calibrated against a highly purified CHO-cell-expressed recombinant human SIGLEC-6/FC Chimera. The interplate and intraplate % CV was below 10%. We did spike-recovery studies and confirmed that there was 90-98% recovery of SIGLEC6 we spiked into human plasma. We note that given this is a research grade ELISA, absolute concentrations may vary with batch, and thus values between cohorts/biobanks should not be directly compared. sFlt-1 and PlGF were measured using a commercial electrochemiluminescence immunoassay platform (Roche Diagnostics). Technicians were blinded to the pregnancy outcomes at the time of running all assays.

1.22. Placental samples from pregnancies 34 weeks gestation

Placental tissues were obtained from women with preterm preeclampsia (birthed 34 weeks gestation) presenting to the Mercy Hospital for Women (Australia). Preterm control placentas were obtained from women who birthed preterm (34 weeks gestation) without preeclampsia (iatrogenic births for medical indications other than preeclampsia). Controls were excluded if there was placental histopathological evidence of infection. All participants birthed by caesarean section. Participant characteristics are shown in Tables S2A-S2C . Ethics approval was obtained from the Mercy Health Human Research Ethics Committee (R11/34) and all participants provided written, informed consent.

Placental tissue was obtained immediately following birth. Maternal and fetal surfaces were removed, and the sample washed in ice-cold sterile phosphate-buffered saline (PBS). Samples for RNA or protein were collected in RNA later stabilisation solution for future analysis.

1.23. Western blot analysis of SIGLEC6 in placental lysates

Total protein was extracted from selected placental tissue. 20-30 mg of frozen preserved placenta tissue was sonicated in 400 µl of cold Radioimmunoprecipitation (RIPA) assay buffer containing Protease Inhibitor cocktail (Sigma) and Halt™ Phosphatase Inhibitor (Thermo Fisher) for 10-20 s. The homogenised tissue was centrifuged at 14000 g for 20 min at 4 °C and clear lysate was collected and stored at -80 °C. Protein concentration was determined using Pierce™ BCA Protein Assay kit. 20 µg of placental lysates were separated on 12% SDS-polyacrylamide gels with wet transfer to PVDF membranes (Millipore, Billerica, MA). Membranes were blocked in 5% skim milk in TBST (Tris-Buffered Saline with 0.1% Tween 20) for 1 h at room temperature, and then incubated at 4 °C overnight with an antibody targeting SIGLEC6 (1:250 Rabbit anti-human SIGLEC6, ab38581, Abcam, Cambridge, UK; RRID: AB_777924), a mouse monoclonal anti-β-actin antibody (1:10,000, Cell Signalling Technologies, Cat#5125, RRID: AB_1903890) and the membranes were then incubated with an appropriate secondary antibody of either Anti-rabbit IgG-HRP antibody (Cell Signalling) or Amersham ECL Mouse IgG-HRP whole antibody (GE Healthcare) for 1 h at room temperature. The signals were visualised using the Amersham™ ECL™ Prime Western blotting detection reagent (GE Healthcare UK) and ChemiDoc MP Image System (Bio-Rad, Hercules, CA, USA). Relative densitometry was determined in all samples using Bio-Rads Image Lab™ 6.0 Software. β-Actin was utilised for normalisation.

1.24. RNA extraction, reverse transcription, and qRT-PCR

Total RNA was extracted from 20 to 30 mg of RNA later preserved placental tissues using RNeasy mini kit (Qiagen) as per the manufacturers instructions. The concentration and quality of all extracted RNA were determined using NanoDrop2000 Spectrophotometer (Thermo Scientific). The integrity of placental RNA samples was assessed using a Bioanalyzer via external service and only RNA samples with a RIN 6 were used.

1.25. Statistics

1.26. Conversion of data to multiples of the median (MoMs)

For SCOPENZ cohorts at 15 and 20 weeks gestation, ELISAs were run in 2 batches because of the large sample numbers. To account for technical variations arising from the two ELISA runs, data were converted into multiple of the median (MoMs) based on the median of cohort according to the batch run. For the samples from Cape Town, data were

converted into MoM according to median gestation to correct for variations in gestation.

1.27. Analyses

Maternal characteristics and birth outcome data were compared for all women who developed preeclampsia or had established preeclampsia against controls according to distribution using students t-test or Mann-Whitney U test for continuous data, and Fishers exact test or Chi square test for categorical data. Placental and circulating biomarker data were compared according to distribution using students t-test or Mann-Whitney U test for 2 groups; and one-way ANOVA or Kruskal-Wallis test for more than 2 groups. For comparisons of more than 2 groups, test groups were compared only to the control group and Dunns test used to correct for multiple comparisons.

We also compared the biomarker performance between SIGLEC6 alone or as a ratio with PlGF compared to sFlt-1 alone, or as a ratio with PlGF.

Unadjusted and adjusted receiver operating characteristic (ROC) analyses were used to compare placental and circulating biomarkers and presented as area under the curve (AUC) and 95% confidence intervals (95% CI). We corrected for the rise in SIGLEC6 concentrations across gestation. To check for potential confounding variables, the associations between SIGLEC6 and preeclampsia were evaluated across studies using logistic regression models, adjusting for smoking (Current vs. Never/Quit/Ex-smoker), BMI, and Parity (1 or more vs. 0) Prior to logistic regressions, the SIGLEC6 pg/ml was transformed to log value and the odds ratios per 50% increase was the output. In addition, the association of SIGLEC6 with preeclampsia severity and number of complications were examined in a similar strategy in the South Africa data, adjusted for BMI and parity.

A p value 0.05 was considered significant. Statistical analyses were performed using GraphPad Prism 8.4.3 (GraphPad Software, San Diego, USA), RStudio 4.1.0 (RStudio Team, Boston, USA), StataMP version 17 (Texas, USA) or SAS Enterprise Guide 8.4.

1.28. Role of funders

The funders had no role in the study design, data collection, data analyses, interpretation or writing of this report.

1.29. Results

1.30. SIGLEC6 is elevated in preterm (34 weeks gestation) preeclampsia

We measured plasma SIGLEC6 concentrations from 41 women with preterm preeclampsia (defined as 34 weeks gestation) who birthed at 34 weeks gestation and compared them to 26 normotensive controls who went on to birth at term (Table S1 shows clinical information). There was no significant difference in mean gestation at sampling between the two groups. In this Australian case-control study, median SIGLEC6 concentrations were 9.5-fold higher in the preeclampsia group compared to controls (p =

6.3 × 10 -9 ; Fig. 2 a, Mann-Whitney U test). Elevated SIGLEC6 was apparent irrespective of gestation at sampling (Fig. 2 b). Fig. 2 SIGLEC6 is increased in preterm preeclampsia. Circulating SIGLEC6 was (a) increased in 41 women who birthed at 34 weeks because of preterm preeclampsia compared to 26 women who birthed healthy babies at term. (b) Circulating SIGLEC6 levels did not significantly alter across gestation for the preeclampsia (n = 41) or control group (n = 26). (c) Placental SIGLEC6 mRNA expression from 62 women with preterm preeclampsia was increased compared to 16 gestational age matched, normotensive preterm controls. Placental SIGLEC6 protein from 82 women with preterm preeclampsia was (d, e) non-significantly increased compared to 20 preterm controls when measured via Western blot (p = 0.058), but (f) significantly increased when placental SIGLEC6 protein was measured via ELISA. All samples were matched for gestation at sampling as shown in Tables S1, S2A-S2C . Data expressed as median ± interquartile range and statistically analysed using a Mann-Whitney U test. ∗∗∗∗p 0.0001. Panel e shows a representative Western blot. PE = preeclampsia, pg = picogramme, ml = millilitre, ug = micrograms.

Mirroring circulating levels, placental SIGLEC6 mRNA expression was increased 3.4-fold in 62 placentas from pregnancies complicated by preterm preeclampsia (birthed at 34 weeks gestation) compared with 16 normotensive, gestational age-matched controls (p = 5.6 × 10 -7 ; Fig. 2 c, Mann-Whitney U test). When measuring placental SIGLEC6 protein, concentrations were non-significantly increased in placentas from women with preeclampsia compared to controls when measured via Western blot and densiometric analysis (p = 0.052; Mann-Whitney U test Fig. 2 d, with representative blot shown in Fig. 2 e), but were significantly increased when measured by ELISA (2.3-fold elevation in the preeclampsia relative to controls, p = 3.1 × 10 -10 ; Fig. 2 d, Mann-Whitney U test).

Thus, SIGLEC6 mRNA and protein expression were significantly increased in placentas from women with preeclampsia. These data suggest the placenta may be the origin of increased circulating SIGLEC6 levels present in preeclampsia (Tables S2A-S2C show clinical information for these placental studies).

1.31. Circulating SIGLEC6 increases further with increasing preeclampsia severity

We measured SIGLEC6 concentrations in plasma from 319 women in South Africa with preeclampsia and varying degrees of maternal organ injury (some with life-endangering disease severity). We categorised them according to whether they i) had preeclampsia without severe features (hypertension ± proteinuria with no other maternal organ affected, n = 111), or suffered significant morbidities of: ii) eclampsia (n = 36), iii) pulmonary oedema (n = 14), iv) developed life-threatening complications (grouped together and named ‘very severe organ injury’-women who developed any of the following: haemolysis, elevated liver enzymes and low platelets (HELLP) syndrome, disseminated intravascular coagulation, or severe renal impairment, n = 23), or v) developed severe hypertension (n = 135). Details around the number of participants that delivered at 34 weeks gestation, between 34 and 36 + 6 weeks gestation and at term are provided in the methods. Baseline clinical characteristics are shown in Table 2 .

Compared to women with preeclampsia without severe features (the reference group), circulating SIGLEC6 levels were significantly elevated among women with preeclampsia

with major maternal complications which signify more advanced pathology (Table 3 , Fig. 3 a): increased 2.34-fold (95% CI 1.63-3.42) for those who had eclampsia (p = 7.7 × 10 -6 , Mann-Whitney U test), unchanged in the small group who developed pulmonary oedema (1.3-fold, 95% CI 0.77-2.3, p = 0.31, Mann-Whitney U test), increased 2.0-fold (95% CI 1.29-3.13) among those with very severe organ injury (p = 0.002, Mann-Whitney U test), and 1.66-fold (95% CI 1.3-2.13) in those who developed severe hypertension (p = 0.00007, Mann-Whitney U test). These fold changes were comparable to circulating sFlt-1 measured in the same samples (Table 3). Table 3 Maternal SIGLEC6 (or as a ratio with PlGF) fold change grouped according to adverse outcomes experienced. Single biomarkers SIGLEC6 MoM sFlt-1 b Crude Adjusted p-value Crude Adjusted p-value Preeclampsia without severe features (n = 111) 1 (ref) 1 (ref) 1 (ref) 1 (ref) Eclampsia (n = 36) 2.34 (1.62, 3.37) 2.34 (1.63, 3.42) 7.7 × 10 -6 1.42 (1.01, 2.01) 1.57 (1.13, 2.13) 0.008 Pulmonary oedema (n = 14) 1.34 (0.78, 2.30) 1.32 (0.77, 2.28) 0.314 1.16 (0.70, 1.93) 1.15 (0.71, 1.88) 0.563 Very severe organ injury: HELLP syndrome or disseminated intravascular coagulation or severe renal impairment (n = 23) 2.09 (1.35, 3.24) 2.01 (1.29, 3.13) 0.002 2.85 (1.89, 4.3) 2.48 (1.67, 3.69) 8.65 × 10 -6 Severe hypertension (n = 135) 1.69 (1.33, 2.16) 1.66 (1.30, 2.13) 0.00007 1.84 (1.46, 2.32) 1.78 (1.43, 2.23) 5.12 × 10 -6 Biomarkers as ratios with PlGF SIGLEC6 MoM/PlGF sFlt-1/PlGF b Crude Adjusted a p-value Crude Adjusted a p-value Preeclampsia without severe features (n = 111) 1 (ref) 1 (ref) 1 (ref) 1 (ref) Eclampsia (n = 36) 7.36 (3.88, 13.96) 8.91 (4.79, 16.58) 2.44 × 10 -11 4.48 (2.34, 8.58) 5.95 (3.28, 10.78) 9.76 × 10 -9 Pulmonary oedema (n = 14) 3.06 (1.19, 7.88) ...

A ratio of a molecule that is upregulated with disease (such as SIGLEC6) with one that is down-regulated (such as PlGF) may provide more accurate discrimination compared with either biomarker alone. Thus, we expressed SIGLEC6 as a ratio with PlGF. Indeed, the relative fold elevations in association with maternal complications of preeclampsia became more pronounced when SIGLEC6 concentrations were expressed as a ratio with placental growth factor (PlGF; see Table 3). Compared to women with preeclampsia without severe features (our reference group), the SIGLEC6/PlGF ratio among women with preeclampsia was increased 8.9-fold (95% CI 4.8-16.6) if they also had eclampsia, 3-fold (95% CI 1.23-7.6) if there was pulmonary oedema, 8.85-fold (95% CI 4.2-18.6) if there was very severe organ injury and 3.25-fold (95% CI 2.1-4.9) if they developed severe hypertension. These fold changes are comparable to the sFlt-1/PlGF ratio (Table 3). Furthermore, we found SIGLEC6 levels were higher in those with preeclampsia and fetal growth restriction, relative to preeclampsia alone (Fig. 3 b, p = 0.0028, Mann-Whitney U test, median is 1.45 fold higher in those with preeclampsia and fetal growth restriction).

We next undertook a simple linear regression between blood pressure and circulating SIGLEC6 in this cohort (Fig. 3 c and d). We found a modest but significant association between systolic and diastolic blood pressure and SIGLEC6-likely associated with the increased severity of disease. We also undertook a simple linear regression looking at the relationship between circulating SIGLEC6 and birthweight centile-revealing a modest, but significant association between higher SIGLEC6 in pregnancies where the baby is born small (Fig. 3 e).

We next compared the diagnostic performance of SIGLEC6 and sFlt-1, as individual markers, or as ratio combinations with PlGF using the same data collected from the participants in South Africa. We did this by generating areas under the receiver operator curve (AUC) and statistically compared whether there were differences (Table S3 shows

the AUCs and Table S4 presents the results of direct statistical comparisons). We did not detect any significant differences between the AUCs for any of the biomarkers/ratios.

We also did a different analysis to see whether a combining sFlt-1, PlGF, and SIGLEC MoM could be a better diagnostic option compared to combining sFlt1 and PlGF. We did logistic regression models on log-transformed values, performing both unadjusted and adjusted analyses (adjusted for gestation age and birthweight). Adding SIGLEC6 MoM to sFlt-1 and PlGF did not significantly improve the sFlt-1/PlGF ratios ability to distinguish between severity levels of preeclampsia. These data suggest that SIGLEC6 does not further add to the predictive capacity of this ratio (Table S5).

Together, our data suggests circulating SIGLEC6 is further elevated when there is severe preeclampsia with maternal organ complications, compared to preeclampsia without severe features.

1.32. Stepwise increases in SIGLEC6 and SIGLEC6/PlGF with increasing numbers of complications

Using the same sample set from South Africa, we next analysed the SIGLEC6 levels based upon the number of severe maternal complications present: 0 (preeclampsia without severe features), 1, 2, or 3 and/or more. The presence of any of the following was considered a severe maternal complication: eclampsia, pulmonary oedema, HELLP, disseminated intravascular coagulation, severe renal involvement, liver involvement (haematoma or rupture, liver enzymes ≥ 500 IU/L), left ventricular failure, stroke or coma.

Of the 319 women with preeclampsia, 208 (65.2%) experienced one or more severe maternal complications, with 21 experiencing ≥3 (Table 4). There were stepwise increases in SIGLEC6 levels with the number of complications experienced; those with three or more severe maternal complications had the greatest change in SIGLEC6 (Table 4). A ratio of SIGLEC6 MoM/PlGF resulted in a more prominent stepwise elevations with increasing number of complications, compared with SIGLEC6 alone: those who had three or more severe maternal complications had a 10.7-fold (95% CI 4.9-23.1) increase in the SIGLEC6 MoM/PlGF ratio, compared to those who had none. This stepwise increases in SIGLEC6 MoM/PlGF ratio. We also observed step-wise increased in the sFlt-1/PlGF ratio with increasing number of severe maternal complications (Table 4). Table 4 Maternal SIGLEC6 (or as a ratio with PlGF) fold change by number of complications (List of complications: 1) eclampsia, 2) pulmonary oedema, 3) ‘very severe organ injury’ [HELLP syndrome or disseminated intravascular coagulation or severe renal impairment], 4) severe hypertension). Number of severe complications SIGLEC6 MoM sFlt-1 b SIGLEC6/PlGF sFlt-1/PlGF b Crude Adjusted a p-value Crude Adjusted a p-value Crude Adjusted a p-value Crude Adjusted a p-value 0 n = 111 1 (ref) 1 (ref) 1 (ref) 1 (ref) 1 (ref) 1 (ref) 1 (ref) 1 (ref) 1 n = 144 1.70 (1.33, 2.16) 1.67 (1.31, 2.13) 0.00005 1.77 (1.41, 2.23) 1.74 (1.40, 2.17) 1.01 × 10 -6 3.47 (2.27, 5.31) 3.46 (2.29, 5.23) 9.34 × 10 -9 3.66 (2.37, 5.64) 3.64 (2.45, 5.42) 6.13 × 10 -19 2 n = 43 2.00 (1.42, 2.82) 1.96 (1.39, 2.77) 0.0002 2.26 (1.64, 3.13) 2.16 (1.58, 2.94) 1.56 × 10 -6 6.22 (3.40, 11.36) 5.80 (3.23, 10.39) 7.93 × 10 -9 7.03 (3.80, 13.02) 6.34 (3.63, 11.17) 3.24 × 10 -10 ≥ 3 n = 21 2.23 (1.41, 3.51) 2.20 (1.39, 3.48) 0.0008 1.21 (0.79, 1.86) 1.21 (0.80, 1.82) 0.36 10.58 (4.76, 23.50) 10.68 (4.94, 23.08) 1.24 × 10 -7 5.75 (2.54, 13.00) 5.67 (2.79, 12.31) 3.96 × 10 -6 Ref = reference group...

1.33. Circulating SIGLEC6 is elevated at 36 weeks gestation before preeclampsia diagnosis

We next assessed whether circulating SIGLEC6 is elevated before preeclampsia is diagnosed at term gestations. Plasma SIGLEC6 concentrations were measured at 36 weeks gestation in unselected pregnant women attending for care (Melbourne, Australia; baseline characteristics shown in Table S6). Circulating SIGLEC6 concentrations at 36 weeks gestation were significantly increased among 41 women (4.1% of the entire cohort) who later developed preeclampsia, compared to 951 who did not (p = 2.9e10 -4 ; Fig. 4 a, Mann-Whitney U test); the Area Under the Curve (AUC) was 0.67 (Fig. 4 b). None of the participants had preeclampsia at the time sampling was done. To determine whether SIGLEC6 levels were influenced by the sex of the baby (assigned at birth), we split the cohort according to newborn sex assigned at birth (Figure S2). In pregnancies carrying either a female (Figure S2A) or male (Figure S2B), levels were significantly elevated in those who later delivered with preeclampsia. When comparing only those who later delivered with preeclampsia, so significant change between groups based on sex assigned at birth were identified (Figure S2C). This suggests that SIGLEC6 levels in the maternal circulation are not influenced by fetal sex (assigned at birth). Fig. 4 SIGLEC6 at 36 weeks predicts preeclampsia. Biomarkers were measured at 36 weeks in 41 women who developed preeclampsia compared to 951 who did not. Circulating SIGLEC6 was (a) elevated at 36 weeks gestation in the women who developed preeclampsia compared to those who did not; an (b) AUC of 0.67. (c) sFlt-1 was also elevated in those who later developed preeclampsia in this cohort; an (d) AUC of 0.79. (e) A ratio of SIGLEC6/PlGF was elevated among those who later developed preeclampsia; (f) an AUC of 0.76. (g) Similarly, the sFlt-1/PlGF ratio was increased in those who later developed preeclampsia relative to those who did not; an (h) AUC of 0.79. Data expressed as median ± interquartile range and statistical...

In these same samples, sFlt-1 was also increased with an AUC of 0.79 (Fig. 4 c and d, Table S7). As an individual marker at 36 weeks gestation, the SIGLEC6 AUC was inferior in performance to sFlt-1 (p = 0.03, DeLongs test). Expressing SIGLEC6 as a ratio to PlGF (SIGLEC6/PlGF ratio, p = 2.71e10 -8 , Mann-Whitney U test) produced an equivalent AUC to a sFlt-1/PlGF ratio (Fig. 4 e-h, Table S8 show the statistical comparisons of the AUCs). However, when we assessed the sensitivity of the biomarkers at a specificity of either 80%, or 90% (Tables S16 and S17), we found that sFlt-1 or a ratio of sFlt-1/PlGF produced the highest sensitivities.

1.34. Circulating SIGLEC6 is elevated at 20, 24-32, and 28 weeks gestation before preeclampsia diagnosis

We next examined how early in pregnancy SIGLEC6 may be raised preceding a diagnosis of preeclampsia in further 4 large plasma sample sets, collected at different gestations: 28 weeks gestation (Melbourne, Australia); 24-32 weeks gestation (Manchester, UK); 20- and 15-weeks gestation (Auckland, New Zealand).

We first measured SIGLEC6 in a case cohort of samples collected at 28 weeks gestation in Melbourne, Australia (participant characteristics shown in Table S9 ; all samples were collected preceding a preeclampsia diagnosis).

Circulating SIGLEC6 at 28 weeks gestation was significantly increased among the 93 women who later developed preeclampsia compared to 190 who did not (p = 3.6e -03 , Fig. 5 a and b, Mann-Whitney U test). We compared the AUCs of SIGLEC6 to sFlt-1 as individual markers, or as ratios with PlGF (Fig. 5 and comparisons shown in Tables S10 and S11). While there were no significant differences in the AUCs (DeLongs test), when we assessed the sensitivities of the biomarkers at a specificity of either 80%, or 90% (Tables S16 and S17), we found that sFlt-1 or a ratio of sFlt-1/PlGF produced the highest sensitivities. Fig. 5 SIGLEC6, sFlt-1, and PlGF across the second trimester. In samples collected in Melbourne, Australia, circulating SIGLEC6 was (a) elevated at 28 weeks gestation in 93 women who developed preeclampsia compared to 190 who did not; an (b) AUC of 0.61. (c) sFlt-1 was also increased with preeclampsia in this cohort; an (d) AUC of 0.65. (e) SIGLEC6/PlGF ratio was increased in this cohort; an (f) AUC of 0.66. (g) sFlt-1/PlGF ratio was also increased in this cohort; an (h) AUC of 0.71. In a different cohort (samples from Auckland, New Zealand), circulating SIGLEC6 was (i) elevated at 20 weeks gestation in 62 women who developed preeclampsia, and 20 women had preeclampsia and birthed small for gestational age infants, compared to 1863 who remained normotensive. The ROC for the preeclampsia/SGA group relative to controls is shown, with an AUC of 0.70. Also in samples collected in Auckland, New Zealand, SIGLEC6 was (j) non-significantly increased at 15 weeks gestation in 84 women who developed preeclampsia, compared to 1923 who did not (p = 0.058). Data expressed as median ± interquartile range and statistically analysed using or Mann-Whitney U tests or a Kruskal-Wallis with Dunns multiple comparisons post hoc test (i). ∗p 0.05, ∗∗p 0.01, ∗∗∗∗p 0.0001. AUC = Area under the curve, ng = nanogrammes, pg = picogrammes.

We next measured SIGLEC6 levels at 24-32 weeks gestation from a high-risk pregnancy group in Manchester, UK. The MAViS clinic is a specialised service providing care for pregnant women with pre-existing vascular conditions (e.g., pre-existing hypertension, diabetes, previous preeclampsia) and at high risk of developing the placental complications of fetal growth restriction and preeclampsia (Table S12 shows clinical information). All samples were taken from participants who did not have preeclampsia at the time of sampling.

In this sample set from the MAViS clinic, SIGLEC6 was increased 2.5-fold (95% CI 1.6-3.9) in 58 participants who later developed preeclampsia, compared to 177 who did not (Table S13). The rise in SIGLEC6 was particularly pronounced in women who later developed preeclampsia with a small for gestational age infant (birthweight 10th centile)-a 5.8-fold increase (95% CI 3.5-9.5). These data suggest SIGLEC6 is further elevated with preeclampsia where there is also a small for gestational age infant.

Finally, we examined samples from the 2nd trimester collected in Auckland, New Zealand, at either 15 or 20 weeks gestation. At 20 weeks gestation, we had samples from 1945 women consecutively recruited (The SCOPE cohort 21 ; baseline information shown in Tables S14 and S15). Compared to 1863 participants who remained normotensive, circulating SIGLEC6 at 20 weeks gestation was significantly elevated among 62 women who later developed preeclampsia (p = 3.4 × 10 -2 , Kruskal-Wallis with Dunns multiple comparison post hoc test), and also elevated (p = 1.8 × 10 -3 , Kruskal-Wallis with Dunns multiple comparison post hoc test) among 20 who later developed preeclampsia and birthed a small for gestation age infant (Fig. 5 i). The AUC for the preeclampsia/SGA group was 0.70 (Fig. 5 i).

At 15 weeks gestation (clinical information in Table S15) we had samples from 2007 participants. There was a non-significant increase (p = 0.054, Mann-Whitney U test) in circulating SIGLEC6 among 84 women who later developed preeclampsia compared to 1923 who did not (Fig. 5 j).

Assessment of the sensitivity for prediction of SIGLEC6, sFlt-1, sFlt-1/PlGF or SIGLEC6/PlGF at either 80 or 90% specificity (Tables S16 and S17) showed modest performance for all biomarkers or combinations with the ratios of two biomarkers giving the best sensitivities.

Together these data from four large sample sets show SIGLEC6 is elevated many months before the diagnosis of preeclampsia.

1.35. Adjustments for potential confounding variables

Although this primary report was designed to look for an association between preeclampsia and SIGLEC6, we also sought to examine the data accounting for potential confounders BMI, parity and smoking status. The odds ratio per 50% increase in SIGLEC6 was calculated. Even after adjusting for these clinical confounders the association between SIGLEC6 and preeclampsia remained largely unchanged (Table S18 and S19).

1.36. Circulating SIGLEC6 rises across gestation during pregnancy

We next set out to report the relative changes in SIGLEC6 across pregnancy from samples serially collected longitudinally from the same pool of participants. We measured levels from 75 uncomplicated pregnancies enrolled in the Microbiome Understanding in Maternity Study (MUMS) from Sydney (participant characteristics are shown in Table S20). Circulating SIGLEC6 levels rose across pregnancy (Figure S3A). Trends were similar for circulating sFlt-1 (Figure S3B).

Last, we measured plasma SIGLEC6 in 12 women who were not pregnant but had chronic hypertension, and 15 who were normotensive. We found no significance difference in SIGLEC6 levels between groups (Figure S4 and Table S21 for participant characteristics). While SIGLEC6 was measurable in all samples, they were much lower than levels seen in the pregnant populations.

1.37. Discussion

We measured SIGLEC6 in multiple international sample sets and found increased circulating SIGLEC6 is associated with preeclampsia and correlated with disease severity.

Levels are elevated among those diagnosed with preterm preeclampsia, compared with controls ($p = 3.4 \times 10^{-9}$). In large numbers of preeclamptic pregnancies in South Africa that experienced a range of maternal complications of varying severity (mild preeclampsia to life-threatening), we show SIGLEC6 levels exhibit fold changes with an increasing number of maternal complications, especially if expressed as a ratio with PlGF. We demonstrate in multiple sample sets that circulating SIGLEC6 is raised many months prior

to disease onset.

A major implication from our findings is that SIGLEC6 could be important in the pathogenesis of preeclampsia and merits further study as new therapeutic strategies may be uncovered.

Screening tests that measure PlGF or the sFlt-1/PlGF ratio are used in some countries as either part of a first trimester prediction model for preterm preeclampsia (a rarer subtype that incurs significant morbidity) 31 ; or as a diagnostic adjunct used when pregnant women present with ambiguous symptoms and signs and the test is used to rule out preeclampsia imminently developing (high negative predictive value). 2 , 3 , 32 Further studies should examine whether SIGLEC6 is elevated during the first trimester, and whether it can help identify who is at increased risk of developing preterm preeclampsia so they can be recommended aspirin to reduce their risk. There are also other clinical situations where a predictive biomarker could improve health outcomes, where PlGF or sFlt-1/PlGF do not perform well. One example is a screening test to predict preeclampsia occurring at term gestation (a rule in test). It accounts for most preeclampsia and the largest share of maternal morbidity. 2 , 33

Throughout this early report, we provide evidence from cohorts and biobanks drawn from multiple countries showing SIGLEC6 is strongly associated with preeclampsia. However, we do not report a test using SIGLEC6 that is better than sFlt-1, or PlGF. This is likely due to the strong correlation between SIGLEC6 and sFlt-1, which makes it unlikely biologically that it would perform better than sFlt-1.

It remains possible that a clinically useful biomarker test that measures SIGLEC6 could be discovered. The central reason for this optimism is that throughout this study we have used a research grade ELISA to measure SIGLEC6 and pitted its performance against commercial platforms to measure sFlt-1 and PlGF. It is highly possible that optimising the reliability of a SIGLEC6 ELISA to commercial standards could uplift its diagnostic performance. Commercial grade ELISAs are optimised to a high level for precision. Via the use of calibrators, it can account for important variables such as batch-to-batch variability, and variability between runs done on different days. Furthermore, we have certainly not exhausted testing all possible biomarker scenarios (e.g., combination tests with ultrasound and/or maternal clinical parameters) and it remains possible that SIGLEC6 could have utility in some clinical settings (noting its strong correlations with increasing disease severity).

The strength of association between elevated SIGLEC6 and preeclampsia raises the possibility it may play a role in disease pathogenesis. It fulfils several key Bradford-Hill criteria for disease causation: a strong association with the disease including a stepwise increase with disease severity (association), and SIGLEC6 is deranged preceding clinical disease onset (prediction). SIGLEC6 is a cell surface receptor meaning if it is found to be a viable therapeutic target it should be readily accessible to drugs. As such, basic science investigations into SIGLEC6 are warranted to determine whether it plays a critical role mediating disease pathogenesis.

SIGLEC6 has a very strong association with preeclampsia, which points to a role as a disease driver. However, unlike PlGF and sFlt-1 where their known angiogenic actions provide obvious leads for their potential role in disease evolution, 34 , 35 there is only rudimentary knowledge of SIGLEC6 biology in pregnancy. There is no obvious explanation

how it could be causing preeclampsia. In fact, there is little known about SIGLEC6 biology overall-most research has interrogated its role in the immune system. 36 , 37 , 38 SIGLEC6 has a far higher expression in placental tissues relative to all other tissues in the body, 39 suggesting it may play a fundamental (though yet to be defined) role in human placental biology. Interestingly, SIGLEC6 seems unique to humans: there is little to no SIGLEC6 present in ape placentas 6 nor indeed, among other species. Furthermore, the mechanism by which it is released from the cell surface remains elusive. In agreement with our findings, SIGLEC6 mRNA 40 and protein expression has been previously reported as increased in placentas of patients with preeclampsia. 10 We demonstrate that circulating SIGLEC6 rises across gestation, in a similar manner to circulating sFlt-1 and such trends are consistent with a placental source.

A particular strength of our study is that we characterised SIGLEC6 in the maternal circulation in very large numbers across seven separate cohorts collected internationally. This provides robust validation of the association between preeclampsia and elevated SIGLEC6. This is often not done for biomarker studies. 2 Some previous studies have reported SIGLEC6 is not detectable in the blood, but they assayed maternal serum, not plasma. 10 , 49 , 50 One study that did use plasma reported proteomic assays of 1125 proteins in a case control study of 33 who would later develop preeclampsia, versus 90 controls. SIGLEC6 was among one of the best performing hits, increased at 28-32 weeks gestation before preeclampsia developed. 16

A limitation of our study was that we used a research grade SIGLEC6 ELISA to measure levels. Research grade ELISAs generally do not have calibrators or QCs that allow comparison between batches. As such, variation in absolute quantification can be affected between batches.

This study identifies SIGLEC6 as a circulating biomarker associated with preeclampsia. Levels closely correlate with clinical disease severity and SIGLEC6 is elevated prior to disease, as early as 20 weeks gestation. Its biology deserves interrogating as it may uncover new therapeutic strategies for a lethal condition that still lacks disease modifying drugs.

1.38. Contributors

TM, SPW, CAC, DS, AH, JEM, LM, RST, LB, FZM, and DMK were involved in the recruitment and characterisation of participant samples utilised in the study. EK and RH were responsible for statistical analysis.

LAB, NJH, PC, TVN, MK, CM, GPW, JM, and NP were involved in experimentation, data collection, and analysis.

1.39. Data sharing statement

Sharing of data will be considered upon correspondence. Correspondence and requests for materials should be addressed to T.J.K-L. or S.T.

1.40. Declaration of interests

ST declares a relationship with Diamedica Therapeutics, receiving consultancy payments to develop an investigational drug unrelated to this current project. All other authors have no conflicts of interest to declare.

1.41. Supplementary data

Supplementary Tables and Figures

1.41.1. Table: Participant cohorts or biobanks in which circulating (plasma) SIGLEC6 was assessed.

nks (number of	Collection site	Years of collection	Cohort/biobank name, or further details
preeclampsia 7 samples)	Melbourne, Australia	2014-2017	Biobank of samples collected at 34 we gestation from those who were diagnosed v preeclampsia or from normotensive contr who delivered at term
se severity (n =	Cape Town, South Africa	2016-2020	Samples were used from three studies: bioba study (PROVE 17), and two randomis clinical trials where samples were taken biobanking: (PIE 18 and PI2 19)
tion from an on cohort-n =	Melbourne, Australia	2015-2016	FLAG cohort 20
station from an on (Prediction eeks n = 2007)	Auckland, New Zealand	2004-2011	SCOPENZ 21
tation from a samples-n =	Manchester, United Kingdom	2011-2017	MAViS 22 study
mples across population (n =	Sydney, Australia	2018-2020	MUMS 23
c hypertension	Melbourne Australia	2016-2018 2020-2021	VicGut 24 pHibre

1.41.2. Table: Maternal characteristics for the samples from South Africa obtained from women with preeclampsia.

n = 36	Pulmonary oedema n = 14	Other (HELLP, DIC, renal) n = 23	Severe hypertension n = 135	p =
	21.5 (17-24.5)	28.5 (23-34)	25 (24-27)	30 (24-34)
-35.0)	23.6 (23.3-27.3)	29.0 (27-31)	30.0 (28-35)	29.0 (24-35.2)
	28 (77.8)	4 (28.6)	9 (39.1)	44 (32.6)
	1 (2.9)	0.0 (0)	0 (0)	2 (1.5)
	23 (65.7)	12 (85.7)	21 (91.3)	116 (87.9)
	11 (31.4)	2 (14.3)	2 (8.7)	14 (10.6)
-33.6)	32.9 (30.3-36.4)	31.0 (28.9-33.7)	30.4 (29.3-31.7)	31.6 (28.9-33.4
-34.1)	32.9 (30.6-36.4)	31.0 (28.9-33.7)	30.9 (29.6-32.0)	32.1 (30.1-34.1
5-2080)	1830 (1205-2702)	1310 (1165-1715)	1270 (1050-1370)	1340 (1085-17
56)	167.5 (151-183)	168.5 (160-185)	168 (160-178)	165 (158-171)
	108.5 (99.5-119.5)	103 (99-116)	104 (98-110)	102 (100-107)

1.41.3. Table: Maternal SIGLEC6 (or as a ratio with PlGF) fold change grouped according to adverse outcomes experienced.

Single biomarkers

Crude
Preeclampsia without severe features (n = 111)
Eclampsia (n = 36)
Pulmonary oedema (n = 14)
Very severe organ injury: HELLP syndrome or disseminated intravascular coagulation or severe renal impairment (n = 23)
Severe hypertension (n = 135)

1.41.4. Table: Maternal SIGLEC6 (or as a ratio with PlGF) fold change by number of complications (List of complications: 1) eclampsia, 2) pulmonary oedema, 3) 'very severe organ injury' [HELLP syndrome or disseminated intravascular coagulation or severe renal impairment], 4) severe hypertension).

of severe ons	SIGLEC6 MoM	sFlt-1 b	SIGLEC6/PlGF	sFlt-1/PlGF b
	Adjusted a	p-value	Crude	Adjusted a
	1 (ref)	1 (ref)	1 (ref)	
	1.70 (1.33, 2.16)	1.67 (1.31, 2.13)	0.00005	1.77 (1.41, 2.2
	2.00 (1.42, 2.82)	1.96 (1.39, 2.77)	0.0002	2.26 (1.64, 3.1
	2.23 (1.41, 3.51)	2.20 (1.39, 3.48)	0.0008	1.21 (0.79, 1.8

1.42. Figures

Figure: Explanation of screening pipeline. In silico analysis of publicly available data repositories allowed us to screen for proteins expressed at high levels in the placenta or on the syncytiotrophoblast surface. To date, we have screened 567 such molecules via enzyme linked immunosorbent assay (ELISA). Of these proteins, 302 were excluded due to poor ELISA quality, due to proteins being undetectable in plasma, or because the ELISAs required high volumes of plasma. We have measured 265 of the proteins in a

case/cohort of samples at 36 weeks gestation-examining the outcome of whether the participants later developed preeclampsia or not. Each ELISA was run individually with its own standard curve and QCs (not multiplexed). Of the 265 proteins, 60 individual proteins were identified as significantly different when measured via ELISA (p 0.05 on students t test or Mann-Whitney U test) and 205 were unchanged. Of the 60, we proceeded to validation to assess whether we could still find significant changes when the molecules were measured in a population cohort (~1000 unselected samples collected at 36 weeks gestation). Of the 60, 17 validated in a population cohort (one of those being SIGLEC6) and we have not tested the other 43. This screening pipeline is ongoing and this flow chart shows how many we have screened as of May 2024.

Figure: SIGLEC6 is increased in preterm preeclampsia. Circulating SIGLEC6 was (a) increased in 41 women who birthed at 34 weeks because of preterm preeclampsia compared to 26 women who birthed healthy babies at term. (b) Circulating SIGLEC6 levels did not significantly alter across gestation for the preeclampsia (n = 41) or control group (n = 26). (c) Placental SIGLEC6 mRNA expression from 62 women with preterm preeclampsia was increased compared to 16 gestational age matched, normotensive preterm controls. Placental SIGLEC6 protein from 82 women with preterm preeclampsia was (d, e) non-significantly increased compared to 20 preterm controls when measured via Western blot (p = 0.058), but (f) significantly increased when placental SIGLEC6 protein was measured via ELISA. All samples were matched for gestation at sampling as shown in Tables S1, S2A-S2C . Data expressed as median ± interquartile range and statistically analysed using a Mann-Whitney U test. ∗∗∗∗p 0.0001. Panel e shows a representative Western blot. PE = preeclampsia, pg = picogramme, ml = millilitre, ug = micrograms.

Figure: SIGLEC6 is increased with disease severity. Relative to participants with preeclampsia and no severe features (n = 111), circulating SIGLEC6 (expressed as multiples of the median (MoM)) was (a) elevated in those who developed eclampsia (n = 36, p = 1.7e10 -3), severe organ involvement (n = 23, p = 4e10 -4) or severe hypertension (n = 135, p = 1.8e10 -6) but not changed in a small group who had pulmonary oedema (n = 14, p = 0.34). When we grouped all patients according to whether they delivered a fetal growth restricted (FGR) infant or not (b) we found circulating SIGLEC6 was elevated in those delivering with FGR (p = 2.7e10 -3). There was a modest and significant association with both diastolic (c) and systolic (d) blood pressure (BP) and SIGLEC6 and higher levels were associated with smaller babies (e) . Data expressed as median ± interquartile range and statistically analysed using a Kruskal-Wallis with Dunns multiple comparisons post hoc test (a) or a Mann-Whitney U test (b). ∗∗p 0.01, ∗∗∗p 0.001, ∗∗∗∗p 0.0001. ng = nanogrammes.

Figure: SIGLEC6 at 36 weeks predicts preeclampsia. Biomarkers were measured at 36 weeks in 41 women who developed preeclampsia compared to 951 who did not. Circulating SIGLEC6 was (a) elevated at 36 weeks gestation in the women who developed preeclampsia compared to those who did not; an (b) AUC of 0.67. (c) sFlt-1 was also elevated in those who later developed preeclampsia in this cohort; an (d) AUC of 0.79. (e) A ratio of SIGLEC6/PlGF was elevated among those who later developed preeclampsia; (f) an AUC of 0.76. (g) Similarly, the sFlt-1/PlGF ratio was increased in those who later developed preeclampsia relative to those who did not; an (h) AUC of 0.79. Data expressed as median ± interquartile range and statistically analysed using Mann-Whitney U tests. ∗∗∗p 0.001, ∗∗∗∗p 0.0001. AUC = Area under the curve, pg = picogrammes, ng =

nanogrammes.

Figure: SIGLEC6, sFlt-1, and PlGF across the second trimester. In samples collected in Melbourne, Australia, circulating SIGLEC6 was (a) elevated at 28 weeks gestation in 93 women who developed preeclampsia compared to 190 who did not; an (b) AUC of 0.61. (c) sFlt-1 was also increased with preeclampsia in this cohort; an (d) AUC of 0.65. (e) SIGLEC6/PlGF ratio was increased in this cohort; an (f) AUC of 0.66. (g) sFlt-1/PlGF ratio was also increased in this cohort; an (h) AUC of 0.71. In a different cohort (samples from Auckland, New Zealand), circulating SIGLEC6 was (i) elevated at 20 weeks gestation in 62 women who developed preeclampsia, and 20 women had preeclampsia and birthed small for gestational age infants, compared to 1863 who remained normotensive. The ROC for the preeclampsia/SGA group relative to controls is shown, with an AUC of 0.70. Also in samples collected in Auckland, New Zealand, SIGLEC6 was (j) non-significantly increased at 15 weeks gestation in 84 women who developed preeclampsia, compared to 1923 who did not ($p = 0.058$). Data expressed as median ± interquartile range and statistically analysed using or Mann-Whitney U tests or a Kruskal-Wallis with Dunns multiple comparisons post hoc test (i). $*p$ 0.05, $**p$ 0.01, $****p$ 0.0001. AUC = Area under the curve, ng = nanogrammes, pg = picogrammes.

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