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Placental Inflammation in Obstetrical Complications

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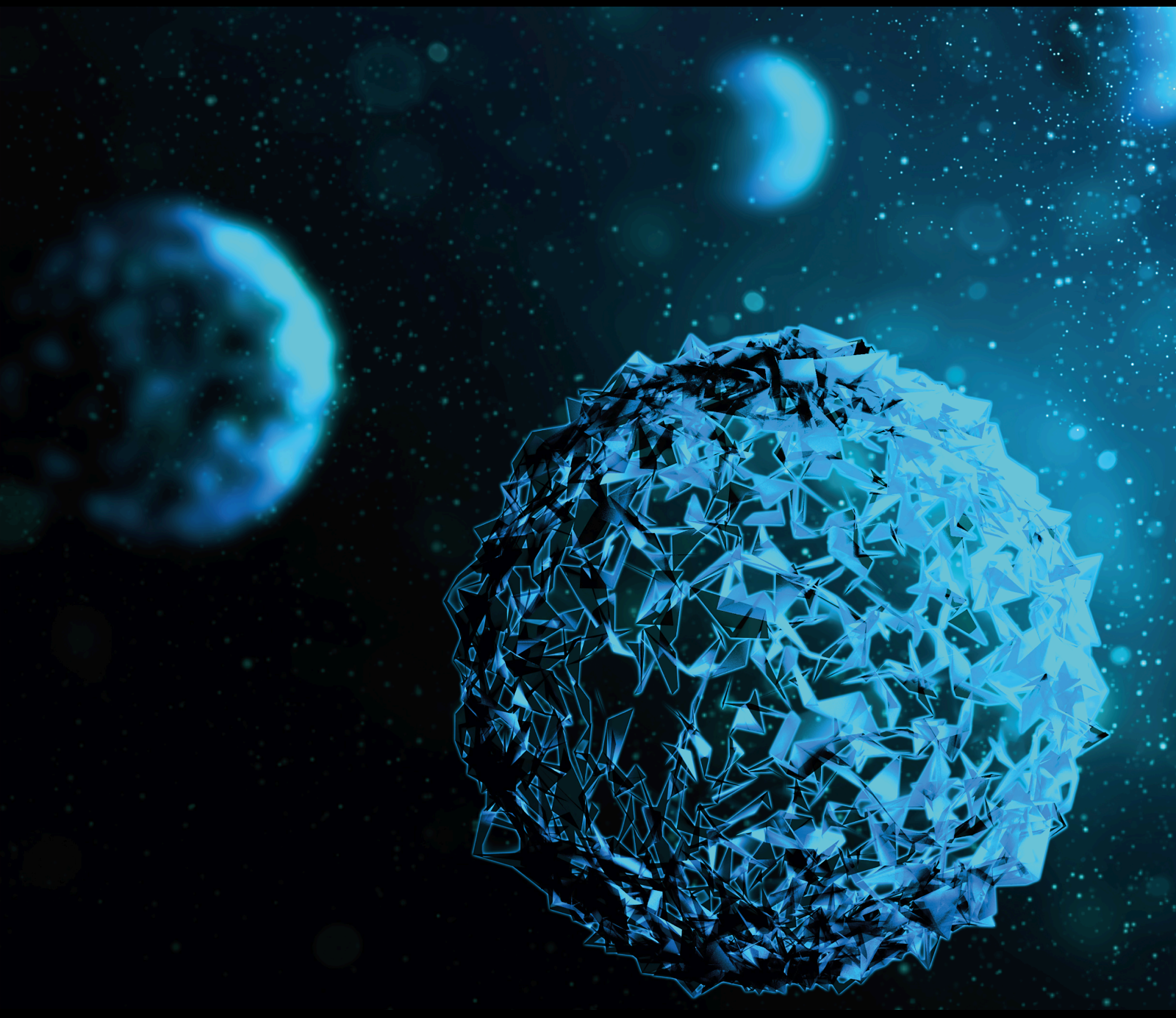

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Hindawi
BioMed Research International
Volume 2020, Article ID 4808072, 10 pages
https://doi.org/10.1155/2020/4808072

Review Article

The Roles of Uterine Natural Killer (NK) Cells and KIR/HLA-C Combination in the Development of Preeclampsia: A Systematic Review

Xiuhua Yang,[1] **Yahui Yang**,[2] **Yiru Yuan**,[2] **Lin Liu**,[2] **and Tao Meng**[1]

[1]*Department of Obstetrics, The First Hospital of China Medical University, Shenyang, Liaoning, China*
[2]*China Medical University, Shenyang, Liaoning, China*

Correspondence should be addressed to Tao Meng; cmumt@163.com

Received 4 December 2019; Revised 18 February 2020; Accepted 20 March 2020; Published 30 March 2020

Guest Editor: Katsuhiko Naruse

Preeclampsia (PE) is termed as a systemic disease that involves multiple organs; however, the exact etiology is still quite unclear. It is believed that the poor remodeling of uterine spiral arteries triggers PE, thereby causing failed placentation and producing inflammatory factors. The decline of blood flow results in lowering the nutrients and oxygen received by the fetus and augmenting the placental pressure in PE. Decidual immune cells, especially uterine natural killer (uNK) cells, are involved in the process of placentation. Decidual NK (dNK) cells significantly contribute to the vascular remodeling through the secretion of cytokines and angiogenic mediators in normal placental development. The abnormal activation of NK cells in both the peripheral blood and the decidua was counted among the causes leading to PE. The correlation existing between maternal killer cell immunoglobulin-like receptor (KIR) and HLA-C in trophoblast cells constitutes a robust evidence for the genetic etiology of PE. The combinations of the two kinds of gene systems, together with the KIR genotype in the mother and the HLA-C group in her fetus, are likely to exactly decide the pregnancy outcome. The women, who have the inappropriate match of KIR/HLA-C, are likely to be prone to the augmented risk of PE. However, the combinations of KIR/HLA-C in PE undergo ethnic changes. The extensive prospective research works in Europe, Asia, and Africa are required for providing more findings in PE patients.

1. Introduction

Preeclampsia (PE) refers to quite a serious obstetrical complication that has high blood pressure and proteinuria, occurring following the 20-week period of pregnancy, and it threatens the life of both the mother and the neonate. In accordance with the statistics of World Health Organization (WHO), one-tenth of the pregnant females suffer from PE, and PE constitutes one-seventh of the deaths in pregnant women [1, 2]. The occurrence of PE in China amounts to 5% [3]. PE is termed as a systemic disease that involves multiple organs including the nervous system, blood system, heart, liver, and kidney [4]. In case of the ineffective control of the symptoms, PE is expected to develop into convulsion or coma, termed as eclampsia. Moreover, severe PE is likely to cause fetal growth restriction (FGR) or even fetal death owing to the placental vascular dysplasia. In treating PE, magnesium sulfate is usually put to use for the purpose of preventing eclampsia [5]. In addition, if systolic blood pressure amounts to higher than 160 mmHg or diastolic blood pressure is above 110 mmHg, antihypertensive drugs are usually put to use intravenously, such as labetalol [6]. Angiotensin-converting enzyme (ACE) inhibitors cannot be utilized in pregnancy owing to their teratogenic function on the neonate [7]. Owing to the fact that the current treatment is incapable of effectively alleviating the symptoms of PE, we require further exploring the pathogenesis of this disease, aimed at finding a better treatment.

Even though a number of factors have been discovered as correlated with the occurrence of PE, the exact etiology is still quite unclear. These causes count on not only environmental factors but also immunological factors, genetic factors, vascular endothelial cell damage, blood system abnormalities, and some unidentified factors [8–10]. In PE,

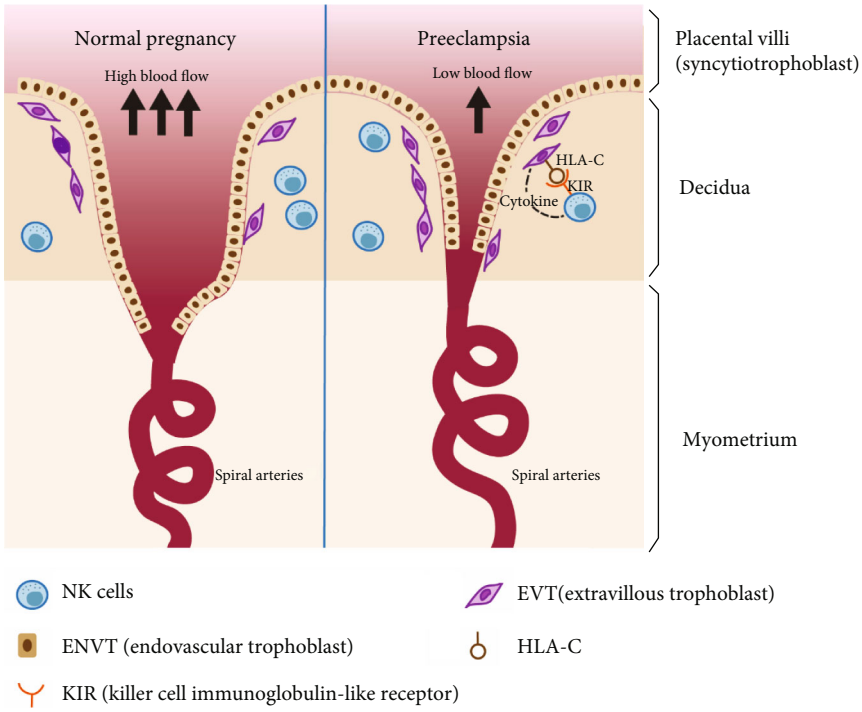

Figure 1: Preeclampsia (PE) is related to the poor placentation in the early pregnancy. In normal early pregnancy (left picture), extravillous trophoblast cells (EVT) invade deeply enough in the myometrium and also migrate into the endothelium of maternal spiral arteries. This ensures that there is abundant blood flow at the maternal fetal interface. However, in PE patients (right picture), the depth of trophoblast invasion is decreased with insufficient remodeling of trophoblast cells. Blood flow is also reduced in PE. Inappropriate combination of KIR/HLA-C in PE will inhibit the functions of NK cells including secreting angiogenic cytokines. As a result, uterine NK (uNK) cells in these women have low functional activity and they do not support placental growth as needed.

trophoblast cells fail in invading optimally [11]. It is believed that the poor remodeling of uterine spiral arteries triggers PE, thereby causing the failed placentation and producing inflammatory factors. PE patients have immune inflammation as well as the generation of autoimmune antibodies [12]. Inflammatory mediators result in the activation of maternal endothelial cells, which have the potential of causing hypertension and proteinuria [13, 14]. In the present review, we provided the summary of the roles of uterine natural killer (NK) cells and killer cell immunoglobulin-like receptor (KIR)/HLA-C combination in the development of PE according to the literature published in the past few years. Also, the current manuscript aims at identifying the theoretical basis for the treatment of immune inflammation in PE, together with improving the outcome for the neonates and the women having PE.

2. Reduced Blood Flow during Placentation in PE

In the early phase of normal pregnancy, the uterine arteries undergo changes in the structure, thereby increasing the blood flow to the uterus by 100 times [15]. The transformation of uterine arteries has a close correlation with placentation. In the process of placentation, fetal trophoblasts from the placenta immerse into the uterine wall, besides implanting into uterine arteries and penetrating the smooth muscle of the uterus. This change in trophoblasts makes uterine arteries significantly conductive catheters, leading to the decline of the speed and pressure of uterine blood flow into the placenta. The cessation of uterine artery dilation further lowers the velocity of blood flow into the villous space. This provides sufficient time for exchanging the nutrients between the mother and the fetus, in particular, when the demand for nutrients is the highest in the late pregnancy. In addition, some important signaling pathways including YY1/MMP2 play important roles in the invasion of trophoblasts during the first trimester [16].

In PE, trophoblast cells fail in helping with the structural transformation of arteries, thereby causing the artery blood to flow into the villus space without essential conversion; also, it causes the injury of the villus structure. The decline of blood flow results in lowering the nutrients and oxygen received by the fetus and augmenting the placental pressure [17]. Accordingly, one of the main causes of PE is the insufficient remodeling of uterine arteries [18]. Decidual natural killer (dNK) cells and extravillous trophoblasts (EVT) are involved in placental formation [19]. Now, a number of scholars hold the belief that the unusual immune response of the mother to the fetus constitutes a preliminary factor of PE, which causes the systemic inflammatory response in the female [20]. A number of evidence suggest that PE is a result of poor placentation in early pregnancy [21, 22] (Figure 1).

3. The Process of Placentation Involved by Immune Cells in PE

For the purpose of comprehending the mechanism of the decidua regulating placentation, the decidual immune cells have been concentrated on [23, 24]. Considering that the reason of immune cells is from the epidemiological investigation of PE [25], firstly, it refers to a disease, occurring in the first pregnancy, after which the mother could get immunity. Changing the father following a normal pregnancy is likely to induce PE; nonetheless, if the patients, having had PE change their sexual partners, the incidence of PE is going to be lowered [26]. Moreover, the incidence of this disease has memory and specificity, which is consistent with the characteristics of immune diseases. There have been a number of investigations dealing with the family history and genetics of mothers; furthermore, several research works have shed light on the fact that the paternal factor also plays a major role in the incidence of PE, together with its association with the fetal weight [27–29]. Numerous research works have revealed that the relationship between the maternal and fetal immune systems has the potential of determining the outcome of pregnancy. The immune cells in the decidua play quite a critical role at the maternal fetal interface. Since the mother and the fetus form the two different genetic individuals, the invading trophoblasts carry genes and molecules with the paternal source; in immunologic terms, the fetus is alien to the mother.

The hypothesis that decidual immune cells are involved in the placentation is primarily owing to two reasons. Firstly, the cell-cell interaction in the decidua takes place between the two allogeneic individuals. Secondly, the pivotal role of the decidua in placentation is reflected in the investigation of obstetrical complications. In the patients having placenta percreta with the absence of the decidua, the trophoblasts deeply invade the uterine muscle wall. In this event, the placenta is most likely to grow in the scar of the former cesarean section [30]. In the early pregnancy, 70% of neutrophils in the endometrium are uNK cells. These cells have KIRs, combining with HLA-C ligands in the trophoblasts [31]. Owing to the genetic variability of KIR as well as HLA-C, there are a number of varying types of combination of not only maternal KIR but also fetal HLA-C in each of the pregnancies [32]. Moreover, integrating the KIR and HLA-C figures out whether uNK cells are capable of secreting angiogenic cytokines. This field is comparatively newer; nonetheless, the comprehension of this knowledge could offer new perceptions and ideas not only for the diagnosis but also for the treatment of obstetrical complications like PE.

4. uNK Cells in the Pathogenesis of PE

Which type of immune cells is likely to be involved in the development of PE? Our answer is uNK cells, because they account for the majority (70%) of leukocytes in the process of implantation and placentation, and they have receptors that could combine with ligands in the trophoblasts. In spite of T cells, as the effector immune cells in charge of rejecting organ transplants, which account for 10 to 30% of leukocytes in the endometrium in the early phase of pregnancy, no available investigation indicates that the failure of pregnancy is a result of the rejection of T cells to the placental tissue [33]. Precisely, there are no research works that have found that maternal T cells are capable of recognizing and acting on trophoblasts. Approximately 90% of pNK cells are cytotoxic, together with having a $CD56^{dim}CD16^{+}$ surface phenotype, and the remaining 10% are $CD56^{bright}CD16^{-}$ phenotypes with little cytotoxicity [24, 34]. In addition, immune factors were collaborative for characterizing the pregnancy as a mildly inflammatory condition. The proportion of pNK cells undergoes a gradual increase in the early phase of pregnancy, together with a decrease in the middle phase of pregnancy, continuing the decline in the third trimester in a normal pregnancy [35]. Carolis et al. were of the belief that the changes in pNK cells played a pathogenic role in PE [36]. The abnormal activation of NK cells in both the peripheral blood and the decidua was counted among the causes leading to PE [36].

The uNK cells differ with pNK cells in phenotype and function [17]. uNK cells are phenotypic $CD16^{-}CD56^{bright}$ NK cells with little cytotoxicity that have a direct contact with the allogeneic EVT cells. uNK cells are regarded as playing a pivotal function in the adjustment of fetal EVT for the establishment of a fine placentation [35]. The specific uNK cells ($CD56^{+}$, $CD3^{-}$, $CD16^{-}$, and $CD9^{+}$) were similar in the late and early pregnancies, which demonstrated that these uNK cells contributed to the normal development of the fetus all through the entire pregnancy [37]. Mice without uNK cells do not have the compatible vascular formations associated with pregnancy [38, 39].

There are two different kinds of uNK cells that have been confirmed in mice in accordance with their activities towards Dolichos biflorus agglutinin (DBA) [40]. DBA^{+} uNK cells produce angiogenic mediators, while DBA^{-} uNK cells secrete IFN-γ [40]. There was an experiment that had a more rigorous design, making use of the alymphoid mice, achieving the bone marrow from either IFN-$\gamma^{-/-}$ mice or serious combined immunodeficient mice, which were absent for T and B lymphocytes; thereafter, they discovered the fact that the IFN-γ produced by NK cells was quite pivotal for the spiral artery remodeling [41]. In another research work, the researchers made use of BPH/5 mice, having the core characteristics of PE; also, they discovered that there was a decline in the number of dNK cells in their decidua [42]. The reduction of uNK cells had an association with the upregulation of Cox2 and IL-15 at the uterus-placenta interface [42]. Following the addition of the Cox2 inhibitor, lowering the expressions of Cox2 and IL-15, the number of uNK cells recovered [42].

Furthermore, the invasion of trophoblast cells in mice was lower as compared with that in human beings, so trophoblast cells in mice significantly differ with those in humans [43]. Even though the majority of NK receptors in mice are from the Ly49 receptor family, their function seems to have a similarity with KIR in humans. With regard to mouse studies, on the addition of H2-Dd, the vascular remodeling was declined and fetal growth was decreased in comparison with the homotypic mice that lacked merely H2-Dd [44]. This major histocompatibility complex (MHC) molecule has the

potential to bind to the inhibitory receptor Ly49A, besides decreasing the extra uNK subtype cells on their appearance [44]. Being specific, the growth rate of fetus slowed down irrespective of the parental source of the H2-Dd molecule [44]. These findings suggest that some combinations of maternal NK receptors and paternal/maternal MHC groups had the potential of impacting the trophoblast invasion and vascular remodeling. The research works dealing with the pregnant transgenic mice discovered the fact that the uterine spiral arteries of transgenic mice, which lacked uNK cells, were aberrantly straight as well as narrow [45, 46]. In mice studies, adrenomedullin (AM), a pregnancy-related peptide, has been termed as a pivotal factor, facilitating the accumulation and activation of maternal uNK cells to the placenta, together with helping the process of spiral arteries remodeling eventually [47]. The placentas that lack AM or its receptor manifested the decreased fetal vessel branching in the uterus, the failure of spiral artery remodeling, and re-endothelialization, in addition to apparently decreasing the amounts of maternal uNK cells [47].

5. Angiogenic Factors Produced by uNK Cells in PE

The human placenta experiences the elevated degrees of angiogenesis as well as vasculogenesis all through the growth of the fetus [48]. Also, the human placenta experiences the phase of pseudovascularization, which indicates that all through the mechanism of placentation, the cytotrophoblasts of the placenta are transformed from the epithelial type to the endometrial type [49]. PE is featured by the extensive systemic impairment of endothelial cells in the maternal body [50]. Currently, a general belief is held that the incidence of PE is owing to the placental vascular dysplasia; contrarily, this changed placenta is expected to cause the extensive damage of vascular endothelial cells [51]. The declining reconstruction of uterine spiral arterioles is considered the outcome of the defect of the intravascular invasion as well as the damaged formation of pseudovessels [52]. Both the animal and human experiments have discovered the fact that PE takes place when the invasion of trophoblast declines, besides the occurrence of the uterine placental hypoperfusion. For instance, in animal experiments, it was discovered that placental ischemia caused the continuous mechanical contraction of uterine arteries and aorta, thereby causing hypertension, proteinuria, and endothelial hyperplasia of renal tubules [53]. Besides that, the pathological report of severe PE patients sheds light on the fact that the placenta has infarction and the arteries have rigid stenosis [54]. By means of ultrasonic monitoring, it could be discovered that, prior to the medical manifestations emerging in PE patients, the blood flow between the uterus and the placenta undergoes a decline, coupled with the increase in the resistance of uterine blood vessels [55]. Nonetheless, this change was observed as insignificant in one-third of PE patients. The placental ischemia itself is deemed as insufficient for causing PE. A number of factors, promoting or inhibiting the angiogenesis, significantly contribute to the placental development [56].

As indicated by in vitro experiments, dNK cells could secrete two cytokines that include interleukin-8 (IL-8) and interferon-inducible protein-10 (IP-10), promoting the invasion of trophoblast cells [54]. Subsequent to the addition of the monoclonal antibodies of IL-8 as well as IP-10 to the cultured trophoblast cells, the migration capability of cells underwent a decline [54]. In vitro, dNK cells also secreted factors promoting angiogenesis, for instance, vascular endothelial growth factor (VEGF) and placental growth factor (PlGF) [57]. In comparison with peripheral NK (pNK) cells, the secretion of VEGF and PlGF augmented significantly following the addition of IL-15 in dNK cells [57]. The migration of human umbilical vascular endothelial cells (HUVEC) was augmented in dNK cells supplied with IL-15 in vitro, besides the reticular structure appearing earlier; nonetheless, they did not receive the same impacts in pNK cells [57]. As the researchers added Flt1-Fc, which was an inhibitor of the VEGF and PlGF signal pathway, some of these functions were declined [57]. Following the subcutaneous injection of dNK cells and JEG-3 choriocarcinoma cell line into the nude mice, the volume of JEG-3 tumor and the number of blood vessels augmented, which suggested that dNK cells had the potential of promoting angiogenesis [57]. In the earliest phase of arterial recasting, matrix metalloproteinase-7 (MMP-7) and MMP-9 were observed in dNK cells in the specimens of the decidua basalis, which suggested that dNK cells had involvement in the independent stage of arterial recasting [58]. In mouse experiments, not only TGF-β but also PlGF and VEGF contributed to the angiogenesis [59]. These experiments suggest that dNK cells significantly contribute to the vascular remodeling through the secretion of cytokines and angiogenic mediators in the development of the placenta.

6. The Roles of KIR and HLA-C in Immunity, Normal Pregnancy, and Preeclampsia

Under physiological conditions, the acting ways between KIRs and HLA class I ligands determine whether NK cells can play normal functions. The consequence of KIR and HLA combination on NK cell function could change according to the resting status or in an immune state. For instance, if there is under the resting status, inhibitory KIRs make NK cells play a functional role, while activating KIR decreases NK cell abilities when combined with their cognate ligand (called NK cell education). In an immune state, inhibitory KIR could reduce NK cell ability unless HLA class I expression is decreased, while activating KIR could prime NK cell roles. The KIR/HLA combination is very complex and extremely polymorphic. The relationships between KIRs and HLAs are related to many diseases, including infectious diseases, autoimmune diseases, malignant tumors, and transplant reactions [60–63]. In pregnancy, the inhibitory or activating KIRs are capable of regulating the activity of uNK cells, thereby playing an immunomodulatory role at the maternal fetal interface. KIR A do not have stimulatory receptors, whereas KIR B have both stimulatory and inhibitory receptors. In each of the pregnancies, the KIR genes of the pregnant woman are expected to change, since these KIR genes

are inherited and expressed by uNK cells. The paternal HLA-C group is also expected to be different (even from the same father), for the reason that the fetus is likely to inherit any group of HLA-C from the father. Besides that, the mixes of the two kinds of gene systems, together with the KIR genotype in the mother and the HLA-C group in her fetus, are likely to exactly decide the reaction between trophoblast cells and uNK cells.

The correlation existing between KIR/HLA-C and PE constitutes a robust evidence for genetic etiology of PE (Table 1). Until today, the largest study that ever took place in Britain involved 200 patients, who had PE in the experimental group, together with 201 women in the control group with normal deliveries [64]. When the mother had inhibitory KIR (KIR AA genotype), besides the fetus having HLA-C2, it was more likely to have the abnormalities during the spiral artery remodeling and defective placentation, eventually resulting in PE [64]. In comparison with C1, C2 combines more closely with homologous KIR. Moreover, inhibitory KIR has KIR2DL1, capable of strongly inhibiting NK cells. Nevertheless, there is no activating KIR at this time, failing in providing activation signals. Consequently, NK cells in these women manifested low functional activity, besides not supporting placental growth as required.

Nonetheless, an extensive research from Japan did not support this finding [65]. To our understanding, Caucasian men are more likely to carry HLA-C2 allele as compared with Japanese men. Accordingly, for Japanese women, the risk of PE in combination with Caucasian men should be higher as compared with that in combination with Japanese men. However, no expected experimental results have been attained that the incidence of PE in the former combination was lower as compared with that in the latter (1.54% *vs.* 2.67%) [65].

It requires observation that the proportion of KIR AA in patients having PE augmented only when the fetus inherited paternal HLA-C2 [19]. Obstetrical complications had lower likelihood of occurrence in the females, having KIR B genotype, including activating KIR2DS1, which was bound, in particular, to HLA-C2 [19]. uNK cells produce a number of cytokines that include TGF-β, PlGF, and VEGF, which may be of pivotal significance in guiding immune reactions [57, 66]. KIR2DS1 is capable of stimulating uNK cells that augment the angiogenesis and immune response, thereby resulting in healthy pregnancy, whereas inhibiting uNK cells is likely to lower the secretion of cytokines, thereby causing PE [66]. It has been discovered that KIR2DS1-positive females having a fetal HLA-C2 had a preferable trophoblast invasion as well as spiral artery remodeling through the secretion of granulocyte-macrophage colony-stimulating factor (GM-CSF), while in the females having the KIR AA genotype, PE was more likely to take place [67].

The correlation between activating KIR genes and lower risk of PE changes among different populations. KIR2DS5 protectively contribute to Ugandans, which are unique to sub-Saharan Africa (SSA) [68]. Researchers are unaware of the fact of what the ligands of KIR2DS5 are; nonetheless, all of the research works carried out the single European KIR2DS5∗002 allele that refers to an activating KIR frequently observed in the tel-B in European people. Together with that, the KIR2DS5∗006 allele refers to a protective allele that appears in the cen-B in SSA and can be activated while binding to HLA-C2 [68].

It has also been illustrated that the expressions of KIR2DL/S1, 3, and 5 were decreased on the percentage of dNK cells in a case where patients had elevated uterine artery resistance index (RI), indicating poor spiral artery remodeling [65]. This is termed as the mechanical application of PE as a result of the interactions between dNK cells and trophoblasts [69].

In the context of China, women having PE have an evidently larger frequency of KIR AA genotype, primarily containing the inhibitory receptors, in addition to the lower frequency of maternal activating gene KIR2DS1 as compared with normal pregnancies [70]. Furthermore, this finding shows consistency with earlier research works in other populations [66, 67]. It is believed that activating KIR2DS1 refers to a protective determinant, and insufficient activation of uNK cells is expected to lead to decreased invasion of trophoblasts, thereby resulting in PE [70]. Moreover, it was also indicated that if the fetus possessed more numbers of HLA-C2 genes as compared with the mother, the maternal KIR AA genotype was correlated with a higher risk for PE [70]. This research work also supports the hypothesis that immune factors from fathers contribute to the development of PE [71]. In another extensive investigation from China, there were 271 women in the experimental group, together with 295 women in the control group, who were collected with the use of the polymerase chain reaction with sequence specific primers (PCR-SSP) assay [72]. They figured out that PE patients had fewer activating KIR2DS2, KIR2DS3, and KIR2DS5 [72]. Besides that, the gene frequency of total activating KIRs in PE group was evidently smaller in comparison with that of the control group ($P = 0.03$) [72].

PE patient showed more likelihood of being KIR2DL1 positive when the fetus had HLA-C2C2; in addition, in this case, uNK cells were expected to receive the strongest inhibitory signals [72]. Furthermore, the same trend was also discovered in Mexico [37]. 10 normal decidual specimens and 9 decidual samples from PE patients were employed in the process of cesarean section [37]. They discovered that inhibitory KIRs were predominated in PE patients in comparison with normal pregnant women [37].

Besides that, it has also been highlighted that activating maternal KIR-B genotype itself, in combination with fetal HLA-C2, had an evident correlation with decidual acute atherosis in PE patients [73]. In PE patients having acute atherosis, the incidence of this combination amounted to be 60%, whereas, in PE patients not having acute atherosclerosis, the rate was 24.5% ($P = 0.001$) [73]. They held the belief that the appearance of acute atherosis was a result of decidual inflammatory reactions owing to the reactions between fetal HLA-C2 and maternal activating KIRs on dNK cells [73].

Nevertheless, some negative findings were made as well. In a Danish study, 259 pregnant females, who had severe PE or eclampsia in the trial group, together with 259 pregnant females, who did not have PE or eclampsia in the control group, were enrolled [74]. The blood of these pregnant

Table 1: Studies on killer cell immunoglobulin-like receptor (KIR)/HLA-C in preeclampsia (PE) sorted by publication date.

Ethnicity	Authors	The year of publication	The experimental group	The control group	Samples	Conclusions
British	Hiby et al. [64]	2004	PE patients ($n = 200$)	Full-term pregnant women ($n = 201$)	Mothers: blood Babies: umbilical cord blood or mouth swabs	The combination of maternal KIR AA and fetal HLA-C2 was more common in PE.
Japanese	Saito et al. [65]	2006	Couples with Japanese women and Caucasian men ($n = 328$)	2003 database in Japan ($n = 36{,}829$)	—	There was no statistical difference in the incidence of PE between the two groups.
White British	Hiby et al. [20]	2010	PE patients ($n = 742$)	Normal primiparas ($n = 592$)	Mothers: blood Babies: umbilical cord blood or mouth swabs	Maternal KIR AA was related to PE when the fetus had more HLA-C2 inherited from the father. Maternal telomeric KIR B (KIR2DS1) was a protective factor for PE.
Mexico	Sánchez-Rodríguez et al. [37]	2011	PE patients ($n = 9$)	Normal pregnant women ($n = 10$)	Mothers: decidual samples	PE patients tended to have more inhibitory KIRs.
Chinese Han population	Yu et al. [70]	2014	PE patients ($n = 47$)	Normal pregnant women ($n = 54$)	Mothers and fathers: blood Babies: umbilical cord blood	Less PE patients had KIR2DS1, and more PE patients had AA genotype compared with normal pregnant women. More PE patients with KIR AA had fewer HLA-C2 than their babies.
Chinese Han population	Long et al. [72]	2014	PE patients ($n = 271$)	Normal pregnant women ($n = 295$)	Mothers: blood Babies: umbilical cord blood	PE patients had less activating KIRs (2DS2, 2DS3, and 2DS5). The frequency of KIR2DL1 was increased in PE patients when the neonate was HLA-C2C2.
Uganda (sub-Saharan Africans)	Nakimuli et al. [68]	2015	PE patients ($n = 254$)	Normal pregnant women ($n = 484$)	Mothers: blood Babies: umbilical cord blood	The combination of maternal KIR AA and fetal HLA-C2 was related with PE. KIR2DS5 and KIR2DL1 had the protective effect on PE.
European	Johnsen et al. [73]	2018	PE patients ($n = 83$)	Normal pregnant women ($n = 83$)	Mothers: blood, decidua, or muscle Babies: umbilical cord blood or fetal placenta	PE patients with acute atherosis tended to have the combination of maternal KIR-B and fetal HLA-C2 compared with PE patients without acute atherosis.
European	Larsen et al. [74]	2019	Severe PE patients ($n = 259$)	Normal pregnant women ($n = 259$)	Mothers: blood Babies: blood	There was no effect of KIR/HLA-C combination on the risk of severe PE.

women as well as their newborns was gathered [74]. No correlation existing between maternal KIR AA and HLA-C2 in their newborns was observed [74]. With the newborns carrying more HLA-C2 allele as compared with the pregnant women, no difference in maternal KIR AA genotype between the trial cohort and the control cohort was observed [74].

Contradictory results of KIR/HLA-C combination in PE patients are likely owing to the changes in KIR gene and repertoire frequencies between different ethnicities. KIR genotypes also have an extensive variation in geographical distribution. Therefore, the direct comparison of these studies about KIR and HLA correlation with PE is a difficult task because they were conducted in various populations, together with distinct methods.

With regard to the future studies, it is necessary to carry out large-scale prospective randomized controlled research on different ethnic groups in Europe, Asia, and Africa, and researchers should select suitable control groups for their studies, simultaneously collect KIR classification of mothers and HLA-C groups of husbands and neonates, and analyze and judge whether different combination types of KIR/HLA-C are related to the prognosis of mothers and newborns.

Except for class I HLA-C, EVT also express atypical class Ib HLA-E, F, and G [75]. HLA-G can inhibit the effect of NK cells [76]. In the first trimester, the embryo could produce soluble HLA-G [77] and it is important for immunotolerance in maternal fetal interface [78]. Compared with nonpregnant females, the expression of soluble HLA-G in serum of pregnant women at all stages was significantly higher [79] and the soluble HLA-G increased the production of IL-10 [80]. It was found that the expression of soluble HLA-G in the serum and placenta of PE women was significantly lower than that of normal pregnant women [81–85]. It is suggested that soluble HLA-G may be involved in the pathogenesis of PE. Recently, it has been found that class II HLA-DR can be detected in placentas from PE patients ($n = 23$), but not in normal placentas ($n = 14$) [86]. The mechanism of HLA-DR in PE needs to be further explored.

7. Conclusions

To conclude, NK cells are existent in the decidua in abundance in early pregnancy, which are of immense significance for the maintenance of normal pregnancy. In the mechanism of placentation, uNK cells require necessary activation for the purpose of releasing cytokines, promoting angiogenesis, and helping remodel uterine spiral arteries. The women, who have the inappropriate match of KIR/HLA-C, are likely to be prone to the augmented risk of PE. With regard to these women, the RI of the uterine artery could be monitored in early pregnancy, whereas timely and effective intervention could be performed for the prevention of PE.

Because of reproductive failure, more and more couples choose gestational carriers. In 2013, gestational carriers accounted for 2.5% of all assisted reproductive technologies in USA [87]. The incidence of PE in gestational carriers has not been reported, while that of multiple births and preterm birth is relatively high [88]. Accordingly, HLA-C and KIR genotyping could be potentially applicable for selecting the third party gametes or gestational carriers, aimed at avoiding the obstetrical complications including PE. In clinical work, for the high-risk patients of PE, the role of uNK cells in the process of placentation should be taken into account; for the women with high-risk combinations of KIR/HLA-C, the frequency of prenatal examination should be increased.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Hindawi
BioMed Research International
Volume 2019, Article ID 5320902, 8 pages
https://doi.org/10.1155/2019/5320902

Research Article

Upregulation of VEGF and PEDF in Placentas of Women with Lower Extremity Venous Insufficiency during Pregnancy and Its Implication in Villous Calcification

Miguel A Ortega,[1,2] Miguel Ángel Saez,[1,3] Ángel Asúnsolo,[2,4] Beatriz Romero,[1,2] Coral Bravo,[3,5] Santiago Coca,[1,2] Felipe Sainz,[3,6] Melchor Álvarez-Mon,[1,2,7,8] Julia Buján,[1,2] and Natalio García-Honduvilla[1,2]

[1]*Department of Medicine and Medical Specialties, Faculty of Medicine and Health Sciences and Networking Biomedical Research Centre on Bioengineering, Biomaterials and Nanomedicine (CIBER-BBN), University of Alcalá, Alcalá de Henares, Madrid, Spain*
[2]*Ramón y Cajal Institute of Healthcare Research (IRYCIS), Madrid, Spain*
[3]*Pathological Anatomy Service, Central University Hospital of Defence-UAH Madrid, Spain*
[4]*Department of Surgery, Medical and Social Sciences, Faculty of Medicine and Health Sciences, University of Alcalá, Alcalá de Henares, Madrid, Spain*
[5]*Service of Gynecology and Obstetrics, Section of Fetal Maternal Medicine, Central University Hospital of Defence-UAH Madrid, Madrid, Spain*
[6]*Angiology and Vascular Surgery Service, Central University Hospital of Defence-UAH Madrid, Madrid, Spain*
[7]*Immune System Diseases-Rheumatology and Oncology Service, University Hospital Príncipe de Asturias, Alcalá de Henares, Madrid, Spain*
[8]*Internal Medicine Service, University Hospital Príncipe de Asturias, Alcalá de Henares, Madrid, Spain*

Correspondence should be addressed to Julia Buján; mjulia.bujan@uah.es

Received 1 August 2019; Accepted 17 October 2019; Published 9 December 2019

Guest Editor: Katsuhiko Naruse

Pregnancy is a period in a woman's life in which changes can occur that affect different physiological processes. Common conditions during this period include vascular changes, such as lower extremity venous insufficiency (VI). This is an observational, analytical, and prospective cohort study in which 114 pregnant women were analyzed, of which 62 were clinically diagnosed with VI. In parallel, 52 control patients without VI (HC) were studied. The aim of this study was to observe changes in angiogenesis and inflammation markers as well as the presence of calcium deposits. The expression of vascular endothelial growth factor (VEGF), transforming growth factor-β (TGF-β), and pigment epithelium-derived factor (PEDF) was analyzed by immunohistochemistry and RT-qPCR. The presence of calcium deposits was revealed using the von Kossa method. In the placentas of mothers with VI, gene expression of VEGF (34.575 [32.380–36.720] VI vs 32.965 [30.580–36.320] HC) and PEDF (25.417 [24.459–27.675] VI vs 24.400 [23.102–30.223] HC) significantly increased, as was protein expression in the placental villi. An increase in calcium deposits was observed in the placentas of women with VI (72.58% VI/53.84% HC). This study revealed the existence of cellular damage in the placental villi of mothers with VI with tissue implications such as increased calcification.

1. Introduction

The appearance of venous insufficiency (VI) in the lower extremities during pregnancy is a common complication, which is usually detected starting in the second half of pregnancy [1, 2]. VI is a vascular disorder that is defined by changes in the peripheral venous system and is a complication with a high prevalence in pregnancy [3, 4]. The

incidence of this venous pathology increases with the number of pregnancies and fetuses and with family history [5]. In pregnant women, it has been found that venous compression by the fetoplacental organ is a very important inducing factor in the development of VI [6–8].

The repercussions of venous disorders in pregnant women are not fully known, but the different components of the placenta make it one of the most susceptible tissues to these repercussions. Our previous studies have demonstrated that VI is related to structural lesions of the placental villi associated with an increase in hypoxia-inducible factor (HIF) [7, 9]. The association of HIF with vascular diseases during pregnancy is well known; numerous studies have clarified its important role in the pathogenesis of diseases such as preeclampsia [10–12]. One of the key points in the regulation of these processes is the angiogenic inducer vascular endothelial growth factor (VEGF), which plays a role in alterations in placental pathology [13]. Recent studies have indicated a decrease in transforming growth factor-β (TGF-β) in the placenta of women with preeclampsia [14]. Notably, equilibrium between VEGF and pigment epithelium-derived factor (PEDF), both of which are associated with altered angiogenesis and vascular remodeling, is necessary [15, 16].

Cell damage can be associated with a process of connective tissue alterations due to the loss or poor organization of elastic fibers. Among these events, calcification may play a fundamental role in the rigidity of tissues and specifically in the human placenta. The studies by Zhang et al. [17] have shown that PEDF can play an important role in human placenta calcification and damage, being a determining factor in vascular diseases such as preeclampsia. The aim of this study is to observe possible changes in the expression of angiogenesis and inflammation markers as well as the changes that may occur as a result of these processes, such as the presence of calcium deposits.

2. Patients and Methods

2.1. Study Population. An observational, analytical, and prospective cohort study was conducted in which 114 women in the third trimester of pregnancy (32 weeks) were analyzed. Sixty-two were clinically diagnosed with VI. In parallel, 52 control patients without a history of VI (HC) were studied. Having signed an informed consent form, the clinical history of each woman was collected, a general physical exam was conducted, and an examination of lower extremities was conducted using echo-Doppler (Portable M-Turbo Doppler Ultrasound, SonoSite, Inc., Washington, USA) at 7.5 MHz. The VI classification in the women in the study was performed according to the Classification System for Chronic Venous Disorders (CEAP) [18]. The CEAP classification is based on clinical data that collect the broad spectrum of morphological and functional alterations of the venous system. *Inclusion criteria* were women between 18 and 39 years of age in their third trimester of pregnancy with clinical evidence of VI in the lower extremities, with a classification of C1 or higher. *Exclusion criteria* were women diagnosed with diabetes mellitus and endocrine diseases, high blood pressure, autoimmune diseases, active infectious diseases, venous malformations, heart, kidney or lung failure, preeclampsia and/or hemolysis, elevated liver enzymes and low platelets (HELLP) syndrome, intrauterine growth restriction by known causes, women with a body mass index (BMI) $\geq 25\,\text{kg/m}^2$, unhealthy habits, presence of pathological lesions such as placental infarcts, avascular villi, delay in villi maturation, and chronic villitis, the appearance of any screening exclusion criteria during the previous months, and prior evidence of VI.

This study was carried out according to basic ethical principles: autonomy, beneficence, nonmaleficence, and distributive justice. The development of the study followed the standards of Good Clinical Research Practice and the principles enunciated in the last Declaration of Helsinki (2013) and the Convention of Oviedo (1997). The patients were informed of the details of the study, and each provided signed consent. The project was approved by the Clinical Research Ethics Committee of the Gómez-Ulla-UAH Defence Hospital (37/17).

2.2. Placental Tissue Samples. Placental tissue biopsies were obtained once the placenta was expelled. In all cases, 5 fragments of the placenta were obtained using a scalpel to ensure that the samples included multiple cotyledons. These fragments were placed into 2 different sterile tubes: one containing minimum essential medium (MEM) with 1% antibiotic/antimycotic (both from Thermo Fisher Scientific, Waltham, MA, USA) and another containing RNAlater® solution (Ambion, Austin, TX, EEUU). In the laboratory, the samples were processed in a laminar flow bench (Telstar AV 30/70 Müller class II 220 V 50 MHz; Telstar SA Group, Terrassa, Spain) in a sterile environment. The preserved samples were kept in 1 mL of RNAlater® at −80°C until processing for gene expression analysis. The samples conserved in MEM were reserved for histological and immunodetection studies.

2.3. Gene Expression Analysis. RNA was extracted using the guanidinium thiocyanate-phenol-chloroform method described by Ortega et al. [19]. RT-qPCR was carried out in a StepOnePlus™ System (Applied Biosystems—Life Technologies, Waltham, Massachusetts, USA) using the standard curve method. The reaction was performed as follows: 1 : 20 dilution of 5 μl of each sample in nuclease-free water mixed with 10 μl of DNase- and RNase-free water in a MicroAmp® 96-well plate (Applied Biosystems-Life Technologies) for a total reaction volume of 20 μl. All sequences were designed *de novo* (Table 1).

2.4. Histological Studies. The samples that were preserved in MEM were rinsed and hydrated multiple times with antibiotic-free medium to eliminate blood cells and then were cut into fragments, which were fixed in F13 (60% ethanol, 20% methanol, 7% polyethylene glycol, 13% distilled H_2O) according to established protocols [20]. Once included, paraffin blocks were made using molds. Once the paraffin

Table 1: Sequences and binding temperatures for RT-qPCR (temp).

Gene	Sequence fwd ($5' \longrightarrow 3'$)	Sequence rev ($5' \longrightarrow 3'$)	Temp (°C)
GADPH	ATGACGAGGGCCTGGAGTGTG	CCTATGTGCTGGCCTTGGTGAG	60
VEGF	ATGACGAGGGCCTGGAGTGTG	CCTATGTGCTGGCCTTGGTGAG	60
TGF-β1	GCGTGCTAATGGTGGAAAC	CGGAGCTCTTGATGTGTTGAAGA	60
PEDF	AGTTACGAAGGCGAAGTCACCAAGTC	GCCCGGTGTTCCACCTGAGTC	50

solidified, an HM 350 S rotation microtome (Thermo Fisher Scientific, Massachusetts, USA) was used to obtain 5 μm thick sections, which were spread in a hot water bath and collected on glass slides previously treated with 10% poly-lysine for better adhesion of the sections.

2.5. von Kossa Staining. von Kossa staining was utilized for the placenta samples, which allowed calcium deposits to be distinguished (seen as a brown-black color) from the remaining tissue (red color). Staining was performed according to the following protocol. The samples were stained with silver nitrate for 20 minutes, rinsed in 5% sodium hyposulfite for 15 minutes, and rinsed in running water. Then, the samples were stained with safranin for 1 minute, dehydrated in 96% alcohol for 3 minutes, dehydrated in 100% alcohol for 5 minutes, and cleared with xylol for 10 minutes. The samples were then mounted with Cytoseal™.

2.6. Immunohistochemical Studies. The antigen-antibody reaction was detected with the ABC method (avidin-biotin complex) with peroxidase or alkaline phosphatase as the chromogen according to the following protocol. The samples were rinsed 3 times in 1× PBS for 5 minutes each time. Nonspecific binding sites were blocked with 3% bovine serum albumin (BSA) in PBS for 30 minutes at room temperature. The samples were incubated overnight at 4°C in primary antibody diluted in 3% BSA and PBS (Table 2). Then, the samples were rinsed in PBS 3 times for 5 minutes each time. The samples were incubated in biotin-conjugated secondary antibody and diluted in PBS for 1.5 hours at room temperature (Table 3) and then rinsed in PBS 3 times for 5 minutes each time. The samples were then incubated in the avidin-peroxidase conjugate ExtrAvidin®-Peroxidase (Sigma-Aldrich, St. Louis, MO, USA) for 60 minutes at room temperature (diluted 1 : 200 in PBS) for PEDF and VEGF. For TGF-β1, the samples were incubated in the avidin-phosphatase conjugate ExtrAvidin®-Alkaline Phosphatase (Sigma-Aldrich, St. Louis, MO, USA) under the same conditions. The samples were rinsed in PBS 3 times for 5 minutes each time. To expose PEDF and VEGF staining, the samples were incubated in the chromogenic substrate diaminobenzidine (Kit DAB, SK-4100) (Vector Laboratories, Burlingame, CA, USA), which was prepared immediately before exposure (5 mL of distilled water, 2 drops of buffer, 4 drops of DAB, 2 drops of hydrogen peroxide), resulting in a brown stain; to expose TGF-β1, the samples were incubated in alkaline chromogenic substrate for 15 minutes. After exposure to chromogenic substrate, the samples were rinsed in distilled water 3 times for 5 minutes each time to stop the reaction. For contrast, nuclei were stained with Carazzi hematoxylin for 5–15 minutes. The samples were rinsed in running tap water for 10 minutes and then mounted in the aqueous polymer Plasdone. In all immunohistochemical studies, sections of the same tissue were used as a negative control, in which incubation with primary antibody was substituted with incubation in blocking solution.

2.7. Statistical Analysis and Interpretation of Results. For the statistical analysis, GraphPad Prism® 6.0 was used. The Mann–Whitney U test was applied, and the Pearson χ^2 test was used. The data are expressed as the median with interquartile range (IQR). Significance was established at values of $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***). For each of the patients in the established groups, 5 sections and 10 fields per section were randomly selected. Patients were described as positive when the marked average area in the analyzed sample was greater than or equal to 5% of the total, according to the IRS score following the anatomical protocol of Cristóbal et al. [21]. The preparations were examined under a Zeiss Axiophot optical microscope (Carl Zeiss, Germany).

3. Results

3.1. Clinical and Demographic Characteristics. The study included 127 women with gestational VI, but 13 were excluded for not completing the study protocol or for leaving the study voluntarily. The complete study was carried out with 114 patients, including 62 women with gestational VI and 52 women without evidence of VI during pregnancy (mean age (SD) = 32.9 (3.5) VI; 34.1 (5.2) HC). No significant differences were observed between the VI and HC groups with respect to gestational age, number of previous pregnancies, previous abortions, regularity of the menstrual cycle, BMI, or size and weight of the placenta. According to the Classification System for Chronic Venous Disorders (CEAP) diagnosis [18], women with gestational VI had a score ≥ C1.

3.2. Expression of VEGF and TGF-β1. By analyzing mRNA levels with RT-qPCR, a significant increase in VEGF gene expression was observed in the placentas of women with VI compared to that in the placentas of women in the HC group (34.575 RQ (Relative quantity) [32.380–36.720] VI vs 32.965 [30.580–36.320] HC $^*p = 0.0158$) (Figure 1(a)). No significant differences were observed between the established study groups in placenta TGF-β1 expression (27.950 RQ [24.520–30.660] VI vs 28.665 RQ [25.870–31.480] HC $p = 0.2234$) (Figure 1(b)).

Table 2: Primary antibodies that were used and their dilutions.

Antigen	Species	Dilution	Provider	Protocol specifications
VEGF	Mouse monoclonal	1 : 50	Abcam (ab28775)	—
TGF-β1	Rabbit polyclonal	1 : 100	Abcam (ab95866)	—
PEDF	Mouse monoclonal	1 : 500	Abcam (ab115489)	Citrate tampon in heat (pH = 6)

Table 3: Secondary antibodies that were used and their dilutions.

Antigen	Species	Dilution	Provider	Protocol specifications
IgG (mouse)	Goat polyclonal	1 : 300	Sigma (F2012/045K6072)	—
IgG (rabbit)	Mouse polyclonal	1 : 1000	Sigma (RG-96/B5283)	—

The detection of VEGF protein expression by immunohistochemistry revealed high expression levels in the syncytiotrophoblast and cytotrophoblast of placental villi in women with VI (Figures 1(c) and 1(d)). A significant increase in the IRS score for VEGF was established in the placental villi of women with VI (1.500 [0.500–3.000] VI vs 1.000 [0.000–2.500] HC $^{*}p = 0.0498$). For TGF-β1, no differences were observed in terms of protein expression in the placental villi studied (0.750 [0.250–1.500] VI vs 1.000 [0.500–2.250] HC $p = 0.1497$) (Figures 1(e) and 1(f)).

3.3. Expression of PEDF. PEDF gene expression was significantly higher in women with VI (25.417 RQ [24.459–27.675] VI vs 23.102 RQ [23.102–30.223] HC $^{***}p = 0.0003$) (Figure 2(a)). Furthermore, detection of PEDF protein expression using immunohistochemistry revealed significantly increased expression in the extracellular matrix of the high areas of the placental villi in women with VI compared to women in the HC group (Figures 2(b) and 2(c)). Analysis of the PEDF expression score showed a significant increase in protein expression (2.500 [1.000–3.000] VI vs 1.000 [0.500–3.000] HC. $^{***}p < 0.0001$).

3.4. Study of Calcium Deposits. The study of calcium deposits in placental villi was performed using the von Kossa technique. The percentage of calcium deposits was higher in women with VI than in women in the HC group (72.58% VI vs. 53.84% HC). In this case, the Pearson χ^2 test was $^{*}p = 0.038$ (Figure 3(a)). The histological study of calcium deposits revealed dystrophic and metastatic calcifications in the placental villi. In women with VI, the percentage of metastatic calcifications (57.78%/42.22%) was higher than that in the control group (Figures 3(b) and 3(d)). The control group presented a higher percentage of dystrophic calcifications (57.14%/42.86%) (Figures 3(c) and 3(e)).

4. Discussion

VI is a disorder that is difficult to approach, where the systemic and specific repercussions on maternal-fetal health are still unknown. The placenta is the tissue through which the exchange of substances essential for normal fetal homeostasis will occur; therefore, it is a dynamic organ that adapts to changes [22]. Our study is the first to demonstrate that the placentas of women with VI during pregnancy undergo changes in the expression of factors important for tissue function, such as VEGF and PEDF, and that there is a significant increase in calcification deposits in the placental villi.

Our previous studies demonstrated the existence of tissue hypoxia characterized by an increase in HIF protein and gene expression in placental villi in women with VI, which is associated with increased placental apoptosis [7]. The increase in HIF in a hypoxic condition is associated with VEGF activity [23, 24]. The increase in the activity and expression of VEGF in situations of high blood pressure, such as preeclampsia, is well known [25]. Zhang et al. [26] showed how increased VEGF activity in placentas in preeclampsia produced an increase in apoptosis. Therefore, the increase in VEGF expression in the placental villi of mothers with VI may be related to this increase in HIF activity and apoptosis.

PEDF plays an important role in vascular pathology because it is multifunctional with anti-angiogenic, anti-inflammatory, and antithrombotic properties [27, 28]. Increased expression of PEDF in the placentas of women with preeclampsia induces placental vascular reconstruction dysfunction and pathological conditions such as placental ischemia and hypoxia, which may be involved in the pathogenesis and pathogenic development of preeclampsia [16].

The gene expression levels of VEGF and PEDF coincide with those described by other authors, such as Loegl et al. for placentas in the third trimester [29]. Likewise, the tissue distribution for both proteins follows a similar pattern in our studies. The ratio of VEGF to PEDF is 3 : 2 in homeostasis. In placentas under VI, an increase of approximately 4% in the gene expression for both molecules was observed. Protein accumulation increased proportionally. However, from the point of view of tissue, the increase in PEDF in the extracellular matrix could provide the appropriate substrate for the nucleation of calcium. PEDF present in higher concentrations than usual in the extracellular matrix has been described in dermis with connective tissue alterations as especially sensitive sites for calcium nucleation [30]. In addition, in placentas of women with VI, it has been demonstrated that placental villi undergo a change in the collagen fibers in the extracellular matrix, producing an alteration in the collagen I/III ratio [9]. Therefore, all these

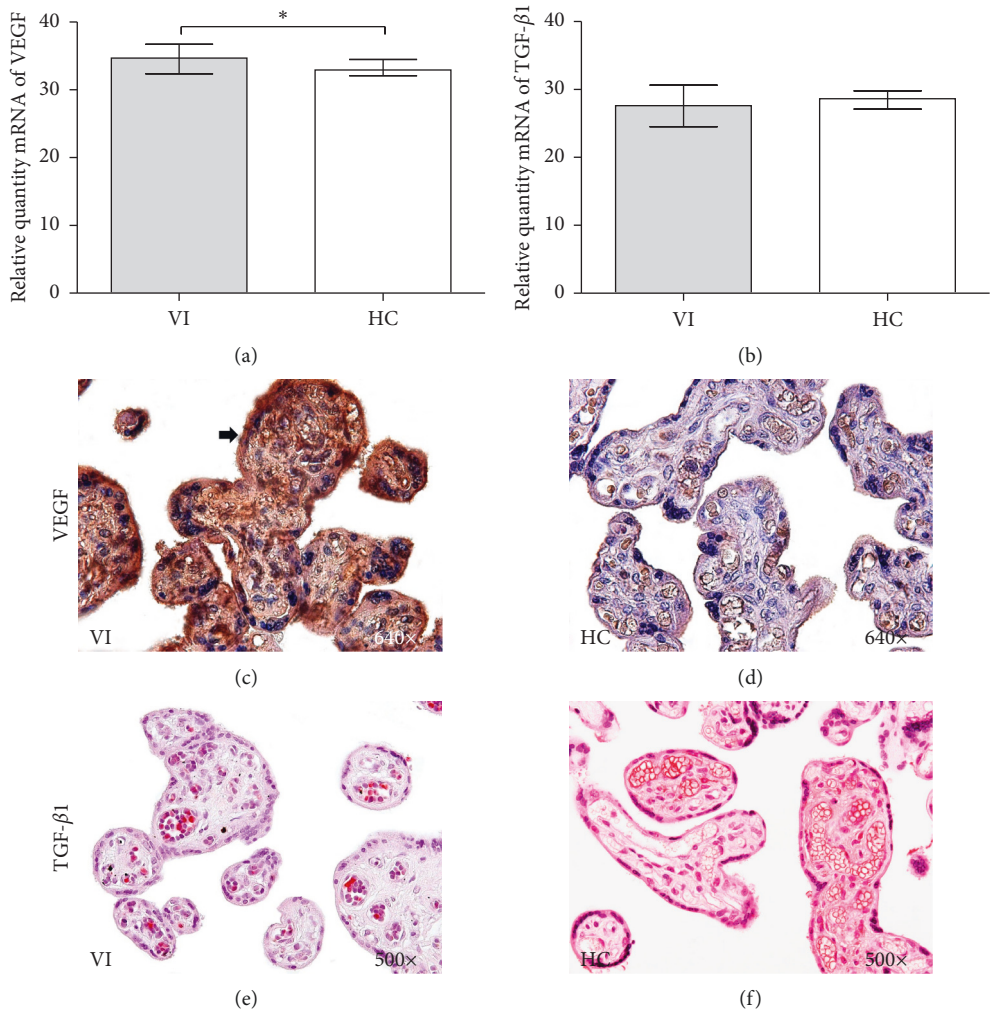


Figure 1: Relative quantity mRNA levels of VEGF (a) and TGF-β1 (b). Histological images of VEGF and TGF-β1 protein expression in placentas of VI (c–e) and HC (d–f). VI = lower extremity venous insufficiency; HC = control patients without VI; $p < 0.05$ (*).

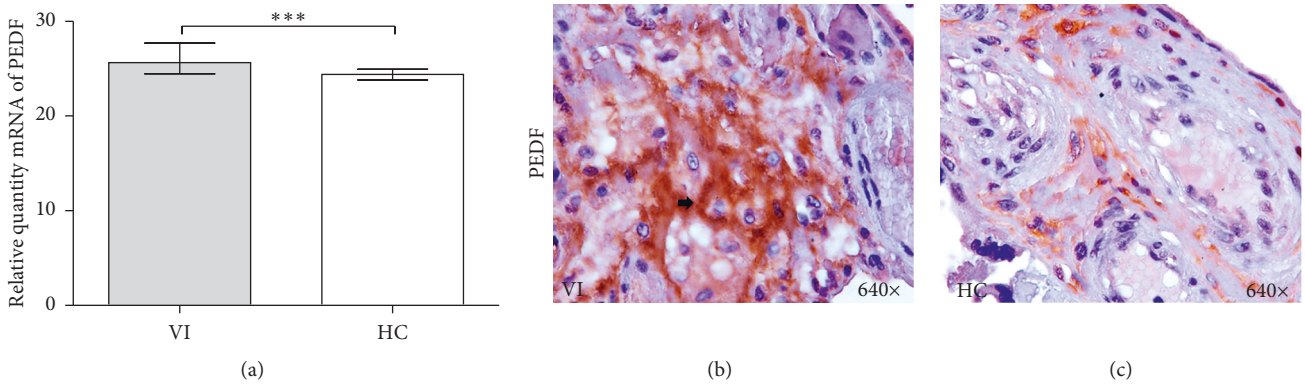
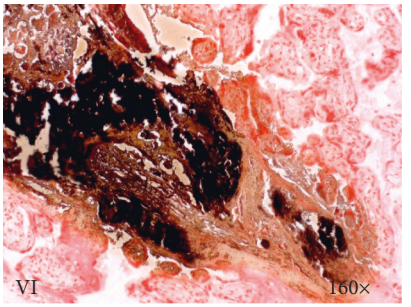


Figure 2: Relative quantity mRNA levels of PEDF (a). Histological images of PEDF protein expression in placentas of VI (b) and HC (c). $p < 0.001$ (***).

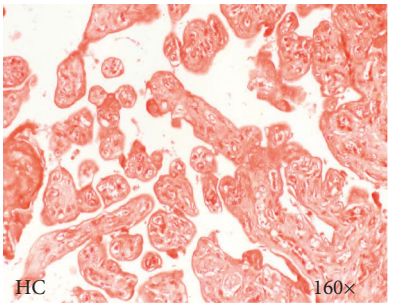
facts seem to focus not so much on the primary role involved in the increase in VEGF/PEDF at the angiogenic level in the VI placenta but, rather, a paracrine effect related to increased calcification. Our results show that in the placentas of women with VI, the dystrophic/metastatic calcification ratio reversed compared to the placentas of women in control group, with an increase in the metastatic calcification in VI placentas.

	Total calcifications (*n*)	Dystrophic (*n*)	Metastatic (*n*)
VI	72.58% (45)	42.22% (19)	57.78% (26)
HC	53.84% (28)	57.14% (16)	42.86% (12)

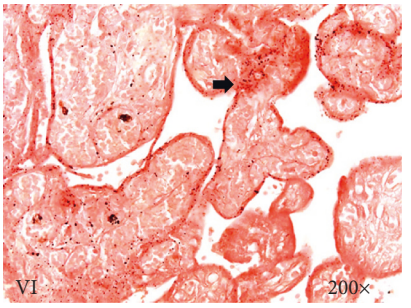
(a)



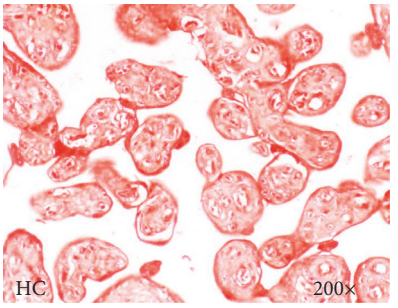
(b)



(c)



(d)



(e)

Figure 3: Percentage of patients with calcium deposits (a). Histological images of calcium deposits, where dystrophic (b) and metastatic (d) calcifications can be observed. VI = lower extremity venous insufficiency; HC = control patients without VI.

The calcification of highly vascularized tissues undergoing hypoxic processes has been described in numerous studies, with consequences on cellular dynamics [31–33]. The process of calcification in the placenta has been described in pathological processes such as preeclampsia and intrauterine growth restriction [34, 35]. The presence of microcalcifications in placental villi seems to have an implication in events such as oxidative stress that occur in situations such as fetal anomalies and mothers with gestational hypertension, gestational diabetes, and placental abruption [36, 37]. Some authors directly relate tissue calcification with changes in cellular metabolism [38, 39]. Therefore, we speculate that the placentas of women with VI may undergo this process as a mechanism to satisfy greater cellular demand. Therefore, all these slight changes induced by possible slowing of the blood flow of the intervillous space, due to poor peripheral blood circulation in the placental environment, can manifest in tissues as increased metastatic placental calcification. This event could affect the normal metabolic exchange of the placenta in women with VI.

Data Availability

The data used to support the findings of the present study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

This study (FIS-PI18/00912) was supported by the Instituto de Salud Carlos III (Plan Estatal de I+D+i 2013-2016) and cofinanced by the European Development Regional Fund "A way to achieve Europe" (ERDF) and B2017/BMD-3804 MITIC-CM.

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