Preeclampsia affects 5% to 8% of pregnancies. It is a significant contributor to global maternal and perinatal morbidity and mortality, responsible for an estimated 60,000 maternal deaths annually and far more neonatal losses. The only effective treatment is delivery of fetus and placenta.

In preeclampsia, the intermittently hypoxic placenta (caused by poor placental implantation) releases excessive antiangiogenic proteins into the maternal circulation. These cause widespread endothelial dysfunction that injures major maternal organs, including the kidneys, liver, and brain.

An antiangiogenic factor central to the pathogenesis of preeclampsia is soluble fms-like tyrosine kinase-1 (sFLT-1), a splice variant of FLT-1 (vascular endothelial growth factor receptor 1 [VEGFR1]). Placentally derived sFLT-1 binds to VEGF and placental growth factor in the maternal circulation, preventing them from activating cognate receptors to promote vascular homeostasis.

At least 4 splice variants of sFLT-1 have been described (Figure 1A). They share the extracellular domain of FLT-1 and differ at their C-terminal sequence. Two sFLT-1 variants are the most abundant and are the 2 main transcripts expressed in placenta. sFLT-1 113 has a unique 28 amino acid C-terminal region and is generically expressed in many tissues, including placenta, endothelium, brain, heart, and kidneys. In contrast, sFLT-1 e15a (also known as sFLT-1 147 or sFLT-1 v2a) is a recently described variant that has a unique 31 amino acid, serine-rich C-terminus. Interestingly, it is primate specific and principally produced in placenta, where ≥80% of all FLT-1 transcripts are spliced to become sFLT-1 e15a. Therefore, sFLT-1 e15a may be the major sFLT-1 isoform contributing to the pathophysiology of preeclampsia.
Figure 1, sFLT-1 e15a is highly expressed in placenta. A, Schematic representation of FLT-1 splice variants. The FLT-1 gene encodes the full-length membrane bound FLT-1 receptor (vascular endothelial growth factor receptor 1), as well as 4 soluble alternative splice variants. Both (B) sFLT-1 e15a and (C) sFLT-1 i13 mRNA expression are significantly elevated in severe early onset preeclamptic placenta (n=18) compared with normotensive preterm controls (n=23). D, The sFLT-1 e15a/FLT-1 ratio was 2-fold higher than the sFLT-1 i13/FLT-1 ratio (P=0.09), suggesting that sFLT-1 e15a is the more abundantly expressed variant in preeclamptic placenta. To assess protein expression and localization of sFLT-1 e15a, we generated a polyclonal antibody (G4635). E, G4635 specifically detects recombinant sFLT-1 e15a, whereas the commercially available sFLT-1 antibody (AF321) detects both sFLT-1 e15a and sFLT-1 i13 recombinant proteins. F-H, Representative micrographs of immunofluorescent staining for sFLT-1 e15a protein showing localization to the syncytiotrophoblast of severe early onset preeclamptic placentas, with limited staining observed in normotensive preterm controls. Isotype controls were clear of staining. Data expressed as mean±SEM. sFLT-1 indicates fms-like tyrosine kinase-1. **P<0.01.
responsible for preeclampsia, a disease essentially unique to humans (with some case reports in primates\textsuperscript{6,7,10}).

Despite the likely importance of sFLT-1 e15a in preeclampsia, it has not been well studied. Initial reports described increased sFLT-1 e15a mRNA with preeclampsia, not protein.\textsuperscript{6,7,11} To our knowledge, functional studies examining sFLT-1 e15a protein bioactivity have been limited to one experiment showing it competitively inhibits VEGF-induced activation (phosphorylation) of VEGFR2 in porcine endothelial cells.\textsuperscript{7} Furthermore, serum levels have not been reported. A possible explanation is the lack of commercially available reagents, notably an sFLT-1 e15a ELISA.

Here, we report the generation and validation of a specific sFLT-1 e15a ELISA. We show serum sFLT-1 e15a levels rise across pregnancy and are significantly elevated in severe preterm preeclampsia. Using purified sFLT-1 e15a protein, we also show sFLT-1 e15a is biologically active: it competes with VEGF to block VEGFR2 signaling, blocks VEGF-induced Akt phosphorylation, and inhibits endothelial cell migration, invasion, tube formation, and angiogenesis. Furthermore, it inhibits VEGF-induced endothelial sprouts from mouse aortic rings cultured ex vivo. Collectively, our work suggests that placentally derived sFLT-1 e15a may play a major role in the pathophysiology of preeclampsia.

Methods
Abbreviated Methods are presented here. Further details are available in the online-only Data Supplement.

Tissue and Serum Sample Collection
Placenta was collected from preterm (<34 weeks) pregnancies not complicated by preeclampsia (n=22) and those complicated by preterm preeclampsia (n=16). Ethics approval was obtained from The Human Research Ethics Committees at Mercy Health (Mercy Hospital for Women, Victoria, Australia). All participants provided written informed consent.

Primary human umbilical vein endothelial cells (HUVECs) and trophoblast were isolated from term placenta and umbilical cords as previously described.\textsuperscript{12,13}

Serum samples were collected longitudinally from 22 women with normal pregnancies at 4 weekly intervals beginning from 16 weeks of gestation until 36 weeks of gestation. Serum samples were also collected from 30 cases of preterm preeclampsia at the same institution. Samples collected at 28 and 32 weeks from the longitudinal cohort (n=22 women) were used as controls to compare sFLT-1 e15a levels with the preeclamptic cohort in this study.

Polyclonal Antibody Development
Unconjugated peptides for sFLT-1 e15a (KNNHKIQQPELEYTSTC) were produced (Auspep; Tullamarine, Australia) and conjugated to keyhole limpet hemocyanin and emulsed in incomplete Freund’s adjuvant and specific pathogen-free rabbits immunized (Invitrogen). Protein A purification was performed to isolate the IgG antibody component from the sera (Invitrogen).

sFLT-1 Variant Protein Production and Purification
For initial optimization of the sFLT-1 e15a ELISA (Figure 2A), sFLT-1 e15a protein was purified in house. FLAG-tagged sFLT-1 variant proteins were produced in a 293F mammalian cell system and FLAG-tagged proteins were purified using anti-FLAG M2 affinity resin (Sigma). For ELISA standards and functional studies, recombinant sFLT-1 e15a protein was obtained by custom order (Genscript, Piscataway, NJ).

Reagents
A commercial sFLT-1 ELISA (R&D systems, NE, Minneapolis) was used to measure total sFLT-1. Commercially available primary antibodies used were sFLT-1 (AF321 and MAB321; R&D Systems), pAkt (ser-473; Sigma, Sydney, Australia), and Akt (Sigma). sFLT-1 i13 and VEGF protein were purchased (R&D Systems).

Western Analysis and Immunofluorescence for sFLT-1 e15a
To assess the specificity of the newly generated sFLT-1 e15a polyclonal, binding was assessed by Western blot following loading of either purified sFLT-1 i13 or sFLT-1 e15a. Immunofluorescence staining using the polyclonal antibody was performed on preterm control and preeclamptic placentas to assess sFLT-1 e15a localization.

Reverse Transcription–Polymerase Chain Reaction
RNA was extracted from tissue or cells using the RNeasy mini kit (Qiagen, Valencia, CA) and converted to cDNA using Applied Biosystems high capacity cDNA reverse transcriptase kit (Life technologies, Mulgrave, Australia) as per manufacturer guidelines. sFLT-1splice variant mRNA expression was determined using variant primer sequences as previously published.\textsuperscript{14} All data were normalized to GAPDH (glyceraldehyde 3-phosphate dehydrogenase) as an internal control and calibrated against the average C\textsubscript{T} (cycle threshold) of the control samples. The results were expressed as fold change relative to controls.

sFLT-1 e15a siRNA Treatment of Primary Trophoblast
Primary trophoblast was transfected with siRNAs targeting sFLT-1 e15a (custom order, Integrated DNA Technologies, San Diego, CA). Seventy-two hours post transfection, RNA was collected to analyze knockdown, and conditioned media were collected to measure sFLT-1 e15a and sFLT-1 by ELISA. Experiments were repeated 3x where each experiment was done in triplicate.

sFLT-1 and sFLT-1 e15a ELISA
Total sFLT-1 levels were determined using a commercially available ELISA (R&D Systems) in accordance with manufacturer’s instructions. We developed an sFLT-1 e15a-specific ELISA by coating the plate with a commercially available anti-FLT-1 antibody (MAB321; R&D Systems) at 4 μg/mL as the capture antibody, and our newly generated sFLT-1 e15a polyclonal antibody (G4655) at 10 μg/mL as the detection antibody. Purified sFLT-1 e15a protein was used to derive standard curves.

Functional Studies to Assess the Bioactivity of sFLT-1 e15a
Bioactivity was assessed using a Ba/F3 assay, where the cells have been engineered to require ongoing VEGFR1 signaling to survive.\textsuperscript{14} Ba/F3 cells were cultured in 12.5 ng/mL of human VEGF\textsubscript{165} (R&D systems) plus serial dilutions of sFLT-1 e15a, heat inactivated sFLT-1 e15a, or recombinant sFLT-1 (R&D systems) ranging from 7.8–1000 ng/mL. Cell viability was assessed at 72 hours by MTS assay (Promega, Fitchburg).

To assess the effects of sFLT-1 on endothelial cell HUVEC migration and invasion, we used the xCELLigence system (Roche). This assay monitors experiments in real time. We examined the migration, and invasion through matrigel of HUVECs toward VEGF. We examined the effects of the following: (1) sFLT-1 e15a, (2) heat-inactivated sFLT-1 e15a, and (3) recombinant sFLT-1 (or i13, purchased from R&D systems). Doses ranged between 125 and 250 ng/mL.

We examined whether sFLT-1 e15a blocks VEGF-induced Akt phosphorylation by treating HUVECs with VEGF for 30 minutes sFLT-1 e15a. The tissues were then collected, and both total and phosphorylated Akt were measured by Western blot followed by densitometric analysis.
We also examined whether sFLT-1 e15a blocked VEGF-induced aortic ring sprouting, as described by others. Aortic rings were collected from C57BL/6 mice and embedded in rat-tail collagen and treated with VEGF±sFLT-1 e15a or recombinant sFLT-1 (R&D systems). At 168 hours post plating, aortic rings were stained with calcein acetoxymethyl ester, and the number of microvessels (or sprouts) per aortic ring was counted.

Results

sFLT-1 e15a Is Increased in Severe Preeclampsia and Is Expressed in Syncytiotrophoblast

We measured sFLT-1 e15a mRNA levels in placenta from 18 cases of preterm preeclampsia and 23 preterm normotensive controls (Table S1 in the online-only Data Supplement shows clinical details). There were no differences in gestational age between the 2 groups. Both sFLT-1 e15a (Figure 1B) and sFLT-1 i13 mRNA (Figure 1C) were increased in preeclampsia compared with controls. By expressing sFLT-1 variants as a ratio with mRNA coding full length FLT-1, we inferred that sFLT-1 e15a transcript abundance is approximately double sFLT-1 i13 mRNA (Figure 1D). Thus, our data confirm previous reports that sFLT-1 e15a mRNA is more abundant than sFLT-1 i13 in placenta.

To detect sFLT-1 e15a protein, we generated sFLT-1 e15a–specific polyclonal antibodies. We confirmed that the sFLT-1 e15a antibody specifically detects recombinant e15a, but not sFLT-1 i13, by immunoblotting (Figure 1E, G4635 is the polyclonal antibody we generated for sFLT-1 e15a). In contrast, we confirmed the commercially available sFLT-1 antibody (AF321) indiscriminately detected both sFLT-1 variants. This is expected because AF321 detects an epitope present on the extracellular portion of sFLT-1, which is present on all variants, as well as FLT-1.

Previously, it was shown by in situ hybridization that sFLT-1 e15a mRNA is expressed in the syncytiotrophoblast. Using the G4635 antibody, we performed immunofluorescence and verified that sFLT-1 e15a protein is indeed highly expressed in the syncytiotrophoblast layer of the placenta (Figure 1F–1H).

Development and Validation of an sFLT-1 e15a–Specific ELISA

We next generated an sFLT-1 e15a–specific sandwich ELISA. We used a commercial sFLT-1 antibody (MAB321) as the capture antibody and biotinylated G4635 (our sFLT-1 e15a polyclonal) as the detection antibody. A dose–response curve was generated using 1:2 serial dilutions of purified sFLT-1 e15a protein. Readings equivalent to blank were obtained when a high concentration of sFLT-1 i13 (1:50) was tested, confirming that the ELISA is specific for sFLT-1 e15a, but does not detect sFLT-1 i13. B, Primary trophoblasts treated with siRNA targeting sFLT-1 e15a resulted in >90% knockdown of sFLT-1 e15a mRNA and a (C) significant reduction in secreted sFLT-1 e15a protein, as measured using our sFLT-1 e15a ELISA. D, There was a small but significant reduction in total sFLT-1 secretion, measured using a commercially sFLT-1 ELISA that detects all sFLT-1 variants. Data expressed as mean±SEM. sFLT-1 indicates fms-like tyrosine kinase-1. *P<0.05 and ****P<0.0001.

Figure 2. Development of an sFLT-1 e15a–specific ELISA. A sandwich ELISA to measure sFLT-1 e15a was developed using a commercial sFLT-1 antibody (MAB321) as the capture antibody and biotinylated G4635 (our sFLT-1 e15a polyclonal) as the detection antibody. A, A dose–response curve was generated using 1:2 serial dilutions of purified sFLT-1 e15a protein. Readings equivalent to blank were obtained when a high concentration of sFLT-1 i13 (1:50) was tested, confirming that the ELISA is specific for sFLT-1 e15a, but does not detect sFLT-1 i13. B, Primary trophoblasts treated with siRNA targeting sFLT-1 e15a resulted in >90% knockdown of sFLT-1 e15a mRNA and a (C) significant reduction in secreted sFLT-1 e15a protein, as measured using our sFLT-1 e15a ELISA. D, There was a small but significant reduction in total sFLT-1 secretion, measured using a commercially sFLT-1 ELISA that detects all sFLT-1 variants. Data expressed as mean±SEM. sFLT-1 indicates fms-like tyrosine kinase-1. *P<0.05 and ****P<0.0001.
commercially available sFLT-1 ELISA detected an 18% reduction in sFLT-1 (Figure 2E). We further validated the specificity of the capture (MAB321) and detection (pAb G4635) antibodies to detect sFLT-1 e15a, by performing an immunoprecipitation using MAB321, followed by Western blotting with G4635 on pre eclamptic placental tissue. A specific band consistent with the expected size of sFLT-1 e15a was identified (Figure S1).

To further validate the robustness of our ELISA, we assessed the intraplate % coefficient of variance (% CV) by measuring sFLT-1 e15a levels in the same samples (3 different samples) and confirmed that it was <10%. We assessed the interplate % CV by measuring sFLT-1 in 3 different serum samples repeated on 3 separate ELISA plates. The interplate % CV was 5.6%, 7.5%, and 4.8%. These % CV suggests that our ELISA is robust and reliable in quantifying sFLT-1 e15a levels. Finally, we tested the accuracy of the ELISA at measuring known amounts of sFLT-1 e15a. Serial dilutions of sFLT-1 e15a were spiked into preeclamptic serum (ranging from 781–25 000 pg/mL) and assessed using the pAb ELISA. As shown in Figure S2, the expected versus actual reads were within 10% of one another, verifying the accuracy of the ELISA at measuring known amounts of sFLT-1 e15a within this range.

Hence, we have generated and validated an ELISA that specifically detects sFLT-1 e15a, but not sFLT-1 i13.

**Serum sFLT-1 e15a Across Normal Pregnancy and in Preterm Preeclampsia**

Using our newly generated sFLT-1 e15a ELISA, we measured sFLT-1 e15a in serum prospectively collected serially from a cohort of 22 women with healthy pregnancies who delivered at term (see Table S2 for clinical details). Similar to trends previously reported for total sFLT-1,5 serum sFLT-1 e15a levels also increased across gestation (Figure 3A).

We then measured sFLT-1 e15a levels in serum from 30 women diagnosed with preterm preeclampsia and 38 samples from healthy pregnancies (selected from 28- and 32-week samples obtained from the longitudinal cohort, ie, healthy women who delivered at term, see Table S2 for clinical details). Serum sFLT-1 e15a in the preeclamptic cohort was increased 10-fold compared with controls (Figure 3B). As expected, the commercial sFLT-1 ELISA (detects all variants) also identified a significant increase in sFLT-1 in the preeclamptic serum compared with controls (Figure 3C).

**sFLT-1 e15a Inhibits VEGF Signaling and Decreases Phosphorylation of Akt**

We next examined whether sFLT-1 e15a protein is bioactive. We coadministered VEGF and sFLT-1 e15a to Ba/F3 cells, which have been engineered to require ongoing VEGFR2 signaling to avoid cell death. Thus, if sFLT-1 e15a is bioactive and able to inhibit VEGF, it would lead to decreased VEGF signaling and consequently increased Ba/F3 cell death. Indeed, we found that sFLT-1 e15a decreased Ba/F3 cell viability in a dose-dependent manner (Figure 4A). This effect was lost when the sFLT-1 e15a was pretreated with heat inactivation. As expected, sFLT-1 i13 was also biologically active. The bioactivity of both sFLT-1 variants appeared comparable.

Akt is an intracellular signal transduction molecule that is phosphorylated with VEGF signaling.6 We found that sFLT-1 e15a blocked VEGF-induced Akt phosphorylation in primary HUVECs (see Figure 4B and 4C). We conclude that sFLT-1 e15a is biologically active and directly antagonizes VEGFR2 signaling, as well as downstream Akt phosphorylation.

**sFLT-1 e15a Inhibits Endothelial Cell Migration and Invasion**

We next examined whether sFLT-1 e15a inhibits endothelial migration (Figure 5A and 5B) and invasion (Figure 5C and 5D) using the xCELLigence system. This assay measures electric impedance across the wells and has an advantage over end point experiments in that it provides a continuous readout of results in real time. VEGF promoted endothelial cell migration (Figure 5A and 5B) and invasion (Figure 5C and 5D), which was blocked by adding either sFLT-1 e15a or sFLT-1 i13 with VEGF. In contrast, heat-inactivated sFLT-1 e15a did not affect VEGF-induced migration or invasion.

**sFLT-1 e15a Inhibits Endothelial Tube Formation**

We next examined whether sFLT-1 e15a inhibited endothelial (HUVEC) tube formation on matrigel (Figure 5E and 5F). VEGF promoted endothelial tube formation (quantified as the number of tubes formed) compared with control, and this was blocked by adding sFLT-1 e15a. sFLT-1 e15a alone also significantly disrupted tube formation compared with cells treated with VEGF alone.
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sFLT-1 e15a Inhibits Murine Aortic Ring Sprouting

We finally examined whether sFLT-1 e15a inhibits angiogenesis using a recently described mouse aortic ring sprouting assay (Figure 6A and 6B). VEGF significantly promoted aortic ring sprouting (assessed as the average number of sprouts/aortic ring) compared with control. However, the number of VEGF-induced sprouts was significantly reduced by administering sFLT-1 e15a. Recombinant sFLT-1 i13 also significantly reduced the number of aortic ring sprouts compared with VEGF alone.

Discussion

The evidence implicating sFLT-1 in the pathophysiology of preeclampsia is compelling. First, there is biological plausibility. That sFLT-1 is a known VEGF ligand binding trap (antagonizing the ability of VEGF to promote vascular homeostasis) is consistent with the fact that endothelial dysfunction is a hallmark of preeclampsia. Mechanistically, adenoviral administration of sFLT-1 to pregnant rats causes hypertension and proteinuria, recapitulating key clinical features of preeclampsia. There is also kidney endotheliosis in the model, a histopathologic finding of glomerular swelling considered pathognomonic for preeclampsia when seen in human kidney biopsies. Finally, women diagnosed with preeclampsia have significantly elevated levels of sFLT-1 that correlate with disease severity, with levels often rising weeks before clinical disease.

The pathophysiological origin of preeclampsia has been recognized for perhaps >2 millennia as a disease, where the culprit(s) are placental factors. This has arisen from the longstanding clinical observation, which is still evident today—that delivery of the placenta 'cures' the disease.

Given all these facts, sFLT-1 e15a—the predominant placental sFLT-1 variant—is likely to be central to the pathophysiology of preeclampsia. Therefore, we set out to characterize sFLT-1 e15a protein given it has remained poorly characterized. Most of the current literature on sFLT-1 detection will have used reagents that indiscriminately detect all sFLT-1 isoforms, including sFLT-1 i13 which is likely to have a significant endothelial source.

In this study, we have characterized the bioactivity and serum levels of sFLT-1 e15a protein. An important strength of this study is that we performed our functional interrogations using exclusively primary human tissues (with the exceptions that we used BaF3 cell line as a bioassay to examine VEGFR2 cell signaling and that we used mouse aortic rings for a novel ex vivo assay).

By generating a new sFLT-1 e15a ELISA, we have shown that levels increase across gestation and are significantly elevated with preterm preeclampsia (10-fold compared with controls). In this set of samples, serum levels of total sFLT-1 were also significantly increased (measured using the existing commercial ELISA which measures all sFLT-1 variants).
It is possible that an sFLT-1 e15a ELISA, which largely detects placenta-derived sFLT-1, may perform better as a biomarker test than the commercial sFLT-1 ELISA (detects all sFLT-1 variants). The reason is that sFLT-1 i13 is secreted from a variety of sources, notably endothelium. It is therefore possible that serum levels of sFLT-1 i13 are increased in other maternal inflammatory states where there is no preeclampsia, such as obesity and gestational diabetes mellitus. However, the cohort we examined is not suited to examine the relative diagnostic performance of the sFLT-1 e15a ELISA and the commercial sFLT-1 ELISA because preterm preeclampsia is known to have high levels of sFLT-1 relative to controls. As such, both ELISAs detected sFLT-1 e15a and total sFLT-1 at extremely elevated levels.

We are undertaking further studies in more appropriate cohorts to compare the performance of the sFLT-1 e15a ELISA to diagnose and predict preeclampsia compared with the commercial sFLT-1 ELISA.

We have confirmed that sFLT-1 e15a protein is bioactive and directly antagonizes VEGF signaling of VEGFR2. Furthermore, we have shown sFLT-1 e15a inhibits endothelial cell migration, invasion, tube formation, and aortic ring sprouting. When considered in light of the fact sFLT-1 e15a is the predominant splice transcript of FLT-1 mRNA in placenta.6
our work suggests that sFLT-1 e15a may be the dominant sFLT-1 splice variant responsible for preeclampsia. It points to the possibility that sFLT-1 e15a is an important molecule centrally involved in the pathogenesis of preeclampsia.

The implication of our work is that those developing therapeutic strategies to decrease sFLT-1 secretion (or inhibit its biological activity) should take into consideration this placenta-derived sFLT-1 e15a variant. For example, for drugs designed to decrease placental sFLT-1 production, it may be optimal to specifically show that they decrease placental production of sFLT-1 e15a or block effects of sFLT-1 e15a on endothelial cells. As noted, it is possible that an ELISA that specifically detects placenta-derived sFLT-1 e15a may have better diagnostic potential than the commercial sFLT-1 ELISA, although this premise requires proper examination.

Perspectives
We have undertaken studies on the newly described sFLT-1 variant, sFLT-1 e15a. We have confirmed that it is vastly increased in preeclampsia and is bioactive; sFLT-1 e15a antagonized VEGF signaling and inhibited endothelial cell migration, invasion, tube formation, and angiogenic sprouting from aorta ex vivo. Given placental derived sFLT-1 appears central to preeclamptic pathogenesis, we suggest that the development of therapeutics and diagnostics for preeclampsia should take sFLT-1 e15a into account. Specifically, there may be significant biomarker potential of measuring serum sFLT-1 e15a. It may be important that candidate therapies are shown to also target placental sFLT-1 e15a either by decreasing placental secretion, neutralizing the biological activity of circulating sFLT-1 e15a, or directly antagonizing its antiangiogenic effects at the level of the endothelium.

Acknowledgments
We wish to thank the research midwives at Mercy Hospital for Women for recruiting women and obtaining clinical samples and to the patients for kindly donating samples. We thank Steven Stacker, Peter MacCallum Cancer Institute, Victoria, and Circadian Technologies (Vegenics Pty Limited, Victoria, Australia) for the kind donation of the Ba/F3 cells. We also thank Dr Sandra Nicholson, Walter and Eliza Hall Institute of Medical Research, for the donation of IL-3 culture media and assistance with protein production.

Figure 6. sFLT-1 e15a neutralizes angiogenic sprouting from mouse aortic rings. A and B, Murine mouse aortas were treated with 30 ng/mL vascular endothelial growth factor (VEGF), which induced significant sprouting. In the presence of 250 ng/mL sFLT-1 e15a or 250 ng/mL sFLT-1, angiogenic sprouting was significantly reduced. Representative images of calcein-stained aortic rings are shown in A. B is aggregate data from n=5 separate mice, mean±SEM. sFLT-1 indicates fms-like tyrosine kinase-1. *P<0.05 and **P<0.01. White arrow points to a sprout.
Sources of Funding
This work was supported by The National Health and Medical Research Council (NHMRC: Project grant #1061977), The Royal Australian and New Zealand College of Obstetricians and Gynaecologists (RANZCOG) Research Foundation Arthur Wilson scholarship (to K.R. Palmer), and The Keith Fitzmaurice Bursary (to K.R. Palmer). The following received salary support from NHMRC: S. Tong (#1050765), T.J. Kaitu'u-Lino (#1062418), N.J. Hannan (#628927), and K.R. Palmer (#607219).

Disclosures
None.

References

Novelty and Significance

What Is New?
• fms-Like tyrosine kinase-1 (sFLT-1) e15a is the major placenta-derived sFLT-1 variant. Given preeclampsia is caused by placental release of sFLT-1, sFLT-1 e15a may be the major variant contributing to preeclampsia.
• This is the first description of serum levels of sFLT-1 e15a in preeclampsia, and serum levels across gestation in normal pregnancies.
• This report demonstrates that sFLT-1 e15a is bioactive and blocks vascular endothelial growth factor receptor 2 signaling.
• This work also shows that sFLT-1 e15a protein is antiangiogenic.

What Is Relevant?
• Preeclampsia a major hypertensive disorder of pregnancy, caused by the placental release of sFLT-1.

Summary
sFLT-1 e15a protein may be the major sFLT-1 variant contributing to the pathogenesis of preeclampsia.
Placental-Specific sFLT-1 e15a Protein Is Increased in Preeclampsia, Antagonizes Vascular Endothelial Growth Factor Signaling, and Has Antiangiogenic Activity
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Hypertension. 2015;66:1251-1259; originally published online September 28, 2015; doi: 10.1161/HYPERTENSIONAHA.115.05883

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/66/6/1251

Data Supplement (unedited) at:
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PLACENTAL SPECIFIC SFLT1-E15A PROTEIN IS INCREASED IN PRE-ECLAMPSIA, ANTAGONIZES VEGF SIGNALING, AND HAS ANTI-ANGIOGENIC ACTIVITY

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Short title: sFLT-1 e15a: serum levels and bioactivity

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Keywords: pre-eclampsia, sFLT-1, anti-angiogenesis, VEGF signaling, pregnancy
Supplementary Methods

Tissue Collection
Women presenting to the Mercy Hospital for Women gave informed written consent for placental tissue collection. Placenta was obtained from preterm pregnancies not complicated by pre-eclampsia (n=22) and those complicated by preterm pre-eclampsia (n=16). Preterm preeclampsia was defined as pre-eclampsia requiring delivery <34 weeks’ gestation. Pre-eclampsia was defined according to the American College of Obstetricians and Gynecologist task force criteria published in 2013 1. Gestationally matched pre-term control placentas were selected from women presenting with pre-term rupture of membranes or spontaneous preterm labor without evidence of infection (histopathological examination of the placentas), hypertensive disease or maternal co-morbidities. We acknowledge the preterm control cohort are not strictly normal, given there was a reason that the pregnancy was delivered at a preterm gestation. However, they represent a more suitable comparison than normal term controls, given that placental and circulating levels of sFLT-1 are known to increase with advancing gestational age.

Placental tissue was obtained immediately following delivery by caesarean section. Placental tissue (excluding fetal membranes) was removed and washed briefly in sterile phosphate-buffered saline (PBS). Samples for RNA or protein extraction were frozen within 15 minutes of delivery and stored at -80°C. A portion of each placenta was also fixed in 10% buffered formalin for histology.

Serum sample collection
Serum samples were collected just prior to delivery with consent from women with severe pre-eclampsia (n=30). Serum samples were also longitudinally collected from a number of women with normal pregnancies at 4-weekly intervals beginning from 16 weeks’ gestation until 36 weeks’ gestation or delivery (n=38). These samples collected longitudinally were also used as the gestationally matched controls. Serum was isolated from whole blood by centrifugation at 1500 g for 10 minutes, aliquotted and stored at -80°C until use.

Ethics
We obtained approval from The Human Research Ethics Committees at both Southern Health (Monash Medical Centre, Victoria, Australia) and Mercy Health (Mercy Hospital for Women, Victoria, Australia) before commencing this study. All participants provided written informed consent prior to participation in these studies. Mice utilized in the aortic ring assay experiments were scavenged after culling, and separate animal ethics approval was not required.

Polyclonal Antibody development
Unconjugated peptides for sFLT-1 e15a (KNNHKIQQEPELYTSTC) were produced (Auspep; Tullamarine, Australia) and conjugated to keyhole limpet hemocyanin and emulsed in incomplete Freund’s adjuvant (IFA) and specific pathogen free (SPF) rabbits immunized (Invitrogen). Protein A purification was performed to isolate the IgG antibody component from the sera (Invitrogen).
sFLT-1 variant protein production and purification

To initially optimize the sFLT-1 e15a ELISA (to generate data for Figure 2A), sFLT-1 e15a protein was purified in house. FLAG-tagged sFLT-1 variant proteins were produced in a 293F mammalian cell system (Invitrogen, Carlsbad, USA). 293F cells were cultured on a orbital shaker in Freestyle 293 expression media (Invitrogen) containing antibiotic/antimycotic solution (1:100; Gibco, Invitrogen) at 37°C in 8% CO₂. Cells were transfected in accordance with manufacturer’s guidelines using Freestyle MAX transfection reagent (Invitrogen) and either sFlt-1 e15a (NM_001160030) or sFlt-1 i13 (NM_001159920)-containing pEFBOS plasmid DNA. 24 hours following transfection LuctraTone™ Lupin (1:40; Millipore, Darmstadt, Germany) and pluronic acid (1:100; Invitrogen) were added. Media was harvested 72 hours following transfection for protein purification.

FLAG-tagged proteins were purified using anti-FLAG M2 affinity resin (Sigma). 25ml polypropylene columns were prepared containing anti-FLAG M2 resin. A series of 4×5mL washes were performed using 0.1M glycine pH 3.5, followed by Tris-buffered saline (TBS). Cell culture media that was pH neutral and containing 0.15M NaCl was then mixed with the resin at 4°C for 4 hours. The resin-media mix was then drained through the columns followed by 3×25mL TBS washes. Competitive elution of FLAG-tagged proteins was achieved using 1mL TBS containing 100µg/mL of FLAG peptide (Sigma) and incubation on ice for one hour. Three further elutions were performed; prior to pooling and concentration using Amicon spin ultraconcentrators (Millipore). Protein concentration was determined by spectrophotometry (Nanodrop 2000; Thermo Scientific, Waltham, MA, USA). Protease inhibitors (1:100; Roche, Indianapolis, USA) and EDTA (2mM) were added. Protein was stored at 4°C for immediate use or aliquotted and frozen at -40°C for subsequent use.

To measure sFLT-1 e15a in patient serum recombinant sFLT-1 e15a protein was obtained by custom order (Genscript, Piscataway, NJ, USA), which was then used for the ELISA standard curve. The same recombinant protein was also utilized for functional studies. According to the manufacture information, Genscript produced the sFLT-1 e15a protein as follows. A recombinant plasmid encoding sFlt-1 e15a (NM_001160030) was transiently transfected in to 293-6E cells and grown in serum-free FreeStyle 293 Expression Medium (Life Technologies). The cells were maintained in Erlenmyer Flasks at 37°C with 5% CO₂. On day 6 after transfection, cell culture supernatant was collected for purification. The target protein was isolated using HisTrap FF Crude 5ml column (GE) at 3ml/min. After washing and elution, factions were collected. Analysis of purified protein by SDS-PAGE and Western blot (using a mouse-anti-his Antibody) identified the target protein with estimated molecular weight of 120kDa.

We produced heat inactivated sFLT-1 e15a protein by heating the custom ordered protein at 95°C for 5 minutes.

Reagents

A commercial sFLT-1 ELISA (R&D systems, NE, Minneapolis, USA) was used to measure total sFLT-1. Commercially available primary antibodies used were: sFLT-1 (AF321 and MAB321; R&D Systems), pAkt (ser-473; Sigma, Sydney, Australia) and Akt (Sigma). sFLT-1 i13 and VEGF protein were purchased (R&D Systems).
Western Analysis

sFLT-1 e15a polyclonal antibody binding ability was tested using purified sFLT-1 i13 and sFLT-1 e15a. For pAkt/Akt 10µg of HUVEC lysate was used. Samples were run on a 7.5% (sFLT-1) or 10% (pAkt/Akt) polyacrylamide gel prior to wet transfer to polyvinylidene difluoride membranes (Millipore). Following blocking, membranes were incubated with antibodies diluted in 1% (sFLT-1) or 5% (pAkt/Akt) skim milk/TBST. sFLT-1 e15a (1:200; G4635), a commercial sFLT-1 (1:1000; AF321), Akt (1:1000) and pAkt (1:1000) antibodies were used. Following exposure to HRP-conjugated secondary antibodies, bands were visualized using an enhanced chemiluminescence detection system (GE healthcare, Little Chalfont, UK) and the ChemiDoc XRS (BioRad, Hercules, USA).

Immunostaining for sFLT-1 e15a

For sFLT-1 e15a immunofluorescent localization, paraffin embedded formalin fixed placental sections from pre-eclamptic (n=6) and preterm control (n=6) pregnancies were dewaxed and rehydrated prior to antigen retrieval using sodium citrate buffer for 20 minutes. Sections were blocked with protein block (DAKO, Carpinteria, USA) before G4635 at 20µg/ml or a matched rabbit isotype control was applied overnight at 4°C. Sections were washed in PBS-T prior to incubation with Alexa Fluor 488 (1:200 dilution; Invitrogen). DAPI nuclear counterstain was applied and sections mounted with fluorescent mounting media (Dako) before visualization with the EVOS fluorescent microscope (Life Technologies).

Immunoprecipitation (IP)

Human placental tissue was collected from ten preeclamptic (PE) patients. Approximately 30mg of tissue was homogenised in 350ul of pre-chilled RIPA (RadioImmunoPrecipitation Assay) buffer containing freshly added protease inhibitor cocktail (Millipore) and 50ug/ml PMFS (RIPA+ buffer), and tissue debris was pelleted by centrifugation at 14,000g for 20 minutes at 4°C. 1ml of pooled lysate or 2ug e15a protein (used as a positive control) were pre-cleared with protein A/G PLUS-agarose beads (Beads, Santa Cruz Biotechnology, USA) for 3 hours at 4°C and then the beads were removed by centrifugation at 1000g for 5 minute at 4°C. The cleared lysate or e15a protein were incubated with 40ul of either mouse anti-human VEGF R1/Flt-1 monoclonal antibody (MAB321, R&D Systems)-conjugated beads or mouse control IgG (Santa Cruz)-conjugated beads, respectively, at 4°C overnight with gentle rotation. The antigen-antibody complexes were washed three times with 400ul of cold RIPA+ buffer and once with cold 10mM Tris-HCl, pH8.0, and were then eluted from the beads by heating at 95°C for 5 minutes in 80ul of electrophoresis SDS-sample buffer. Bead-free immunoprecipitates were loaded on a SDS-PAGE gel for Western analysis. The eluted immuno precipitates were separated on a 7.5% SDS-PAGE gel and then transferred to PVDF membrane (Merck Millipore). Membrane was blocked in 5%BSA in Tris-buffered saline containing 0.05% Tween 20 (TBST) for 1 hour at room temperature and incubated with a rabbit anti Flt1 e15a polyclonal Ab G4635 (GeneScript, 1:500 dilution in Canget solution1) at 4°C overnight. Membrane was washed 4 times with TBST and was then incubated with an anti-rabbit HRP antibody (1:2500 dilution in Canget2, Cell Signaling Technology, USA). Finally, proteins were visualized using an ECL prime detection reagent kit (Amersham, GE Health) and ChemiDoc XRS (BioRad, USA).
RT-PCR

RNA was extracted from tissue or cells using the RNeasy mini kit (Qiagen, Valencia, CA) and quantified using Nanodrop 1000 spectrophotometer (NanoDrop technologies Inc, Wilmington, USA). RNA was converted to cDNA using Applied Biosystems high capacity cDNA reverse transcriptase kit (Life technologies, Mulgrave, Australia) as per manufacturer guidelines.

sFlt-1 splice variant mRNA expression was determined using variant specific primer sets: sFlt-1 i13 forward 5’-ACAATCACAGGTGACCTGCAA-3’ and reverse 5’-TCCGAGCCTGAAGTACCA-3’, sFlt-1 e15a forward 5’-CTCCTGCAGACCTCAGTG-3’ and reverse 5’-GACGATGGTGACGTTGATGT-3’ as published previously 2. GAPDH was used for housekeeping. Run conditions were: 95°C for 20 minutes; 95°C for 1 second, 60°C for 20 seconds repeated for 40 cycles. All RT-PCR was performed on the CFX 384 (Biorad) with results analysed using the comparative Ct method.

All data were normalized to GAPDH as an internal control and calibrated against the average C_t of the control samples. The results were expressed as fold change relative to controls.

Isolation of primary human umbilical vein endothelial cells

The umbilical cord was also obtained from placentas collected and human umbilical vein endothelial cells (HUVECs) isolated. Within 30 minutes of delivery the cord was infused with 10ml (1mg/ml) of collagenase. Cells were cultured in M199 media (Life Technologies) containing 10% fetal calf serum, 1% antibiotic-antimycotic, 1% endothelial cell growth factor (Sigma) and 1% heparin, maintained at 37°C in 5% CO_2, and used between passages 2 to 4.

Primary Trophoblast Isolation

Primary trophoblast were isolated from term placenta as previously described 3. Primary trophoblasts were cultured in DMEM with 10-20% FCS and 1% antibiotic-antimycotic (Life Technologies) and maintained at 37°C in 5% CO_2, 8% O_2.

sFLT-1 e15a siRNA treatment of primary trophoblast

siRNA targeting sFlt-1 e15a (custom order, Integrated DNA Technologies, San Diego, CA) was added at 50nM using 1ul Lipofectamine RNAiMAX to primary trophoblast (Invitrogen). The transfection was performed in accordance with Invitrogen’s RNAiMAX recommended protocol. 72 hours post-transfection, RNA was collected for analysis of knockdown and conditioned media collected for ELISA analysis of sFLT-1 e15a and sFLT-1. Experiments were repeated three times with triplicates performed at each experiment.

sFLT-1 and sFLT-1 e15a ELISA

Total sFLT-1 levels were determined using a commercially available ELISA (R&D Systems) in accordance with manufacturer’s instructions. An sFLT-1 e15a specific ELISA was established using 96-well MaxiSorp immunoplates (Nalgene Nunc, New York, USA) coated with 4µg/mL of anti-FLT-1 antibody (MAB321; R&D systems) in PBS. Plates were incubated overnight at room temperature. This capture antibody is raised against the extracellular domain of FLT-1 and is capable of binding FLT-1 and all sFLT-1 isoforms. Following three washes in PBS-Tween 20 (Sigma), the wells were blocked in 1% BSA/PBS at room temperature for one hour. Three further
washes were performed prior to the addition of 100µL of sample. The plate was then incubated for two hours at room temperature. Three washes were performed before detection antibody was added and incubated for two hours at room temperature.

sFLT-1 e15a polyclonal antibody (G4635) was biotinylated and used as the detection antibody at a concentration of 10c/mL. Polyclonal antibodies were biotinylated using EZ-Link-Sulpho-NHS-LC biotin (Thermo Scientific) according to manufacturer’s instructions. Three PBS-T washes were then performed prior to the addition of 100µL of streptavidin-HRP (R&D systems) per well. The plate was incubated at room temperature for 20 minutes before a final series of washes. 100µL of TMB (Sigma) was then added until an appropriate colour change had occurred or 30 minutes passed. The reaction was then stopped (using 1M H₂SO₄) prior to reading at OD450 with an OD570 subtraction on the X-mark microplate reader (BioRad). Results were analysed using the Microplate Manager 6 software (BioRad).

Purified sFLT-1 e15a protein was used for standard curves to enable quantification. For serum analyses, control serum samples were run on all plates to enable inter-run comparisons.

**Ba/F3 Bioassay**

Ba/F3 cells modified to express a chimeric protein receptor utilizing the extracellular domain of mVEGFR2 and the Epo intracellular kinase domain led to cells requiring either IL-3 or VEGF as an additional growth factor to maintain viability. These cells were kindly donated by Vegenics Pty Ltd (South Yarra, Victoria, Australia) and Professor Stephen Stacker (Peter MacCallum Cancer Research Institute, Victoria, Australia). The addition of sFLT-1 to the Ba/F3 bioassay should antagonistically bind VEGF and induce cell death. Experiments were undertaken in a 96-well tissue culture plate. Modified Ba/F3 cells were passaged, counted and washed three times in PBS to remove any IL-3. The cell pellet was then resuspended in DMEM/10% FCS to give 1×10⁴ cells per well. To this varying media conditions to be tested were added giving a final well volume of 100µL. Control wells always included were an IL-3 alone positive control (10% WEHI-3D media), VEGF (R&D systems) alone positive control, and no growth supplements as a negative control. Cells were cultured in 12.5ng/mL of human VEGF₁₆₅ (R&D systems) plus serial dilutions of sFLT-1 e15a, heat inactivated sFLT-1 e15a or recombinant sFLT-1 (R&D systems) ranging from 1000ng/ml – 7.8ng/ml. 72 hours later cell viability was assessed by MTS assay (Promega, Fitchburg, USA). Plates were read at OD490 using the X-mark microplate reader (BioRad).

**xCELLigence monitoring of HUVEC migration and invasion**

HUVEC migration and invasion was assessed using the xCELLigence system, which allows real time monitoring of cell behavior. The xCELLigence platform works via the presence of gold electrodes on the bottom of the well insert that continuously measure electrical impedance across the cellular monolayer. When more cells migrate or invade, electrical impedance will increase, resulting in an elevation in cell index.

For migration and invasion, VEGF (12.5ng/ml, R&D Systems) was plated into the bottom of a CIM plate (Acea Biosciences, San Diego, USA) in the presence of either 125 or 250ng/ml of sFLT-1 e15a (Custom order, Genscript), or 250ng/ml of sFLT-1 (R&D Systems). For invasion experiments, matrigel was plated for 30mins at 37°C. For both migration and invasion, 40,000 HUVEC cells were plated and electrical
impedance was measured continuously for 24h. Experiments were repeated three times with separate HUVEC isolations and triplicates performed for each treatment.

**Endothelial tube formation using primary HUVECs**

Primary HUVECs were plated on matrigel overnight in media containing 2% FCS to allow formation of tubal structures. Wells were then treated with either control media (PBS), media containing VEGF at 1ng/ml, media containing VEGF at 1ng/ml + sFLT-1 e15a at 250ng/ml, or media containing sFLT-1 e15a at 250ng/ml alone. All wells were also treated with calcein to allow fluorescent visualization. Four hours after treatment was administered, four images were captured per well, and the number of tubules determined.

**Mouse aortic ring assay**

Aortas were collected from five wild-type C57BL/6 female mice and cleaned of extraneous fat, tissue and branching vessels. Each aorta was gently flushed with 1ml of Opti-MEM and then cut into 0.5mm wide rings. For each treatment, 5 aortic rings/mouse were included. Aortic rings were transferred to 5ml of Opti-MEM and serum starved overnight at 37°C, 20% O₂ and 5% CO₂. The following day, type I rat-tail collagen was diluted on ice to 1mg/ml in DMEM, and the pH adjusted to slightly basic by the addition of 5-N NaOH. Each aortic ring was embedded in 50µl of collagen in a 96 well plate and incubated at 37°C for 1h prior to the addition of 200µl of Opti-MEM supplemented with 2.5% FBS, 1% penicillin-streptomycin and either 30ng/ml VEGF (RnD Systems), 30ng/ml VEGF + 250ng/ml rhFLT (RnD Systems) or 30ng/ml VEGF + 250ng/ml sFlt-1 e15a. At both 48h and 120h, 100µl of media was removed from each well and replaced with 100µl of fresh media. At 168h aortic rings were stained with calcein AM and imaged at ×4 magnification with an EVOS fluorescent microscope (Life Technologies). The numbers of microvessels per aortic ring were manually counted. n = 5 aortic rings/mouse/treatment.

**Statistical Analysis**

Continuous variables were compared using a student’s T-test for parametric data or Mann-Whitney U test for non-parametric data. To assess parity, mode of delivery and ethnicity, a Fisher’s Exact Test was used. P<0.05 was considered significant. Statistical calculations were performed using GraphPad.Prism5 software (GraphPad Software, La Jolla, CA).
Supplementary references


### Supplementary Tables

#### Clinical characteristic

<table>
<thead>
<tr>
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<th>Preterm normotensive controls (n=23)</th>
<th>Pre-eclampsia (n=18)</th>
<th>p-value</th>
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<tr>
<td>Maternal Age - Years</td>
<td>28.6 ±1.4</td>
<td>29.5 ±1.3</td>
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<td>GA at delivery - Weeks</td>
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<td>Birthweight - Grams</td>
<td>1898 ± 261</td>
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<td>Primiparous – n</td>
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<td>12 (66.6%)</td>
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<tr>
<td>Ethnicity</td>
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<tr>
<td>- White</td>
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<tr>
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<td>2 (11%)</td>
<td></td>
</tr>
<tr>
<td>- Other</td>
<td>-</td>
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<td>Systolic BP – mmHg</td>
<td>117.2 ±2.3</td>
<td>171.0 ±3.6</td>
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<td>Diastolic BP - mmHg</td>
<td>68.36 ±2.2</td>
<td>101.3 ±2.6</td>
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</table>

#### Supplementary Table S1: Baseline clinical characteristics for preterm normotensive controls and pre-eclamptic women used to assess sFlt-1 variant mRNA expression in placenta. GA = gestational age, BMI = body mass index and BP = blood pressure. Blood pressure shown is highest recorded during the hospital admission when the women were delivered. Data is presented as mean ± SEM or number (%).

#### Clinical characteristic

<table>
<thead>
<tr>
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<th>Controls (n=22)</th>
<th>Pre-eclampsia (n=30)</th>
<th>p-value</th>
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<tbody>
<tr>
<td>Maternal Age - years</td>
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<td>0.92</td>
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<td>GA at delivery - weeks</td>
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<td>BMI – kg/m²</td>
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<td>28.6±1.6</td>
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<td>Birthweight - Grams</td>
<td>3469±88</td>
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<td>Primiparous - n</td>
<td>9 (40%)</td>
<td>18 (60%)</td>
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#### Supplementary Table S2: Baseline clinical characteristics for preterm and preeclamptic women used to assess sFlt-1 serum levels. GA= gestational age, BMI = body mass index. Data is presented as mean ± SEM or number (%). Controls were serum samples taken at 28 and 32 from this same cohort of 22 women, where all these women progressed to a healthy delivery at term.
Supplementary Figure S1: Immunoprecipitation pull down of sFLT-1 e15a using MAB321 and G4635. To assess the specificity of the antibody used as the capture antibody (MAB321) and capacity for G4635 (polyclonal antibody developed against sFLT-1 e15a) to identify sFLT1-e15a, a pull down experiment utilising preeclamptic placental lysate and MAB321, followed by Western blot probing with G4635 was carried out. As expected, a specific band corresponding to sFLT1-e15a was obtained. Titrated amounts of sFLT-1 e15a recombinant antibody was included in the western blot as a positive control.
Supplementary Figure S2: Spike in of sFLT-1 e15a into preeclamptic (PE) patient serum. To assess the capacity of the newly generated ELISA to measure absolute sFLT-1 e15a values, known amounts of sFLT-1 e15a were spiked in to preeclamptic serum. Graphed is a comparison between the expected (calculation of the PE serum read plus the known amount spiked in), and the actual values determined using the ELISA. We conclude that the ELISA reads accurately within this dose range.