Author: Penning, Maria Elisabeth (Marlies)
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II Preeclampsia is associated with the presence of transcriptionally active placental fragments in the maternal lung

Aletta J. Buurma*, Marlies E. Penning*, Frans Prins,
Joke M. Schutte, Jan Anthonie Bruijn,
Suzanne Wilhelmus, Augustine Rajakumar,
Kitty W.M. Bloemenkamp, S. Ananth Karumanchi,
Hans J. Baelde

Hypertension

*These two authors contributed equally

Abstract

Preeclampsia is associated with increased levels of the circulating anti-angiogenic factor sFlt-1 and with an excessive shedding of placenta-derived multinucleated syncytial aggregates into the maternal circulation. However, it remains unclear whether these aggregates are transcriptionally active in the maternal organs and can therefore contribute to the systemic manifestations of preeclampsia.

In this study, we measured placental sFlt-1 mRNA levels in preeclamptic- and control placentas and performed RNA in situ hybridization to localize the main placental expression site of sFlt-1 mRNA. Because the maternal lung is the first capillary bed that circulating syncytial aggregates traverse, we studied the presence and persistence of placental material in lungs of preeclamptic and control subjects. To confirm the placental origin of these aggregates in maternal lungs, immunohistochemistry for the placenta-specific marker hCG and Y-chromosome in situ hybridization were performed.

Using human placental tissue, we found that syncytial knots are the principal site of expression of the anti-angiogenic factor sFlt-1. In addition, autopsy material obtained from women with preeclampsia (n=9), showed significantly more placenta-derived syncytial aggregates in the lungs than in control subjects (n=26). Importantly, these aggregates still contained the anti-angiogenic factor sFlt-1 following their entrapment in the maternal lungs.

The current study confirms the important role of syncytial knots in placental sFlt-1 mRNA production. Additionally, it shows a significant association between preeclampsia and larger quantities of sFlt-1 containing syncytial aggregates in maternal lungs, suggesting that the transfer of syncytial aggregates to the maternal compartment may contribute to the systemic endothelial dysfunction that characterizes preeclampsia.

Introduction

Preeclampsia is a severe, pregnancy-specific syndrome that is characterized by endothelial dysfunction and presents with hypertension and proteinuria after the 20th week of gestation. Therapeutic options are limited beyond delivery of the fetus and placenta and therefore, preeclampsia remains one of the major causes of fetal and maternal morbidity and mortality worldwide, and particularly in developing countries.[1]

The widespread endothelial dysfunction that characterizes preeclampsia is believed to be due to an imbalance between pro- and anti-angiogenic factors.[2, 3] The placenta is a major source of circulating anti-angiogenic factors during both normal and preeclamptic pregnancies.[3-6] In preeclampsia in particular, the outermost layer of the placenta—the syncytiotrophoblast—forms “knots” that contain high amounts of the anti-angiogenic protein sFlt-1.[7] These syncytial knots are released into the maternal circulation, thereby becoming syncytial aggregates that can become lodged in maternal organs.[8-10] Importantly, a recent study showed that upon their release, circulating syncytial aggregates remain transcriptionally active and likely serve as an autonomous source of sFlt-1 protein within the maternal circulation.[7]

We hypothesized that in preeclampsia, syncytial knots are the primary placental site of sFlt-1 production and that increased numbers of sFlt-1-containing syncytial aggregates are retained in the maternal lungs. To test this hypothesis, we first studied the expression of sFlt-1 in both normal and preeclamptic placentas. Next, we used placenta- and fetus-specific markers to investigate the presence of sFlt-1-containing syncytial aggregates in the lungs of women with preeclampsia and control subjects.

Methods

PATIENT SELECTION AND TISSUE COLLECTION

Placentas were obtained from preeclamptic[11] (n=32) and control (n=37) subjects who delivered at the Leiden University Medical Center (LUMC), the Netherlands from 2007 through 2010. All women gave written informed consent. Parallel to the collection of placenta material, autopsy samples from women who died during pregnancy were obtained via a nationwide search using the Dutch PALGA system, a histopathology and cytopathology network and archive that includes all pathology laboratories within the Netherlands.[12] The paraffin-embedded lung samples obtained from nine preeclampsia patients and 26 pregnant control subjects were provided by collaborating laboratories. The control subjects were women who died due to a cause other than a hypertensive disorder of pregnancy. The cause of death in each case was confirmed using the records of the National Maternal Mortality Committee of the Dutch Society of Obstetrics and Gynecology. To investigate the effect of pregnancy on maternal aggregates, an additional control group (n=11) comprised of non-pregnant, non-hypertensive women was included. All tissues were coded and handled anonymously in accordance with the Dutch National Ethics Guidelines (Code for Proper Secondary Use of Human Tissue, Dutch Federation of Medical Scientific Societies). This study was approved by the ethics committee of the LUMC.

PLACENTAL sFlt-1 mRNA EXPRESSION

SYBR Green quantitative PCR was performed to quantify the placental sFlt-1 mRNA levels. The expression of sFlt-1 was normalized to the expression of hypoxanthine phosphoribosyltransferase (HPRT) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). All cDNA samples were measured in duplicate. In addition, *in situ* hybridization was performed to identify the cells in the placenta that synthesized sFlt-1 mRNA. Accordingly, an RNA probe was prepared to specifically recognize sFlt-1 but not Flt-1 mRNA. Four placentas per group were examined.

IMMUNOHISTOCHEMISTRY

To test for the presence of placental material in the maternal lungs, lung tissues from preeclamptic women were stained immunohistochemically for the trophoblast-specific marker hCG. If hCG-positive syncytial aggregates were observed, sequential sections were stained for Flt-1 protein to determine whether these syncytial knots still contained this anti-angiogenic protein.[7] The control group was also screened using hCG staining to determine the specificity of these syncytial aggregates to preeclampsia. Sections were incubated with an anti-human beta-hCG antibody (1:1600, DakoCytomation) or an anti-human Flt-1 antibody (1:100, R&D Systems). Binding of the primary antibody was visualized using the appropriate secondary antibodies with diaminobenzidine as the chromogen. Placental tissue served as a positive control.

Y CHROMOSOME IN SITU HYBRIDIZATION

A DIG-labeled DNA probe that specifically recognizes the Y chromosome[13] was used to determine whether the putative syncytiotrophoblast aggregates in the maternal lungs were of fetal origin. Sections of lungs from women who had carried a male fetus were incubated with the DIG-labeled probe. To visualize the probe, the sections were incubated first with a mouse-anti-DIG monoclonal antibody (Sigma-Aldrich) followed by goat-anti-mouse IgG Alexa-647 (Invitrogen).

QUANTIFICATION OF STAINING

The number of sFlt-1 mRNA positive syncytial knots was counted by two independent observers who were blind with respect to the groups. Two observers also scored the lung sections for the absence or presence of hCG. When hCG-positive multinucleate aggregates were present, the sequential sections were tested for the co-localization of hCG with Flt-1 protein and the Y chromosome.

Results

CLINICAL DATA

Placentas were investigated from women with preeclampsia (n=32) and pregnant controls (n=37). Gestational age in the women with preeclampsia (mean 30.6 weeks, SD 1.3 weeks) was significantly lower than the controls (mean 39.6 weeks, SD 1.7 weeks; p<0.05). Clinical data of the women whose lungs were investigated is provided in Table 1. Furthermore, in these women, the presence of pulmonary edema was investigated at the clinical, gross and microscopic levels. Neither the presence of clinical symptoms of pulmonary edema nor evidence of pulmonary edema on either gross or microscopic examination differed significantly between the groups.

INCREASED PLACENTAL sFlt-1 mRNA EXPRESSION IN PREECLAMPSIA

To compare the levels of sFlt-1 mRNA in the preeclamptic and control placentas, quantitative PCR was used to measure sFlt-1 mRNA. On average, the placental sFlt-1 mRNA levels were six-fold higher in the preeclamptic placentas than in the placentas obtained from control subjects (p<0·001, Mann-Whitney test). The preeclamptic placentas had more intense sFlt-1 staining (measured using *in situ* hybridization) than control placentas, particularly in the syncytial knots (Figure 1). In addition, the number of syncytial knots was significantly higher in the preeclamptic women than in the control subjects (*p*<0·05, Figure 1). As expected, the sense control probe was negative in all samples (Figure 1).

THE PRESENCE OF HCG POSITIVE AGGREGATES IN MATERNAL LUNGS IS SIGNIFICANTLY ASSOCIATED WITH PREECLAMPSIA

Because hCG was highly expressed within the syncytial knots, we considered hCG to be a suitable specific marker to study the presence of syncytiotrophoblast aggregates in maternal lungs. hCG-positive multinucleate aggregates were observed in the lungs of six of the nine preeclamptic women. Following the observation that syncytial aggregates were present in the lungs of women with preeclampsia, we also stained control lung sections for hCG. Syncytial aggregates were observed in the lung samples of six of the 26 pregnant control subjects. Importantly, the women with preeclampsia had a significantly higher number of syncytial aggregates per 100 mm^2 lung tissue (*p*<0·05, Mann-Whitney test, Figure 2). Syncytial aggregates were found in the pregnant control subjects whose gestational age was 10-40 weeks and in preeclamptic women with a gestational age of 32-39 weeks. We performed a separate analysis to exclude any potential effect of gestational age. Because the shortest gestational age in the preeclampsia group was 30 weeks, we performed an analysis in which we excluded the control subjects with a gestational age shorter than 30 weeks. Importantly, even though the gestational age of the resulting subset of controls was now similar to the preeclamptic women, the number of syncytial aggregates remained significantly higher in the preeclampsia group ($p < 0.05$; see Figure S1 in the online supplement). Aggregates were observed in subjects who died up to 13 days after delivery. The lung samples obtained from the additional control group of non-pregnant, non-hypertensive women contained no syncytial aggregates. The number of aggregates was not associated with gestational age, maternal age or the severity of preeclampsia.

SYNCYTIOTROPHOBLAST AGGREGATES IN THE MATERNAL LUNG RETAIN THE sFlt-1 PROTEIN

To test our hypothesis that syncytial aggregates retain sFlt-1 protein after transferring to the maternal compartment and becoming entrapped in the maternal lung, we stained the hCG-positive aggregates in the maternal lung samples for Flt-1 protein. Staining sequential sections for Flt-1 and hCG revealed that these proteins were co-localized within the aggregates (Figure 2). In the preeclampsia group, 56% of all hCG-positive aggregates were also positive for Flt-1; in contrast, in the control group, 26% of the syncytial aggregates were positive for Flt-1 protein ($p<0.05$; see Figure 3).

Y CHROMOSOME IN SITU HYBRIDIZATION STRONGLY SUPPORTS THE IDEA THAT MULTINUCLEATE AGGREGATES ARE OF FETAL ORIGIN

To confirm our hypothesis that the multinucleated syncytial aggregates in the maternal lung were of placental—and therefore fetal—origin, we performed Y chromosome *in situ* hybridization in lung samples obtained from women who were carrying a male fetus. A sequential section was used to investigate co-localization with hCG and Flt-1. We observed Y chromosome positive aggregates in the maternal lung samples, and sequential sections showed co-localization between the Y chromosome and both hCG and Flt-1 (Figure 2).

Discussion

Here, we report that multinucleate aggregates in the maternal lungs originate from the syncytiotrophoblast, and that these aggregates retain the anti-angiogenic protein sFlt-1. Syncytial knots—which become syncytial aggregates upon release from the placenta—are rich in sFlt-1 mRNA and protein, suggesting that these structures are the primary placental site of sFlt-1 production. The systemic spread of these syncytial aggregates was confirmed by the presence of hCG-positive multinucleate aggregates in the lungs of pregnant women, and the number of syncytial aggregates in the maternal lungs was significantly higher in the women with preeclampsia. Co-localization of hCG with both the Y chromosome and sFlt-1 levels strongly supports the idea that these aggregates are of fetal origin and shows that these aggregates contain sFlt-1 even after their release from the placenta.

Our finding that syncytial knots are the primary placental site of sFlt-1 mRNA synthesis is in agreement with the observations that syncytial knots have the highest placental levels of sFlt-1 protein and that these knots are more numerous in the setting of preeclampsia.[7, 14] Syncytial knots detach readily from the placenta, becoming syncytial aggregates that circulate in the maternal blood.[7] It has long been known that circulating placental material—most likely trophoblast cells—can reach maternal organs, particularly the lungs.[10] Using co-localization of hCG with the Y chromosome, we show that the placental multinucleate aggregates in the maternal lung were derived from the syncytiotrophoblast. Interestingly, these placenta-derived aggregates in the maternal lung still contained sFlt-1 protein. This observation supports the idea of circulating syncytial aggregates as a mechanism of sFlt-1 release into the maternal circulation. Importantly, we also found that preeclampsia was associated with a significantly higher number of syncytial aggregates within the maternal lung tissue. During both preeclamptic and normal pregnancies, the placenta is the principal source of sFlt-1. However, previous research has shown that approximately 25% of all sFlt-1 is derived from shed syncytial aggregates.[7] It has been estimated that near the end of pregnancy, approximately 3 grams of this placental material is shed into the maternal circulation (i.e. into the maternal lungs) daily.[15] Therefore, the cumulative quantity of these circulating aggregates—and their relative contribution to total sFlt-1 production—should not be underestimated.

The lungs of the preeclamptic women contained a significantly higher percentage of sFlt-1-positive syncytial aggregates than the control samples. This observation further supports the idea that preeclampsia is associated both with an increased number of circulating syncytial aggregates and with increased sFlt-1 expression within these aggregates. By releasing sFlt-1, these aggregates may contribute to the systemic endothelial dysfunction that is characteristic of preeclampsia. This finding is also consistent with the observation that preeclamptic placentas contain more syncytial aggregates that are heavily loaded with sFlt-1 than placentas obtained following uneventful pregnancies.

In addition, the presence of syncytial aggregates in maternal organs—particularly in the early stages of pregnancy—may play a key role in the development of immune tolerance. As early as gestational week 10, we observed syncytial aggregates in maternal lungs. Because preeclampsia rarely presents prior to 20 weeks of gestation,[11] we could not investigate the presence of syncytial aggregates in the lungs of preeclamptic women early in pregnancy. We did, however, observe syncytial aggregates in the lungs of preeclamptic women at gestational week 32 and later, and other groups have reported the presence of trophoblast fragments in maternal blood in earlier stages of preeclamptic pregnancy.[15] Altogether, circulating syncytial aggregates are present early in pregnancy, and we and others[16] have found a strong association between increased shedding of syncytial aggregates and preeclampsia. Thus, one may speculate that the release and transfer

of syncytial aggregates to the maternal compartment is an early event in the pathogenesis of preeclampsia. However, the relative contribution of sFlt-1 expression in these aggregates in early pregnancy is unclear.

The presence and persistence of fetal cells in maternal organs may also have both short-term and long-term implications for postpartum maternal health. Syncytial aggregates that remain in the maternal lungs may undergo further disaggregation, forming smaller microparticles. These sFlt-1-loaded microparticles may—via their release into the systemic maternal circulation—contribute to endothelial dysfunction in maternal organs other than the lungs. We found that even 13 days after delivery, hCG-positive syncytial aggregates can be detected within the maternal lungs. This finding supports the idea that placenta-derived syncytial aggregates may be involved in the post-partum complications that are associated with preeclampsia. Preeclampsia usually resolves rapidly after delivery, and its resolution is reflected by a parallel decrease in sFlt-1 levels.[17] However, in a subset of women, the symptoms and complications of preeclampsia can persist or present several days following delivery. Syncytial aggregates remain transcriptionally active up to 48 hours after delivery, and estimates suggest that during pregnancy, 25% of all circulating sFlt-1 is derived from circulating syncytial aggregates.[7] Therefore, we propose that these aggregates may play an important role in postpartum (pre)eclampsia.

It must be acknowledged that the placentas in our study were not obtained from the same women from whom we obtained the lung tissues. Therefore, the preeclampsia phenotype of the women whose lung tissues were investigated might have been more severe than the phenotype of the women who provided the placentas. As a consequence of this potential mismatch between phenotypes, we were unable to correlate placental sFlt-1 production to the portion of sFlt-1-loaded syncytial aggregates in the maternal lungs. To overcome this complication, an animal model could be used to study the association between placental sFlt-1 production and lung pathology.

Trophoblast cells are likely not the only fetal cell population that is present in the maternal lung. A previous study using mice suggested that fetal cells in the maternal lung are comprised of a mixture of cell types that includes trophoblasts, mesenchymal stem cells, and cells from the immune system.[18] We have now confirmed the presence of trophoblast cells in the human maternal lung. In the long run, the release of vital cells from the placenta may result in chimerism, as fetal cells can be retained in the maternal blood and organs for decades after delivery.[19, 20] Because retained fetal cells have stem cell-like properties,[21] it can be speculated that these cells provide a mechanism through which maternal health can be affected for decades after pregnancy.

PERSPECTIVES

In conclusion, we have demonstrated that multinucleate aggregates in the maternal lungs originate from the syncytiotrophoblast, that their presence is significantly associated with preeclampsia and that these aggregates retain the anti-angiogenic protein sFlt-1. Further studies are needed to determine the relevance and relative contribution of trophoblast cells—and other cell types—to maternal health. Likewise, understanding what drives the formation, detachment and transfer of syncytial knots to the maternal compartment—and why these knots produce sFlt-1—are important questions to be investigated. Nevertheless, this report highlights the importance of investigating further the role that syncytial aggregates play in preeclampsia and its complications.

NOVELTY AND SIGNIFICANCE

What is new?

- Within the placenta, syncytial knots are the principal site of expression of the anti-angiogenic factor sFlt-1.
- Placenta-derived syncytial aggregates that become lodged in the maternal lungs retain the anti-angiogenic factor sFlt-1.
- Preeclampsia is associated with significantly higher quantities of sFlt-1 loaded syncytial aggregates within the maternal lung.

What is relevant?

- Although the precise etiology of preeclampsia and its complications remains unknown, the condition is associated with excessive shedding of placental material into the maternal circulation.
- Placenta-derived syncytial aggregates within the maternal lungs contain sFlt-1 and may contribute to the systemic endothelial dysfunction that characterizes preeclampsia.
- Furthermore, retained fetal cells within the mother may have stem cell-like properties, thereby providing a mechanism through which maternal health can be affected for decades after pregnancy

Summary

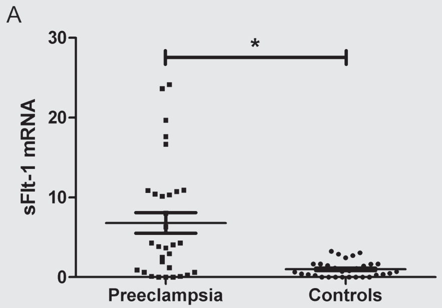
The current study confirms the important role of syncytial knots in placental sFlt-1 mRNA production. In addition, it demonstrates a significant association between preeclampsia and the presence of increased quantities of sFlt-1 containing syncytial aggregates in maternal lungs. These observations suggest that the transfer of syncytial aggregates into the maternal compartment likely contributes to the systemic endothelial dysfunction that characterizes preeclampsia.

Table 1

Characteristics	PE (n=9)	Controls (n=26)
Maternal age at death (years)	33.3 (4.6)	32.0 (5.2)
Death postpartum (%) †	100	42.3
GA at birth (w) *	35.2 (3.0)	38.5 (3.2)
Death Postpartum (h)	107.4 (157.2)	96.8 (180.0)
Death during pregnancy (%) †	0	57.5
GA at death (w)	-	22.3 (10.6)
Death-Autopsy time (h)	19.7 (13.8)	25.7 (14.7)
Gender offspring		
Male (%)	57.1	47.8
Female (%)	42.9	52.2
Parity	0.7 (1.1)	1.0 (1.2)

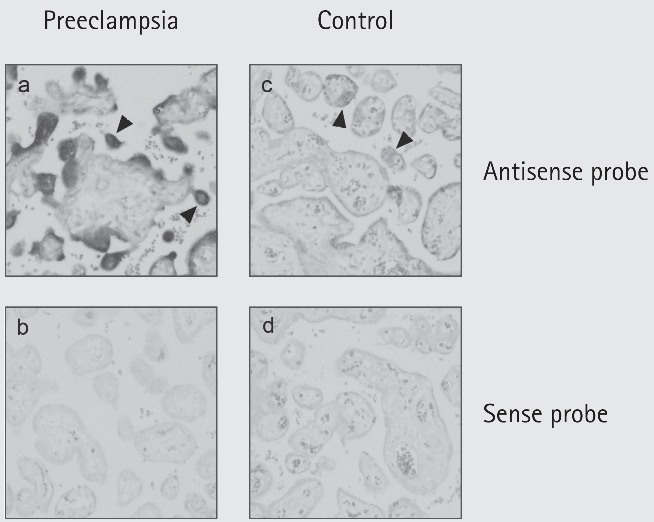
*Table 1: Data are given as mean ± SD. * p<0.05 † p<0.01, PE= preeclampsia*

Figure 1A



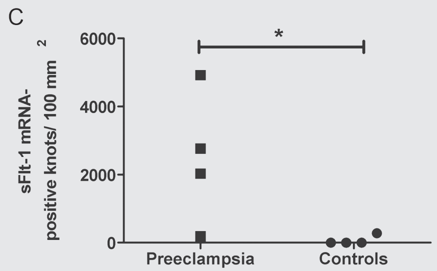
*Panel A shows the relative sFlt-1 mRNA levels in the placentas of women with preeclampsia and control subjects with mean+SEM (*p<0·001).*

Figure 1B



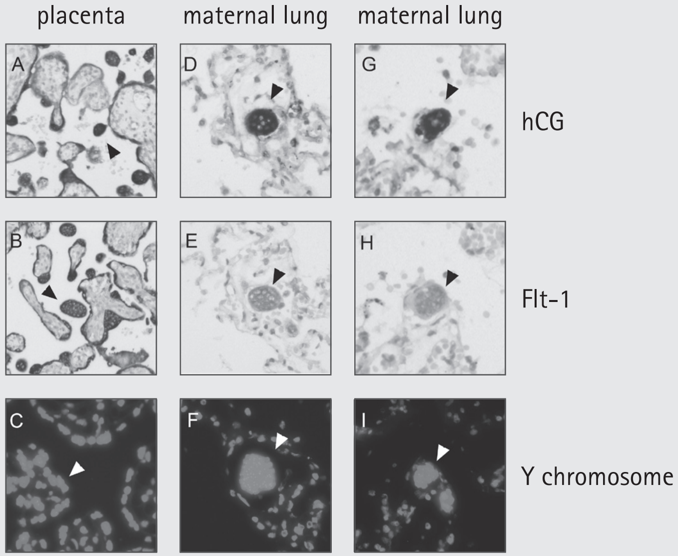
Panel B (full colour version inside cover) shows typical examples of sFlt-1 mRNA in situ hybridization in the placenta of a woman with preeclampsia (a and b) and a control subject (c and d). The term "syncytial knots" describes multinucleated structures that are loosely attached to the tips of placental villi in situ. Each column represents an individual placenta, and the various RNA probes are shown horizontally. The antisense probe (a and c) revealed that the syncytial knots (arrowheads) were the primary placental site of sFlt-1 mRNA production in both placentas. The sense probe (b and d) was used as a negative control.

Figure 1C



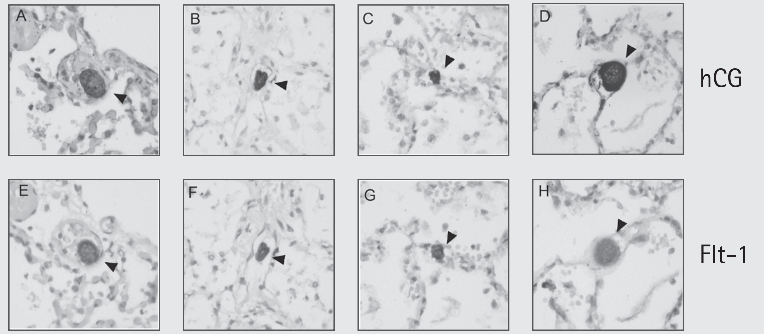
*Panel C shows the density of sFlt-1 mRNA-positive syncytial knots in the placentas of preeclamptic women and control subjects. *p<0·05.*

Figure 2A



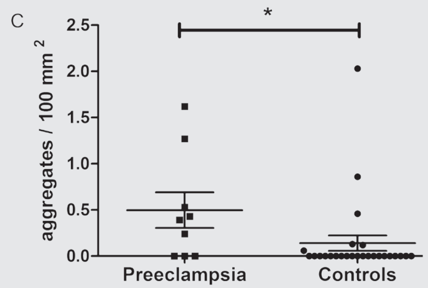
Panel A (full colour version inside cover) shows sections of placenta and sequential sections of maternal lungs that were stained for hCG or Flt-1 (using immunohistochemistry) or the Y chromosome (using in situ hybridization). To simplify the terminology, we use "syncytial knots" to describe multinucleated structures that are loosely attached to the tips of placental villi in situ and "syncytial aggregates" to describe detached multinuclear structures within the maternal lungs. The left column shows a placenta obtained from a preeclamptic patient, and the middle and right columns represent the lungs obtained from two women who were pregnant with a boy and died due to preeclampsia. The various stains are shown horizontally. Subpanels a-c show that within the preeclamptic placenta, the hCG (a) and Flt-1 (b) proteins are most abundantly present within the syncytial knots (arrowheads). In addition, Y chromosome in situ hybridization (c) shows the presence of the Y chromosome in the nucleus (visible as red puncta). Subpanels d and g show the presence of hCG-positive aggregates (arrowheads) in the lungs of two women who died due to preeclampsia. Subpanels e and h show the Flt-1 staining patterns in sections that were sequential to the sections shown in panels d and g, respectively. These images demonstrate that within the maternal lungs, hCG-positive aggregates also contain Flt-1 protein. The next sequential sections were used to perform Y chromosome in situ hybridization (subpanels f and i). These images show that the multinucleate aggregates contain Y chromosomes (arrowheads), indicating that it is very unlikely that these aggregates are not of fetal origin. Altogether, the figure demonstrates co-localization of hCG, Flt-1 and the Y chromosome in the multinucleate aggregates (arrowheads).

Figure 2B



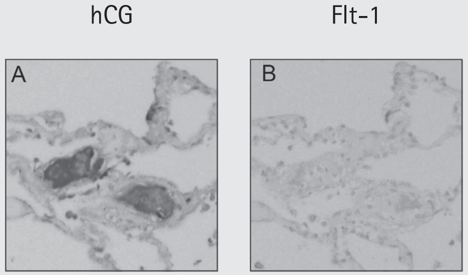
Panel B shows additional examples of multinucleate aggregates in maternal lungs, with co-localization of hCG (subpanels a-d) and Flt-1 (subpanels e-h). Each row shows matched sequential sections.

Figure 2C



*Panel C summarizes the number of hCG-positive syncytial aggregates within the maternal lungs of preeclamptic women (n=9) and control subjects (n=26). *p<0·05.*

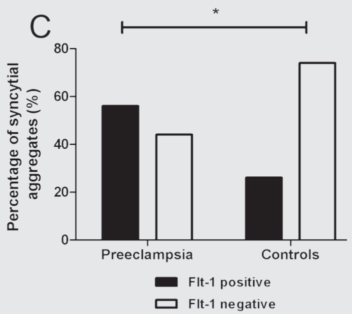
Figure 3AB



Panel A (full colour version inside cover) shows the presence of a hCG-positive aggregate in the lung of a control subject.

Panel B (full colour version inside cover) shows negative Flt-1 staining in a section that was sequential to the section shown in Panel A. These images demonstrate that within the lungs of control subjects, the minority of the hCG-positive aggregates also contain Flt-1 protein.

Figure 3C



*Panel C shows that within the preeclampsia group, 56% of all hCG-positive aggregates were also Flt-1 positive, whereas in the control group 26% of the syncytial aggregates showed positivity for Flt-1 protein. *p<0·05.*

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