CHAPTER VI

PREECLAMPSIA AND PLACENTAL FRAGMENTS IN THE MATERNAL LUNG

ALETTA BUURMA • MARLIES PENNING • FRANS PRINS
JOKE SCHUTTE • JAN ANTHONIE BRUIJN
AUGUSTINE RAJAKUMAR • KITTY BLOEMENKAMP
ANANTH KARUMANCHI • HANS BAELDE

HYPERTENSION, IN PRESS

Summary

Preeclampsia is associated 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. Using human placental tissue, we found that these syncytial aggregates are the principal site of expression of the anti-angiogenic factor sFlt-1 that has been pathogenically linked to the disease's signs and symptoms. In addition, in autopsy material obtained from women with preeclampsia (n=9), we observed significantly more placenta-derived syncytial aggregates in the lungs than in control subjects (n=26). Importantly, these placental aggregates still contained anti-angiogenic factors following their entrapment in the 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.[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. 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 9 preeclampsia patients and 26 pregnant control subjects were provided by collaborating laboratories. 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. 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 multinucleated aggregates were present, the sequential sections were tested for the co-localization of hCG with Flt-1 protein and the Y chromosome.

Results

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 6-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

multinucleated 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 6 of the 26 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. Aggregates were observed in subjects who died up to 13 days after delivery.

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).

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 multinucleated 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 multinucleated 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 strongly supports the idea that these aggregates are of fetal origin and that they remain transcriptionally active 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 multinucleated 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. By releasing sFlt-1, these aggregates might contribute to the systemic endothelial dysfunction that is

characteristic of preeclampsia.

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.

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; however, in a subset of women, the symptoms and complications of preeclampsia can persist or present in the days following delivery. Because syncytial aggregates remain transcriptionally active up to 48 hours after delivery,[7] we speculate that these aggregates may play a

role in the development of postpartum (pre)eclampsia.

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.[17] 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.[18,19] Because retained fetal cells have stem cell-like properties,[20] it can be speculated that these cells provide a mechanism though which maternal health can be affected for decades after pregnancy. 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.

PLACENTAL SFLT-1 MRNA LEVELS

FIG 1A

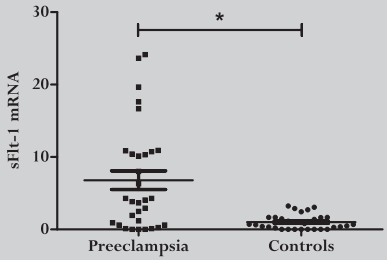

Figure 1A shows the relative sFlt-1 mRNA levels in the placentas of women with preeclampsia and control subjects with mean+SEM (*$p<0.001$).

SFTL-1 MRNA IN SITU HYBRIDIZATION

FIG 1B

(full colour version inside cover)

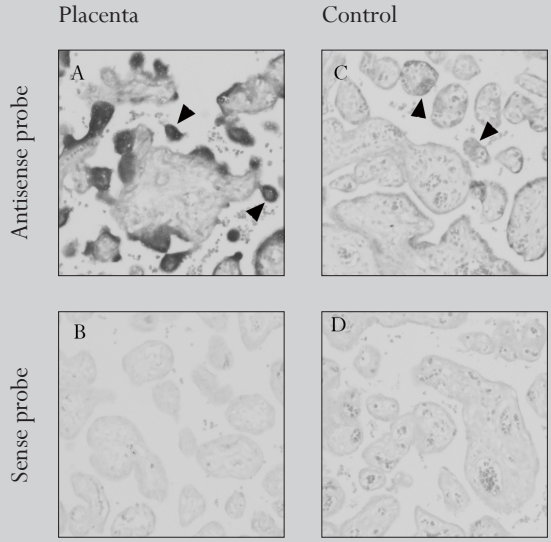

Figure 1B shows typical examples of sFlt-1 mRNA *in situ* hybridization in the placenta of a woman with preeclampsia (A and B) and a control subject (C and D). The term "syncytial knots" describes multinucleated structures that are loosely attached to the tips of placental villi *in situ*. Each column 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.

FIG 1C DENSITY OF SFLT-1 MRNA POSITIVE SYNCYTIAL KNOTS

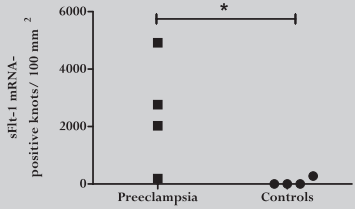

Figure 1C shows the density of sFlt-1 mRNA-positive syncytial knots in the placentas of preeclamptic women and control subjects. *$p<0.05$.

FIG 2A STAININGS OF THE PLACENTA AND MATERNAL LUNGS

(full colour version inside cover)

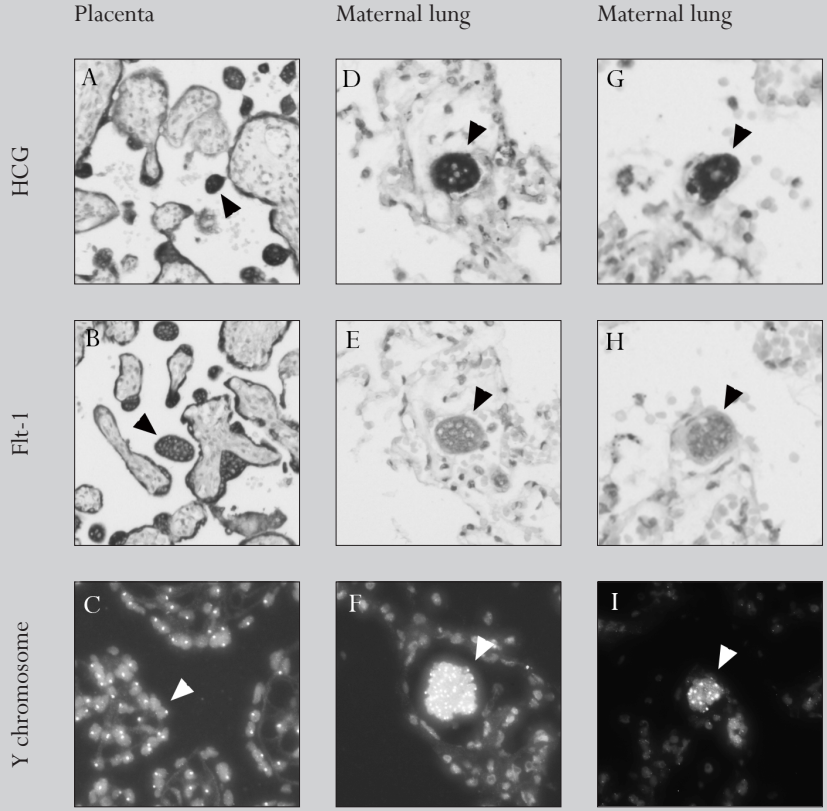

Figure 2A shows sections of placenta and sequential sections of maternal lungs that were stained for hCG or Flt-1 (using immunohistochemistry) or the Y chromosome (using *in situ* hybridization). To simplify the terminology, we use "syncytial knots" to describe multinucleated structures that are loosely 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 multinucleated 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 multinucleated aggregates (arrowheads).

FIG 2B CO-LOCALIZATION OF HCG AND FLT-1

(full colour version inside cover)

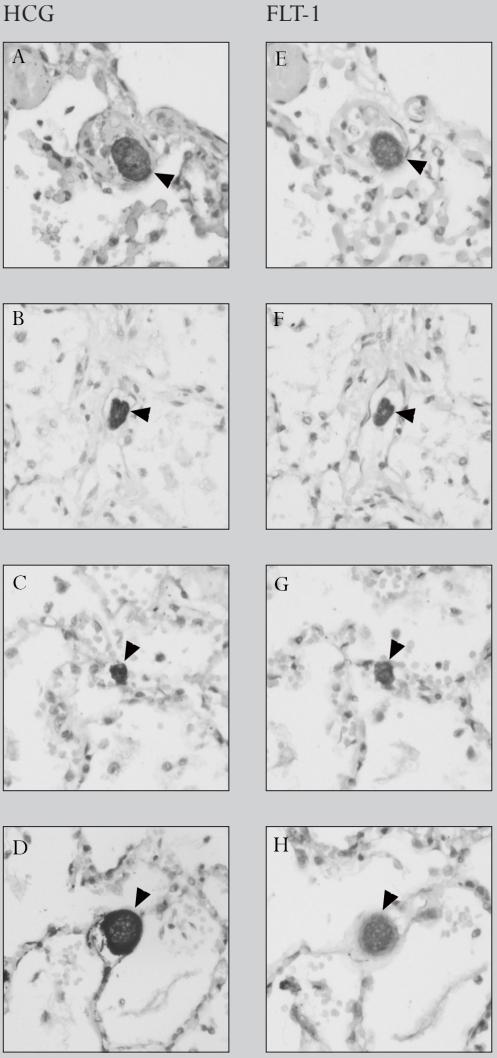

Figure 2B shows additional examples of multinucleate aggregates in maternal lungs, with co-localization of hCG (subpanels A-D) and Flt-1 (subpanels E-H). Each row shows matched sequential sections.

DENSITY OF SFLT-1 MRNA POSITIVE SYNCYTIAL KNOTS FIG 2C

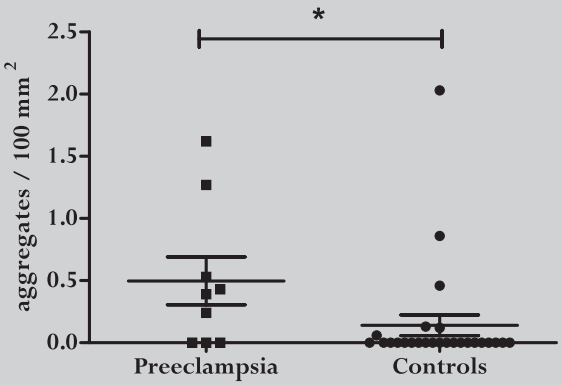

Figure 2C summarizes the number of hCG-positive syncytial aggregates within the maternal lungs of preeclamptic women (n=9) and control subjects (n=26). *$p<0.05$. The control subjects were women who died due to a cause other than a hypertensive disorder of pregnancy (e.g., pulmonary embolism, heart attack or arrhythmia).

REFERENCES

1. Steegers EA, von Dadelszen P, Duvekot JJ, Pijnenborg R. Pre-eclampsia. *Lancet* 2010; 376:631-44.
2. Levine RJ, Maynard SE, Qian C, Lim KH, England LJ, Yu KF, *et al.* Circulating angiogenic factors and the risk of preeclampsia. *N Engl J Med* 2004; 350:672-83.
3. Maynard SE, Min JY, Merchan J, Lim KH, Li J, Mondal S, *et al.* Excess placental soluble fms-like tyrosine kinase 1 (sFlt1) may contribute to endothelial dysfunction, hypertension, and proteinuria in preeclampsia. *J Clin Invest* 2003; 111:649-58.
4. Ahmad S, Ahmed A. Elevated placental soluble vascular endothelial growth factor receptor-1 inhibits angiogenesis in preeclampsia. *Circ Res* 2004; 95:884-91.
5. Bujold E, Romero R, Chaiworapongsa T, Kim YM, Kim GJ, Kim MR, *et al.* Evidence supporting that the excess of the sVEGFR-1 concentration in maternal plasma in preeclampsia has a uterine origin. *J Matern Fetal Neonatal Med* 2005; 18:9-16.
6. Clark DE, Smith SK, He Y, Day KA, Licence DR, Corps AN, *et al.* A vascular endothelial growth factor antagonist is produced by the human placenta and released into the maternal circulation. *Biol Reprod* 1998; 59:1540-48.
7. Rajakumar A, Cerdeira AS, Rana S, Zsengeller Z, Edmunds L, Jeyabalan A, *et al.* Transcriptionally active syncytial aggregates in the maternal circulation may contribute to circulating soluble fms-like tyrosine kinase 1 in preeclampsia. *Hypertension* 2012; 59:256-64.
8. Chua S, Wilkins T, Sargent I, Redman C. Trophoblast deportation in pre-eclamptic pregnancy. *Br J Obstet Gynaecol* 1991; 98:973-79.
9. Redman CW, Sargent IL. Circulating microparticles in normal pregnancy and pre-eclampsia. *Placenta* 2008; 29 Suppl A:S73-S77.
10. Schmorl G. Pathologisch-anatomische Untersuchungen uber Puerperal-Eklampsie. Leipzig: Verlag FCW Vogel, 1893.
11. Brown MA, Lindheimer MD, de Swiet M, Van Assche A, Moutquin JM. The classification and diagnosis of the hypertensive disorders of pregnancy: statement from the International Society for the Study of Hypertension in Pregnancy (ISSHP). *Hypertens Pregnancy* 2001; 20:IX-XIV.
12. Casparie M, Tiebosch AT, Burger G, Blauwgeers H, van de Pol A, van Krieken JH, *et al.* Pathology databanking and biobanking in The Netherlands, a central role for PALGA, the nationwide histopathology and cytopathology data network and archive. *Cell Oncol* 2007; 29:19-24.
13. Lau YF. Detection of Y-specific repeat sequences in normal and variant human chromosomes using in situ hybridization with biotinylated probes. *Cytogenet Cell Genet* 1985; 39:184-87.
14. Nevo O, Soleymanlou N, Wu Y, Xu J, Kingdom J, Many A, *et al.* Increased expression of sFlt-1 in in vivo and in vitro models of human placental hypoxia is mediated by HIF-1. *Am J Physiol Regul Integr Comp Physiol* 2006; 291:R1085-R93.
15. Johansen M, Redman CW, Wilkins T, Sargent IL. Trophoblast deportation in human pregnancy--its relevance for pre-eclampsia. *Placenta* 1999; 20: 531-9.

16. Askelund KJ, Chamley LW. Trophoblast deportation part I: review of the evidence demonstrating trophoblast shedding and deportation during human pregnancy. *Placenta* 2011; 32:716-23.
17. Pritchard S, Wick HC, Slonim DK, Johnson KL, Bianchi DW. Comprehensive analysis of genes expressed by rare microchimeric fetal cells in the maternal mouse lung. *Biol Reprod* 2012; 87:42.
18. Bianchi DW, Zickwolf GK, Weil GJ, Sylvester S, DeMaria MA. Male fetal progenitor cells persist in maternal blood for as long as 27 years postpartum. *Proc Natl Acad Sci U.S.A* 1996; 93:705-08.
19. O'Donoghue K, Chan J, de la Fuente J, Kennea N, Sandison A, Anderson JR, *et al.* Microchimerism in female bone marrow and bone decades after fetal mesenchymal stem-cell trafficking in pregnancy. *Lancet* 2004; 364:179-82.
20. Khosrotehrani K, Johnson KL, Cha DH, Salomon RN, Bianchi DW. Transfer of fetal cells with multilineage potential to maternal tissue. *JAMA* 2004 ;292:75-80.