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On the nature of immunologically challenging pregnancies

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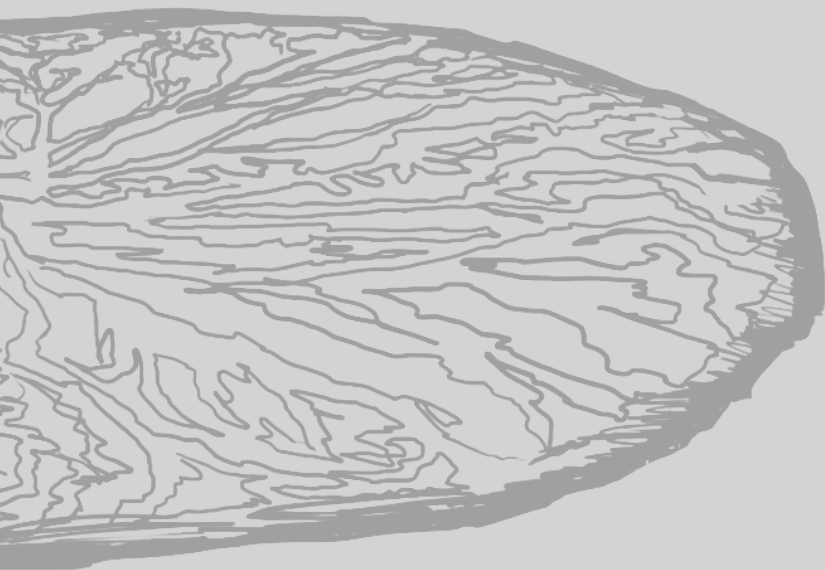
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On the nature of immunologically challenging pregnancies

Manon Bos



Colophon

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On the nature of immunologically challenging pregnancies

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On the nature of immunologically challenging pregnancies

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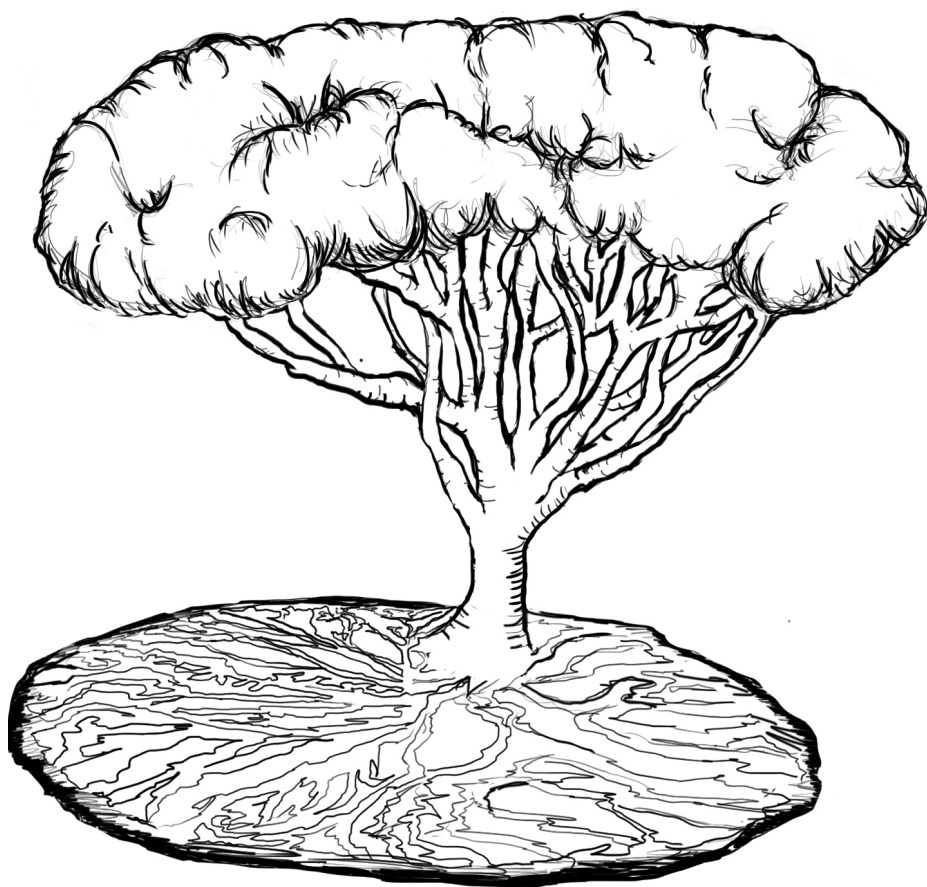
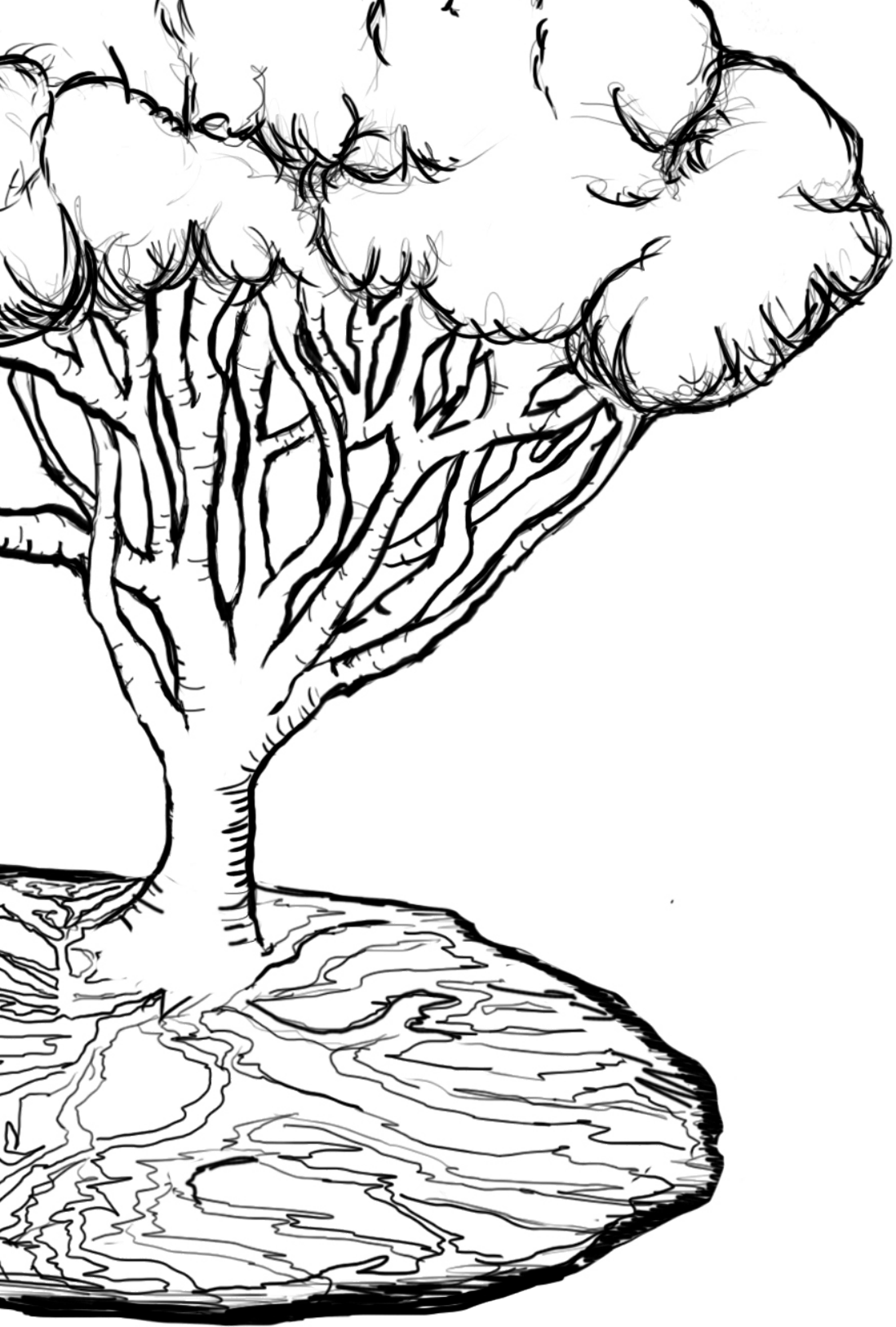


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Introduction

The immunological paradox of pregnancy

From an immunological point of view, pregnancy is an enigmatic status for the mother. The fetus can be considered semi-allogenic towards the mother, since both maternal and paternal genes are expressed in the fetus. Therefore, it is reasonable that there must be some maternal tolerance towards the fetus during pregnancy. The first to recognize this apparent immunological paradox of pregnancy in viviparous animals was Peter Brian Medawar in 1953.¹ He stated: "The immunological problem of pregnancy may be formulated thus: how does the pregnant mother contrive to nourish within itself, for many weeks or months, a fetus that is an antigenically foreign body?"¹ Furthermore, Medawar offered possible explanations: mother and fetus are anatomically separated, the fetus is antigenically immature, or the mother is immunologically tolerant for the fetus.¹ Later, one of Medawar's former colleagues, Rupert Billingham, proposed an additional explanation: The uterus is an immunologically privileged site.² More recent insights show that the ideas of Medawar and Billingham could not entirely encompass the complexity of reproductive immunology and cannot fully explain the immunological paradox of pregnancy.³ First, the fetus and the mother are not anatomically separated. Fetal trophoblasts are in direct contact with maternal cells in the intervillous space and decidua. Furthermore, the placenta is not a cell-impermeable barrier. The trafficking of cells between the fetus and the mother is tightly regulated such that fetal and maternal cells can move in two directions through the placenta.⁴ A significant amount of placental cells and cell fragments are released in the maternal circulation throughout pregnancy.⁵⁻⁹ Interestingly, these fetal cells remain in the maternal circulation post-partum,¹⁰ and these chimeric cells can remain in maternal tissues until many years after pregnancy.¹¹⁻¹⁴ Second, the fetus is not antigenically immature. The fetus and placenta are recognized by the maternal immune system by the expression of human leukocyte antigens (HLAs), and this immune recognition seems essential for normal pregnancy.¹⁵⁻¹⁷ Third, the mother is not completely immunologically tolerant of the fetus. The mother makes antibodies directed against the fetus. These alloantibodies are directed against HLAs or antigens on erythrocytes or platelets.¹⁸⁻²⁰ The presence of alloantibodies against red blood cells or platelets is associated with serious complications, such as haemolytic disease of the fetus and fetal neonatal alloimmune thrombocytopenia. However, maternal antibodies against fetal HLAs are also found in uncomplicated pregnancies.²¹ Fourth, the uterus is not an immunologically privileged site; ectopic pregnancies are seen in the fallopian tubes and abdominal cavity. Ectopic pregnancies in the abdominal cavity can even reach full term and result in a viable fetus.^{22, 23} In addition, maternal tolerance of the fetus is not caused by systemic immune suppression; the mother is still capable of sufficient immunoreactions.

However, the immune system does react slightly differently during pregnancy.^{24, 25} For example, T-cell-mediated autoimmune diseases show remission during pregnancy,²⁶ and pregnant women are more susceptible to influenza, hepatitis, herpes simplex virus and malaria infection.²⁷ It is proposed that the placenta plays an important role in modulating the immune response in pregnant women.²⁴ Furthermore, the placenta may also have an important regulatory function in protecting the fetus from infectious diseases, since maternal to fetal transmission of viruses is rather the exception than the rule.^{25, 28} Notwithstanding the growing knowledge of reproductive immunology, pregnancy remains a fascinating paradoxical status. When considering some frequently occurring pregnancy complications, such as pre-eclampsia and miscarriage, these placenta-related pregnancy complications are not frequently observed in other species and are almost unique to humans.^{29, 30} The general introduction of this thesis will provide an overview of the fetal-maternal interface, discuss the physiology and pathophysiology of the placenta and address immunologically challenging pregnancies such as pre-eclampsia, oocyte donation and chronic intervillitis of unknown aetiology.

The immune system, coagulation, and endothelial cells

A pregnant woman's body experiences anatomical and physiological changes to adapt to pregnancy in such a way that homeostasis is maintained and that fetal growth is facilitated. Adaptation to pregnancy starts just after conception and evolves throughout pregnancy under the influence of hormones produced by the placenta.³¹ Most of these physiological changes return to pre-pregnancy levels within weeks after delivery.^{32, 33} To understand maternal adaptation to pregnancy, this general introduction starts with the introduction of the immune system, haemostasis and the role of endothelial cells in immunology and haemostasis.

The immune system

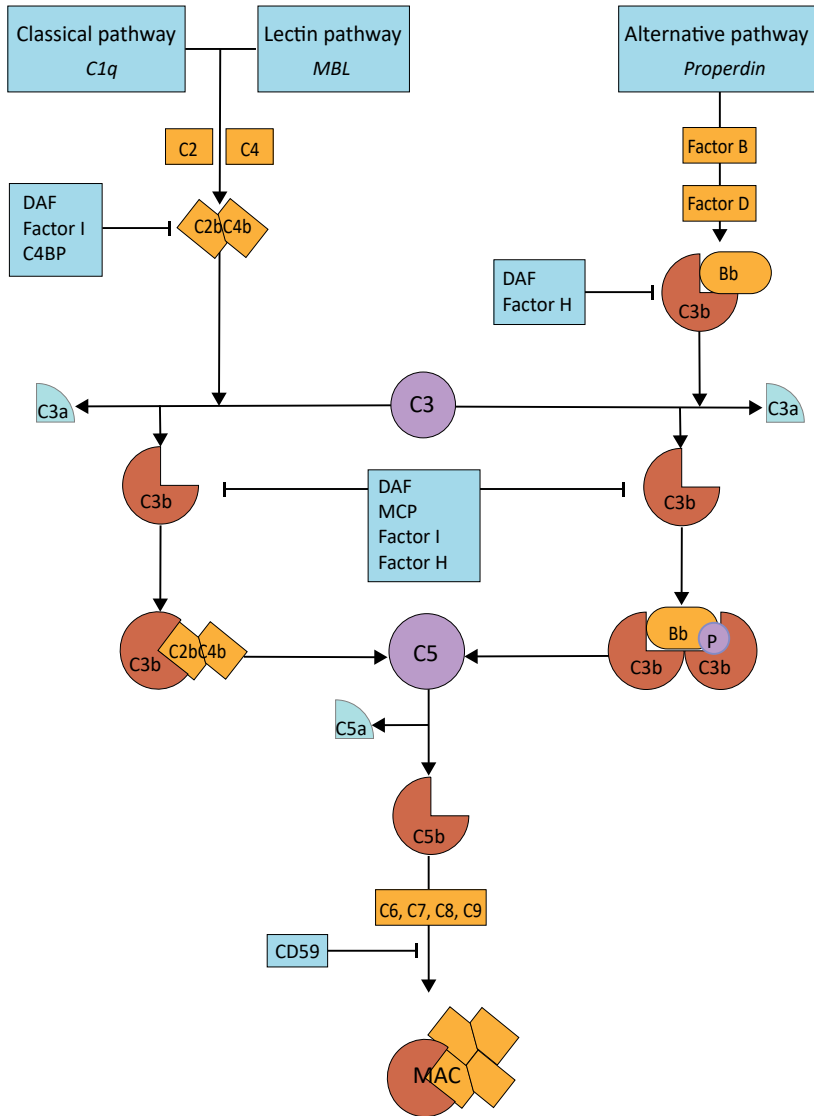
The immune system protects the human body against pathogens and cancerous cells. To achieve this, two lines of defence exist: a non-specific, fast-reacting innate immunity and a more specific, slower-adapting immune system.

Innate immunity

The first line of defence against microorganisms is formed by epithelial barriers. When a microorganism passes an epithelial barrier and enters the human body, the complement system mostly activates the immune system. The complement system can recognize pathogens and mark these pathogens for destruction. Three different pathways of complement system activation are described: the alternative pathway, the lectin pathway and the classical pathway of complement activation (Figure 1).

Complement system activation eventually results in the lysis of cell membranes due to membrane attack complex formation. The anaphylatoxins C3a and C5a

Figure 1 The complement system: activation and regulation



The classical pathway is activated via immune complexes, the lectin pathway via microbial carbohydrates and the alternative pathway via pathogen surfaces. All pathways of complement activation result in *C3* cleavage, *C5* cleavage and membrane attack complex (MAC) formation. The complement system is inhibited by *CD59*, decay-accelerating factor (*DAF*), membrane cofactor protein (*MCP*), *Factor I*, *Factor H* and *C4* binding protein. Figure based on: ³⁵.

induce inflammation, e.g., they increase vascular permeability and the activation of neutrophils and monocytes. Given the forceful effect of complement system activation, the complement system is tightly regulated by complement regulatory proteins.³⁴ These proteins are either soluble or membrane-bound and inhibit the activation of the complement system and the amplification and formation of the membrane attack complex. These factors are highly expressed in tissues that are in direct contact with blood, such as endothelial cells and placental cells. Other complement regulatory proteins are circulating in the blood.

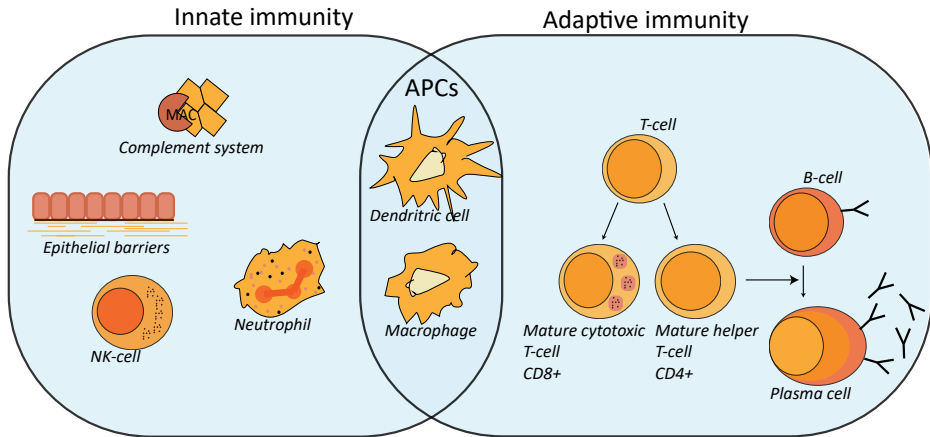
The opsonisation and destruction of microorganisms via the complement system inhibits the spread of a microorganism throughout the body. Apart from the complement system, other types of plasma proteins also prevent the spread of microorganisms. For instance, damage to endothelial cells results in the activation of the coagulation system, which also immobilizes microorganisms.

Neutrophils and macrophages form the first line of cellular defence against microorganisms (Figure 2). Both of these cells are phagocytes, cells of the innate immune system that engulf and kill microorganisms and so destroy invading pathogens. The recognition of a pathogen can occur via C3b deposition on a pathogen but also via other receptors that recognize pathogen/microorganism-specific molecules, such as toll-like receptors. Furthermore, natural killer (NK)-cells provide an early response against intracellular infections. When a cell becomes distressed, it starts to express surface proteins that activate the NK-cells and hence stimulate NK-cells to kill the infected cell. Therewith, dendritic cells and macrophages are antigen-presenting cells. After phagocytosis, antigen-presenting cells present antigens to T-cells in lymph nodes, provoking the adaptive immune response.

Adaptive immunity

Antigen-presenting cells internalize pathogens and degrade them into small peptides. Some of the peptides are expressed on the surface by the HLA molecules. T-cell receptors can bind to the HLA-peptide complex if they have the right receptor. The adaptive immune response is pathogen-specific. T-cell receptors have an almost infinite number of different versions and can therefore also recognize an almost infinite number of different HLA-peptide complexes. When a T-cell in the lymph node recognizes the pathogenic peptide presented by the HLAs, the T-cell will be stimulated to proliferate and differentiate. Two different types of immune responses can be provoked by HLA class-I and HLA class-II molecules. Class-I HLA molecules present peptides derived from intracellular infections and activate cytotoxic T-cells (CD8+ T-cells). Pathogen-specific cytotoxic T-cells will be able to recognize the infected cells and kill these cells. Class-II HLA molecules present antigens derived from extracellular infections. The presentation of a pathogen by an antigen-presenting cell on an HLA class-II molecule results in the proliferation and differentiation of T-helper cells (CD4+ T-cells). T-helper cells facilitate the activation of B-cells, which will differentiate into plasma cells. The antibodies made by these plasma cells can neutralize pathogens. Furthermore, antibodies enable the opsonisation of pathogens and complement activation, which makes phagocytosis more efficient. A

Figure 2 Innate and adaptive immunity



Epithelial barriers prevent the intrusion of microorganisms. When a microorganism enters the human body, the complement system can mark microorganisms for destruction (C3b deposition). The first line of cellular defence is formed by NK-cells and neutrophils. Antigen-presenting cells, dendritic cells and macrophages initiate the adaptive immune response. Figure based on: ³⁶.

successful adaptive immune response terminates infection and provides long-lasting immunity by the development of memory T-cells and memory B-cells.

Haemostasis

Haemostasis is the tightly regulated balance between pro-coagulation and anti-coagulation. Haemostasis is maintained in the body by interactions between the coagulation system, fibrinolytic system, platelets, and endothelial cells. Haemostasis has three phases: primary haemostasis, coagulation, and fibrinolysis (**Figure 3**).³⁷ Primary haemostasis is the result of the interplay between the vascular endothelium and platelets.³⁷ Endothelial surfaces ensure haemostasis by their anti-thrombotic properties, such as the synthesis and secretion of platelets and coagulation inhibiting factors and activators of fibrinolysis. Platelets have a pivotal role in haemostasis by forming the initial haemostatic plug that provides a surface for the binding of activated coagulation factors. When endothelial cells are damaged and the underlying extracellular matrix is exposed, platelets bind with their adhesion receptors to Von Willebrand factor and collagen. The adhesion of platelets results in the activation of these platelets, which further reinforces platelet aggregation and the acceleration of the coagulation cascade.

The coagulation cascade is activated by two distinct pathways. The extrinsic pathway of coagulation is induced after vascular damage. When a vessel is damaged, tissue factor is released from the subendothelial space, activating the extrinsic pathway. The contact of blood with an artificial surface results in the activation of Factor XII, initiating the intrinsic pathway. The two pathways of coagulation both result in the conversion of fibrinogen to fibrin. The extrinsic pathway is the most predominant coagulation pathway after vascular damage, whereas the intrinsic pathway is almost dispensable in the context of normal haemostasis.³⁸ The activation of the intrinsic pathway, however, activates pro-inflammatory signalling.³⁹

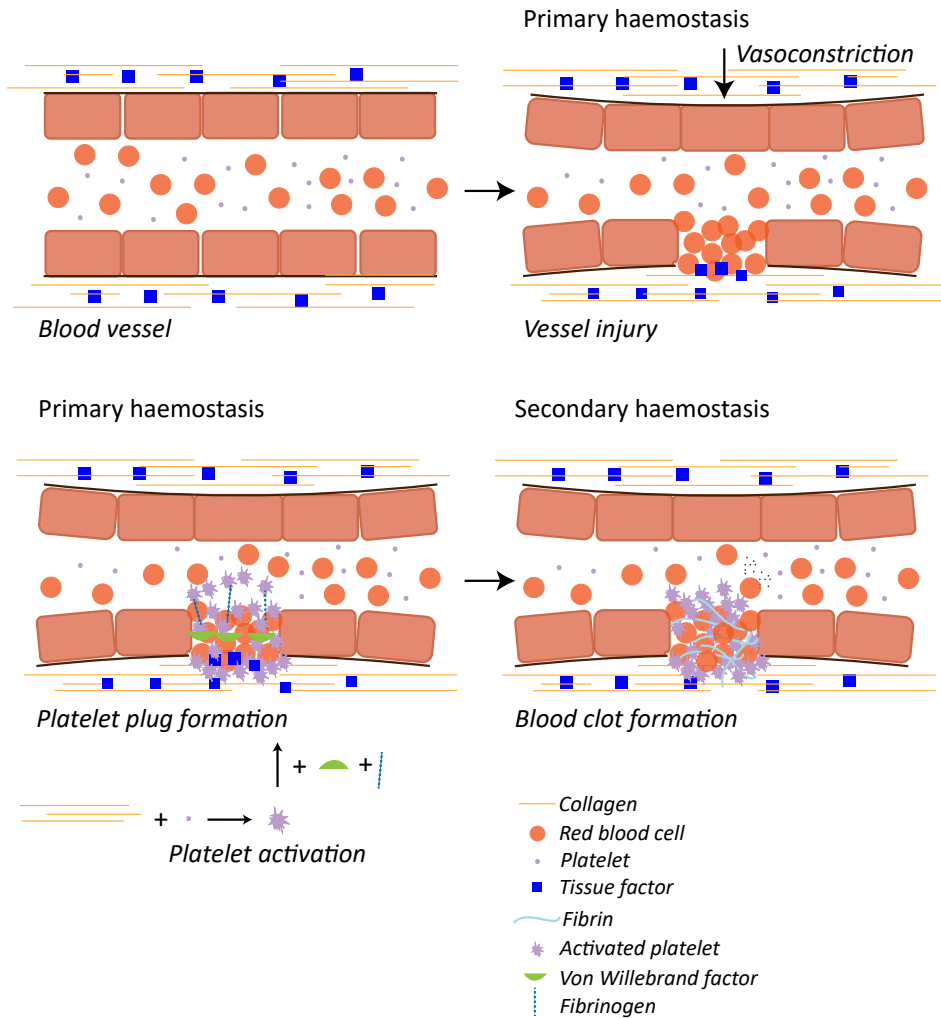
To limit fibrin formation to the site of injury, several control mechanisms exist, for example, tissue factor pathway inhibitor, protein c system and anti-thrombin. When the coagulation cascade is activated, the fibrinolytic system is activated as well. Fibrinolysis ensures the limitation of the clot size since fibrinolysis dissolves a fibrin clot into fibrin degradation products. Plasmin causes fibrinolysis, and its activity is regulated by anti-plasmin (alpha-2 anti-plasmin). Furthermore, fibrinolysis is also limited by thrombin-activatable fibrinolysis inhibitor (TAFI), a proenzyme that is activated by thrombin and decreases the affinity of plasminogen to fibrin.

Endothelial injury, together with abnormal blood flow and hypercoagulability, can result in the loss of haemostasis and the formation of a thrombus.

Endothelium

Endothelial cells form the lining of blood vessels, and these cells are essential for maintaining haemostasis. When the endothelium is damaged or stressed, endothelial cells become highly pro-thrombotic.⁴² Tissue factor is an important protein for transforming the anti-coagulant endothelial surface to a pro-coagulant

Figure 3 Haemostasis



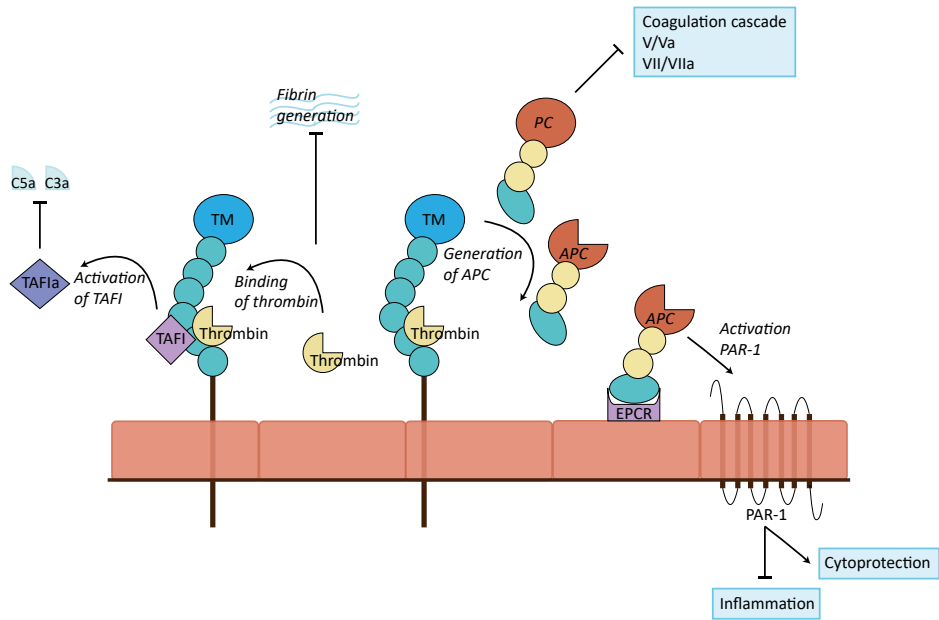
After an injury to a vessel, vasoconstriction occurs, and the blood is exposed to the subendothelial matrix. Platelets become activated after binding to collagen and Von Willebrand factor, and the initial platelet plug is formed. This is primary haemostasis. When the coagulation system is activated, a blood clot forms. This is secondary haemostasis. Fibrinolysis eventually results in the degradation of the blood clot. Figure based on: ^{40, 41}.

endothelial surface. The expression of tissue factor by endothelial cells can be induced by thrombin, endotoxins, shear stress and hypoxia.⁴² Therewith, endothelial cells orchestrate an inflammatory response by attacking immune cells and thereby enabling immune cells to pass through cell-cell junctions. Endothelial cells can also change their phenotype in response to pathophysiological stimuli, known as endothelial activation. The endothelium can become activated in response to microorganisms, inflammation, haemodynamic stresses, lipid products, hyperglycaemia, complement components, and hypoxia. When endothelial cells are activated, they start to express adhesion molecules (vascular cell adhesion molecule (VCAM)-1, intercellular adhesion molecule (ICAM), E-selectin, and P-selectin) and produce cytokines (IL-1 and IL-6), chemokines, growth factors, vasoactive molecules and coagulation-regulating molecules. The cell permeability also increases. Under normal circumstances, the intercellular cell junctions are impermeable. However, under inflammatory conditions, these junctions loosen, and as a result, fluid and leukocytes can leak from vessels. Normal endothelial function is characterized by a balanced response towards different triggers. Excessive endothelial activation results in endothelial dysfunction. Endothelial dysfunction is characterized by impaired vasoreactivity, a thrombogenic endothelial surface and abnormal adhesiveness for inflammatory cells.

Thrombomodulin, a mediator of inflammation, coagulation and cell survival

Thrombomodulin is a glycoprotein that covers three distinct pathways that are important for endothelial maintenance: inflammation, coagulation and cell survival (Figure 4). All endothelial cells throughout the human body express thrombomodulin.⁴³ Thrombomodulin is essential for the development and maintenance of endothelial cells; thrombomodulin knockout models are lethal in the embryonic phase and result in massive thrombosis.^{44, 45} Thrombomodulin is best known for its anti-coagulative effects via the binding of thrombin, resulting in the inactivation of thrombin. The thrombomodulin-thrombin complex also facilitates the activation of protein C (APC), together with endothelial protein C receptor (EPCR), thereby further inhibiting the coagulation cascade.⁴⁶ The thrombomodulin-thrombin-EPCR complex can activate the protease-activated receptor (PAR)-1 receptor. When PAR-1 is activated by thrombomodulin, this results in the inhibition of inflammatory cell signalling and the activation of cytoprotective signalling. Apart from the inhibition of inflammation via PAR-1, the thrombomodulin-thrombin complex also inhibits inflammation via the activation of TAFI. Activated TAFI (TAFIa) inhibits the anaphylatoxins C3a and C5a.

Under inflammatory circumstances, thrombomodulin is cleaved by metalloproteases. The breakdown product of thrombomodulin, soluble thrombomodulin, can be detected in the serum.⁴⁶ This cleaved thrombomodulin product is believed to be non-functional and is elevated in many inflammatory diseases. Therefore, soluble thrombomodulin is a useful biomarker for endothelial dysfunction.^{47, 48} Moreover, treatment with recombinant soluble thrombomodulin seems to reduce the mortality in patients with diffuse intravenous coagulation due to sepsis.⁴⁹

Figure 4 The different functions of thrombomodulin

Thrombomodulin is essential for the maintenance of endothelial cells. Thrombomodulin inhibits coagulation, inhibits inflammation and is important for cell survival. Figure based on: ^{50, 51}.

Maintenance of the fetal-maternal interface

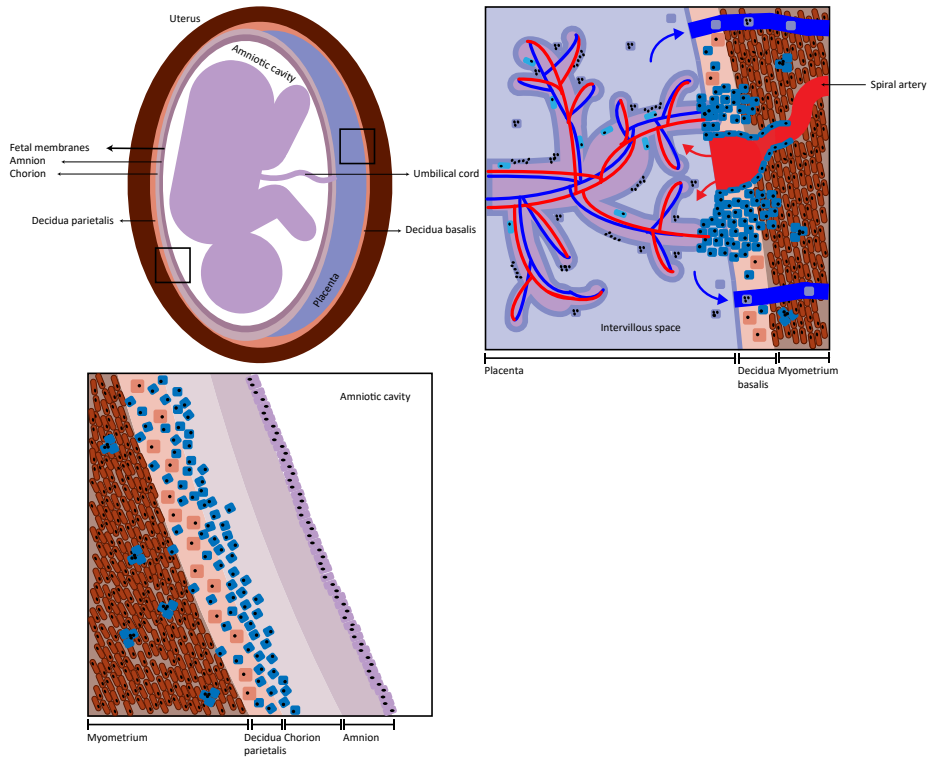
During pregnancy, maternal and fetal cells are in direct contact. This contact occurs at three different locations: the fetal-maternal interface, consisting of the maternal peripheral blood; the decidua basalis; and the decidua parietalis. The maternal peripheral blood is in direct contact with the fetal trophoblasts in the intervillous space of the placenta to facilitate the exchange of oxygen and nutrients. Furthermore, fetal cells and fetal cell debris are found in the maternal circulation and are in direct contact with the maternal vascular bed. In the decidua basalis, the maternal side of the placenta, and in the decidua parietalis, the maternal side of the fetal membranes, maternal immune cells, and fetal trophoblasts interact (Figure 5).

Placental development

Approximately four days after conception, when the morula becomes a blastocyst, the trophoblast, which forms the outer layer of the blastocystic cavity, develops. The trophoblast is the forerunner of most cells in the placenta and fetal membranes. From the inner cell mass of the blastocyst, the embryo, umbilical cord and amnion develop. Shortly after formation, the blastocyst implants in the endometrium, and right after implantation, the placenta starts to form. Endometrial cells react to the invasion of trophoblasts by swelling and filling with glycogens and lipids. This process is known as decidualization, and henceforth, the former endometrium is referred to as the decidua. Vacuoles are formed when the trophoblasts invade further into the decidua. These vacuoles fuse to lacunae and will eventually form the intervillous space. The syncytiotrophoblast pillars that divide the lacunae are known as trabeculae. Soon after the implantation of the blastocyst, the entire blastocyst is surrounded by the syncytiotrophoblast. The first villi develop when a core of cytotrophoblast is surrounded by the syncytiotrophoblast. As the villi further develop, embryonic vessels arise from embryonic mesenchymal cells that spread throughout the inner surface of the cytotrophoblast layer. Embryonic vessels in the villi eventually connect to the vessels in the umbilical cord and foetal circulation. The umbilical cord connects the fetus with the placenta and thus enables fetal-placental circulation. The maternal blood enters the placenta via the spiral arteries and flows through the intervillous space alongside the fetal villi. Maternal blood leaves the placenta via the placental veins. Approximately 18 days after conception, the outline of the placenta is complete. As pregnancy proceeds, villi continuously develop to increase surface area to enable sufficient transport of nutrients and oxygen (Figure 6).

The trophoblast population can be divided into two types: villous trophoblasts and extravillous trophoblasts. Villous trophoblasts are divided into two subtypes: cytotrophoblasts and syncytiotrophoblast. Syncytiotrophoblasts originate from cytotrophoblasts and are a specialized epithelium that forms the outer lining of the villi. Syncytiotrophoblasts have several functions, including the transport of oxygen and nutrients and the synthesis of several hormones. Extravillous trophoblasts are all trophoblastic cells that are located on the outside of the villi. These extravillous

Figure 5 Fetal-maternal interface



Fetal and maternal cells are in direct contact at the decidua parietalis, the decidua basalis and in the intervillous space. Fetal cells and placental cell debris are also found in maternal circulation. Figure based on: ^{52, 53}.

trophoblast cells invade the decidua and spiral arteries. Interstitial extra villous trophoblasts ingress as far as the inner third of the myometrium, forming giant cells. The endovascular invasion of extravillous trophoblasts results in the displacement of vascular smooth muscle cells and the replacement of maternal endothelial cells from uterine spiral arteries.

Maintenance of the fetal-maternal interface

The fetal-maternal interface in humans is unique due to its direct contact of maternal blood with syncytiotrophoblast in the intervillous space. Extravillous trophoblasts invade the maternal spiral arteries, decidua, and myometrium.

Trophoblasts are a unique kind of cell

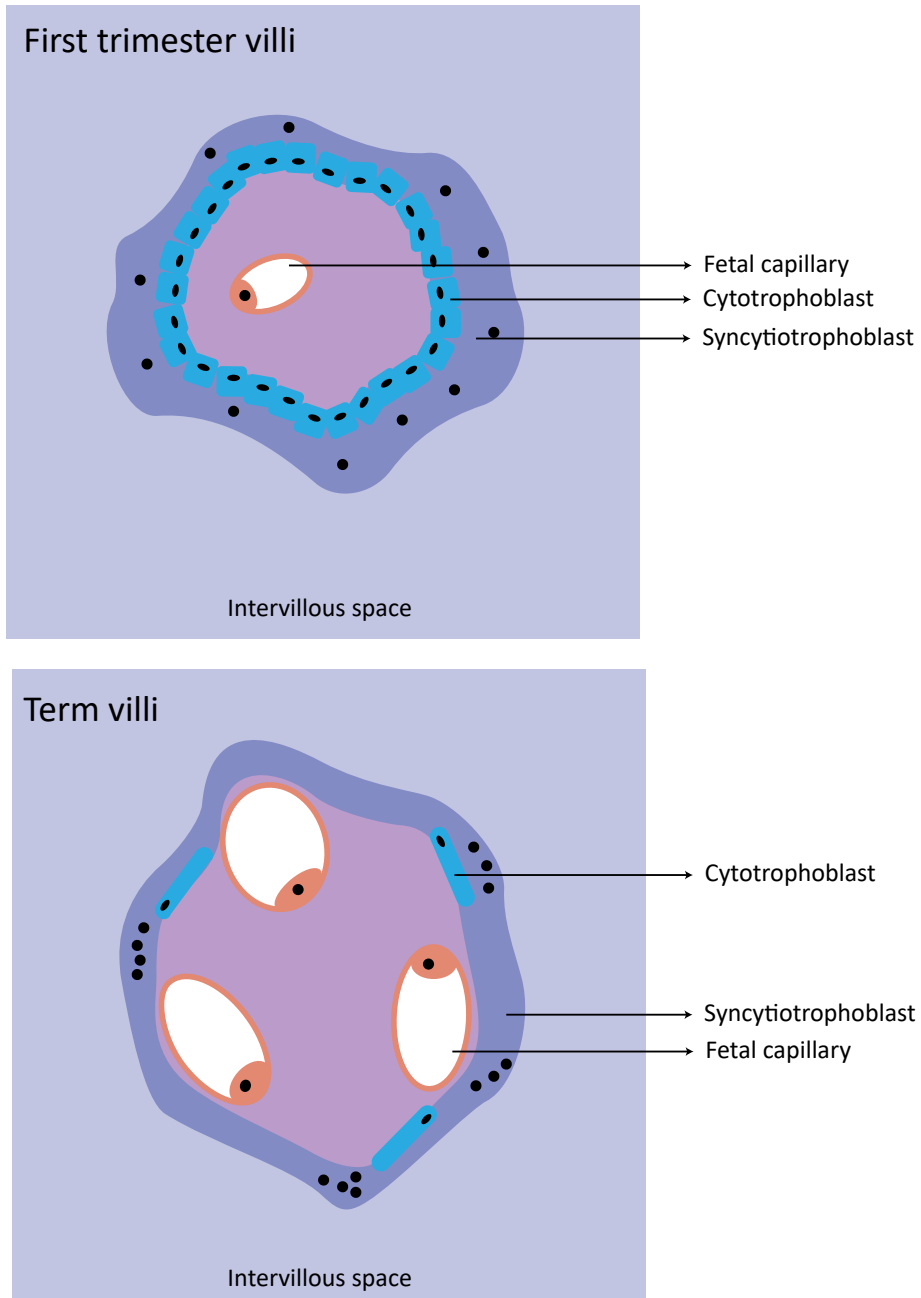
When considering the immune regulation at the fetal-maternal interface, it is important to understand that trophoblasts are a unique kind of cell that undermine some of the principles of classical immunology.⁵⁴ First, trophoblasts are of fetal origin, which makes these cells semi-allogeneic, expressing paternal genes as well as maternal genes. Second, trophoblasts are normally healthy cells with some peculiar features because trophoblasts are extraembryonic cells instead of somatic cells. Therefore, trophoblasts express endogenous retroviral products, oncofetal proteins and imprinted genes.⁵⁵⁻⁵⁸ Unmethylated DNA is also present in trophoblasts, and trophoblasts frequently form giant cells.⁵⁵⁻⁵⁹ Third, the invasion of trophoblasts happens not without damage to the implantation site and trophoblasts themselves. For example, spiral artery remodelling is characterized by fibrinoid necrosis, and necrosis is observed at the location where anchoring villi attach. Extravillous trophoblasts at remodelled spiral arteries have a spidery appearance, which also suggests cell stress.⁵⁴ On the other hand, when comparing damage to the spiral arteries and decidua to tumour invasion, placentation appears to be a relatively mild and organized process.⁵⁴ In general, one could conclude that the maternal uterine immune system has to adapt to a highly invasive, selectively destructive extraembryonic cell with an apparently normal phenotype.⁵⁴

Since syncytiotrophoblasts rather than endothelial cells line the maternal vascular compartment in the placenta, these cells should express factors to maintain haemostasis in the intervillous space. The flow in the intervillous space is almost zero, and physiological changes in several coagulation factors in the maternal serum during pregnancy result in a mild pro-thrombotic state in the intervillous space.⁶⁰ Similar to endothelial cells, trophoblasts express haemostasis regulating factors such as thrombomodulin and endothelial protein C receptor.^{43, 61, 62} However, trophoblast cells express tissue factor under normal conditions, while endothelial cells express tissue factor only under pathological conditions, e.g., in an activated state.⁶³ Interestingly, tissue factor activity as well as thrombin are essential for placental development. Thrombin inhibits proliferation and influences the invasiveness of trophoblasts.^{44, 64}

Immune modulation by trophoblasts

Experiments with mice in the early 1960s already showed that trophoblasts have

Figure 6 First trimester and term villi



Development of villi throughout pregnancy to optimize the surface area for the exchange of oxygen and nutrients between the fetal and maternal circulation.

immune-modulating properties; transplanted embryonic tissues were rejected while the transplantation of trophoblast cells resulted in the proliferation of these cells and a vascularized tumour.⁶⁵ Several mechanisms result in the modulation of the maternal immune response to trophoblasts. One of the mechanisms trophoblasts use to escape and modulate the maternal immune response is a selective expression pattern of HLAs. Villous trophoblasts and the syncytiotrophoblast do not express HLAs, and extravillous trophoblasts express only a specific set of HLA class-I molecules; HLA-C, HLA-E, HLA-F and HLA-G.⁶⁶ The expression of HLA molecules on extravillous trophoblasts dampens the cytotoxicity of NK-cells, macrophages, and T-cells and thus mediates the immune response of maternal uterine immune cells in the decidua. Trophoblasts also express immune inhibitory molecules that induce the apoptosis of activated T-cells, e.g., Fas ligand (FasL), tumour necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) and indoleamine 2,3-dioxygenase (IDO).⁶⁷⁻⁶⁹ In addition, tolerance induction by trophoblasts occurs via the inhibition of effector T-cells as well as by promoting regulatory T-cells. Programmed cell death protein 1 (PD-1) is an inhibitory receptor that is expressed by different activated immune cells. When PD-1 recognizes its ligand (PD-L1 or PD-L2), this results in reduced proliferation, a dampening of effector function and promoting differentiation towards regulatory T-cells.^{68, 70} The secretion of human chorionic gonadotropin (hCG) also stimulates the differentiation of T-cells to regulatory T-cells.⁷¹ CD200, also known as the OX-2 tolerance signal, is expressed by all trophoblasts and results in tolerance.⁷² Carcinoembryonic antigen-related cell adhesion molecule-1 (CEACAM1) is expressed by extravillous trophoblasts and inhibits the cytolytic properties of decidual NK-cells.^{68, 73}

Components of the innate immune system also play an important role in immune modulation by trophoblasts. Toll-like receptors are expressed by trophoblasts.^{74, 75} The activation of these toll-like receptors of trophoblast cells results in the production of pro-inflammatory and anti-inflammatory cytokines.⁷⁶ Complement regulatory molecules determine the extent and effects of complement system activation in the placenta and are sufficient to prevent excess complement activation in normal pregnancy.⁷⁷⁻⁸¹

Maternal immune cells throughout pregnancy

The maternal immune cells that populate the decidua and the maternal immune cells in the peripheral blood directly contact fetal cells. Approximately 35% of the decidua consists of maternal immune cells.^{82, 83} Throughout pregnancy, relatively many macrophages are found in the decidua.⁸⁴ The number of decidual macrophages increases gradually during pregnancy.⁸⁵ These macrophages are mainly of an anti-inflammatory subtype and produce anti-inflammatory cytokines and angiogenic factors.^{86, 87} Macrophages in the decidua are different from typical anti-inflammatory macrophages since they are activated by M-CSF and interleukin (IL)-10.⁸⁸ Furthermore, decidual macrophages show a limited inflammatory response and prevent T-cell activation.^{89, 90} In the peripheral blood of pregnant women, the number of monocytes is increased compared to non-pregnant women.⁹¹ Furthermore, relatively few dendritic cells (1-2%) are present in the decidua,⁸⁴ and

most of these dendritic cells are immature.⁹² This may be caused by the production of factors by decidua stromal cells, which inhibit dendritic cell maturation.⁹³ Mature dendritic cells are very potent antigen-presenting cells and can activate T-cells and B-cells, where immature dendritic cells are more tolerogenic. The maturation status of dendritic cells, therefore, determines their immune response.⁹⁴ Neutrophils in the endometrium and decidua have an important role in infertility, pre-eclampsia, and intrauterine fetal demise.^{95, 96} A specific population of neutrophils with pro-angiogenic properties is found in the decidua, and their numbers increase over gestation.⁹⁷ NK-cells are also found in the decidua, but these cells are different from the circulating NK-cells since they express higher levels of the killer-cell immunoglobulin-like receptor (KIR). KIR receptors are important for the functioning of NK-cells, and the KIR receptor binds to HLAs on NK-cells.^{98, 99} The combination of the KIR genotype and the fetal HLA-C genotype regulates trophoblast invasion and spiral artery remodelling. During the first trimester, the number of NK-cells increases in the decidua, and from the second trimester onwards, it decreases again.^{100, 101}

Few T-cells are found in the decidua in the first trimester, and approximately 10% of the total decidua leukocyte population consists of T-cells.⁸⁴ When a pregnancy reaches term, almost 50% of the immune cells in the decidua are T-cells.¹⁰² Regulatory T-cells are found in the decidua, and reduced numbers of regulatory T-cells are found in the placenta of women with a spontaneous miscarriage or women with pre-eclampsia.¹⁰³ In the peripheral blood, the number of effector T-cells is increased in pregnant women. The number of regulatory T-cells was not different from non-pregnant controls.¹⁰⁴ B-cells are virtually absent in the decidua, and their role in pregnancy is not precisely known.^{105, 106} The role of B-cells in pregnancy is best known from auto-antibodies and alloantibodies, which are involved in several pregnancy complications. The presence of auto-antibodies directed against anti-phospholipids is associated with miscarriages and pre-eclampsia.^{107, 108} Antibodies directed against angiotensin also play a pivotal role in pre-eclampsia.¹⁰⁹ Angiotensin antibodies could result in hypertension through activation and stimulation of the angiotensin-I receptor and can contribute to complement activation.¹¹⁰⁻¹¹² Furthermore, alloantibodies directed against fetal human thrombocyte antigens and fetal antigens on red blood cells result in fetal complications as IgG antibodies can pass through the placenta.¹⁸ B-cells can also produce pregnancy-protective antibodies; the cytotoxic effect of lymphocytes towards trophoblasts is hampered after the addition of maternal serum. When the IgG fraction is removed from this serum, the protective effect is significantly reduced.¹¹³

In conclusion, at the fetal-maternal interface, maternal cells are in direct contact with fetal cells. Trophoblasts are important in the modulation of the immune response at the fetal-maternal interface and in the maintenance of haemostasis in the intervillous space.

Immunologically challenging pregnancies

Every pregnancy is unique due to maternal, paternal and fetal factors. Presumably, immunology differs between pregnancies, and some pregnancies could be immunologically more challenging for the mother than others. For example, in oocyte donation pregnancies, the fetus is completely allogeneic compared to the mother. Hence, oocyte donation pregnancies provide an interesting setting to evaluate the effect of immunogenetic differences between the mother and fetus on maternal adaptation to pregnancy. Furthermore, several pregnancy complications have an immunological component. This thesis will cover oocyte donation pregnancies with and without pre-eclampsia, naturally conceived pregnancies complicated by pre-eclampsia and chronic intervillitis of unknown aetiology (CIUE).

Pre-eclampsia

Pre-eclampsia is a pregnancy-related syndrome, and approximately 3-5% of pregnancies are complicated by pre-eclampsia.^{114, 115} Pre-eclampsia is defined as the new onset of hypertension (≥ 140 mmHg systolic or ≥ 90 mmHg diastolic) after 20 weeks of pregnancy and the new onset of proteinuria, other maternal organ dysfunction or signs of uteroplacental dysfunction.¹¹⁴ It is important to note that proteinuria is no longer required for the diagnosis of pre-eclampsia.¹¹⁵

Clinical impact

The course of pre-eclampsia is generally relatively mild. However, the symptoms can worsen very abruptly, and complications such as renal insufficiency, liver dysfunction, and neurological complications may develop. Pre-eclampsia is an important cause of maternal, fetal and neonatal mortality and morbidity.¹¹⁶ Serious maternal morbidity is observed in 15% of women with severe pre-eclampsia, and severe pre-eclampsia contributes to unfavourable perinatal outcomes.^{117, 118} The only definitive cure for pre-eclampsia is the birth of the fetus and placenta. Any further treatment focuses on the prevention and treatment of the more severe complications of pre-eclampsia. Due to the erratic course of pre-eclampsia, women with mild symptoms must also be monitored closely. Anti-hypertensive drugs, such as calcium channel antagonists and beta-blockers, are used to lower blood pressure when blood pressure exceeds 110 mmHg diastolic or 160 mmHg systolic. Magnesium sulfate (MgSO_4) is given to prevent eclampsia.^{115, 119} Clinicians aim to postpone delivery in women with pre-eclampsia before 37 weeks of gestation to enhance fetal outcomes. However, delivery is necessary when the maternal or fetal condition worsens, e.g., in cases of uncontrollable maternal blood pressure, eclampsia, placental abruption or fetal distress.^{114, 115, 119}

Women who have had pre-eclampsia are at risk of developing cardiovascular disease and kidney disease later in life. For instance, women who have suffered from pre-eclampsia have a 5- to 12-fold increased risk for developing end-stage renal disease later in life.¹²⁰ A nationwide study from Norway identified pre-eclampsia as an independent risk factor for the development of end-stage renal disease.¹²¹⁻¹²³ A systematic review and meta-analysis showed that 31% of women

who had pre-eclampsia developed microalbuminuria 7.1 years post-partum. This is four times higher than for women with uncomplicated pregnancies.¹²⁴

Two-stage hypothesis of pre-eclampsia

The placenta is a crucial component of the development of pre-eclampsia. The two-stage model of pre-eclampsia has been proposed as an explanation for the relationship between the placenta and the maternal syndrome.¹²⁵⁻¹²⁸ The first stage of pre-eclampsia starts with problems in the placenta; subsequently, the placenta starts to produce excess levels of several factors, such as anti-angiogenic factors soluble FMS-like tyrosine kinase 1 (sFlt-1) and soluble endoglin (sEng), pro-inflammatory cytokines, syncytiotrophoblast microparticles and reactive species.^{129, 130} These different placental factors contribute to generalized endothelial dysfunction in the mother. The symptoms of stage two of pre-eclampsia are a suggestion of generalized endothelial dysfunction that expresses itself in hypertension, proteinuria, liver problems, a hyper-coagulable status and neurological problems.^{129, 131} Animal models confirm the two-stage hypothesis. Rats with reduced placental perfusion, a so-called reduced uterine perfusion pressure (RUPP) model in which one of the uterine arteries is clipped, will develop hypertension.¹³² Furthermore, animals exposed to sFlt-1, sEng or TNF- α will develop a pre-eclampsia-like phenotype characterized by hypertension and proteinuria.¹³³⁻¹³⁵ sFlt-1 is a splice variant of vascular endothelial growth factor (VEGF) receptor 1 and functions as a decoy receptor for VEGF. Binding of VEGF to sFlt-1 results in a relative shortage of VEGF and thus an angiogenic imbalance. The importance of VEGF inhibition in the pathophysiology of pre-eclampsia is further confirmed by studies with animals exposed to anti-VEGF antibodies (e.g., bevacizumab exposure) and VEGF inhibition (e.g., sunitinib exposure), as these animals also developed a pre-eclampsia-like phenotype characterized by hypertension and proteinuria.^{136, 137} Moreover, the treatment of patients with anti-VEGF antibodies or angiogenesis inhibitors results in pre-eclampsia-like symptoms as well.¹³⁸⁻¹⁴¹

Pre-eclampsia and placental dysfunction

The placenta plays an important role in the development of pre-eclampsia; the presence of a placenta alone could cause pre-eclampsia. This is the case in molar pregnancies and when a piece of placenta remains in utero post-partum.^{142, 143} In general, the placenta of a pregnancy complicated by pre-eclampsia is characterized by inadequate extravillous trophoblast migration, decreased uteroplacental vascular perfusion and intrauterine hypoxia.¹⁴⁴ The histopathological examination of the placenta of women with pre-eclampsia shows variable changes: infarction, accelerated villous maturation together with increased syncytial knot formation, unconverted placental bed spiral arteries and fetal nucleated red blood cells as a sign for fetal hypoxia.¹⁴⁴ The disturbances in both coagulation and immunology play an important role in the pathophysiology of placental dysfunction in pre-eclampsia. Atherosclerosis, a lesion characterized by macrophage foam cells in the vessel wall, is frequently observed in decidual arteries when a pregnancy is complicated by pre-eclampsia.¹⁴⁴ The PAR-1 receptor is more highly expressed in trophoblasts in a pre-eclamptic placenta.¹⁴⁵ The degree of placental infarction is associated with

pre-eclampsia.^{146, 147} Furthermore, the placenta of women with pre-eclampsia is characterized by complement deposits¹⁴⁸ and increased complement factors in the maternal serum as well.^{81, 149} The importance of complement system activation is further revealed in a pre-eclampsia mouse model in which symptoms and placental dysfunction were prevented by C3 inhibition.¹⁵⁰ On the other hand, C1q is necessary for trophoblast invasion, and pregnant C1q-deficient mice also develop symptoms of pre-eclampsia.¹⁵¹ Immune cell subsets in the placenta of women with pre-eclampsia seem to point to a more pro-inflammatory phenotype. The dominance of pro-inflammatory macrophages in the decidua is associated with pre-eclampsia.^{152, 153} Fewer NK-cells are found in pre-eclamptic placentas, which seems to be related to fetal growth restriction rather than to pre-eclampsia.^{154, 155} Pregnancies with a combination of a maternal KIR AA genotype (predominantly inhibitory KIR receptors) and a fetal HLA-C2 genotype have an increased risk of developing pre-eclampsia.¹⁵⁶ This genotype combination is also associated with reduced fetal birth weight.¹⁵⁷ Interestingly, placental findings in pre-eclampsia vary highly and can display, especially in late-onset cases of pre-eclampsia, no distinct pathological findings at all.¹⁴⁴ Therefore, in the placenta of growth-restricted babies, signs for placental dysfunction are also found, while not all women with a growth-restricted baby develop pre-eclampsia.¹⁵⁸ Placental findings in women with pre-eclampsia cover a broad spectrum of disrupted placentation—from hypercoagulation and inflammation to an entirely normal placenta. Henceforth, the two-stage hypothesis for the development of pre-eclampsia is not entirely correct, and the results of placental dysfunction are not the only contributing factors to the clinical development of pre-eclampsia.

Generalized endothelial dysfunction and pre-existing factors

The maternal response to the anti-angiogenic and pro-inflammatory products produced by the placenta also differs. For example, the sFlt-1 concentrations overlap between women with pre-eclampsia and women with uncomplicated pregnancies.¹⁵⁹ This could partly be explained by the production of other factors in the placenta that affect endothelial function, e.g., sEng and several cytokines. Soluble endoglin is a decoy receptor for TGF- β , which is similar to VEGF, an essential factor for the maintenance of the endothelium. TNF- α , which is also produced by the placenta, can activate endothelial cells as well. On the other hand, it is likely that pre-existing maternal factors modify the physiological adaptation to pregnancy. Conditions that are known to influence the endothelium seem to be associated with pre-eclampsia; diabetes mellitus increases the risk of pre-eclampsia four times, pre-pregnancy hypertension results in a sevenfold higher risk of pre-eclampsia and obesity increases the risk of pre-eclampsia by a factor of three.¹⁰⁷ Pre-eclampsia also has a strong genetic component. For example, a positive family history for pre-eclampsia is associated with a threefold-increased risk of pre-eclampsia.¹⁰⁷ Women with inherited thrombophilia have a higher risk of pre-eclampsia¹⁶⁰, and different genes that have a function in the coagulation system and innate immune system are linked to pre-eclampsia.¹⁶¹⁻¹⁶³ Apart from important pre-existing factors that influence haemostasis and endothelial function, it is also well known that the maternal immune system is an important driver for the development of pre-eclampsia.

Pre-eclampsia is associated with primigravida, while subsequent pregnancies with the same father are protected.¹⁶⁴ Moreover, pre-eclampsia occurs more often in subsequent pregnancies with a different father.¹⁶⁵ Blood transfusions, previous abortions or miscarriages, and prolonged semen or seminal fluid exposure also seem to have a protective role.¹⁶⁶⁻¹⁷⁰ Several studies have also shown that oocyte donation is an independent risk factor for pre-eclampsia and other hypertensive complications of pregnancy.¹⁷¹⁻¹⁷⁶ In conclusion, pre-eclampsia is a multifactorial disease with placental, vascular and immunological risk factors, and these factors most likely interact with each other.

Thrombomodulin in pre-eclampsia

Thrombomodulin could be a possible link between the dysregulation of coagulation and inflammation in women with pre-eclampsia. The soluble thrombomodulin levels are increased in the serum of pre-eclamptic women compared to controls.¹⁷⁷ These increased levels of soluble thrombomodulin in pre-eclampsia occur most likely due to placental or vascular endothelial cell activation or damage rather than due to ineffective clearance by the kidney or liver.¹⁷⁸ Furthermore, placental thrombomodulin mRNA expression and thrombomodulin protein expression are decreased in women with pre-eclampsia.¹⁷⁹ In this study, it was also shown that placental thrombomodulin mRNA expression is associated with maternal body mass index and blood pressure.¹⁷⁹ An association with body mass index could suggest a link with pre-existent endothelial dysfunction, and an association with blood pressure could suggest a link with the extent of endothelial dysfunction.¹⁷⁹ sFlt-1 mRNA expression was associated with thrombomodulin mRNA expression, and cell culture experiments revealed that angiogenic imbalance could cause thrombomodulin downregulation.¹⁷⁹ Furthermore, treatment with recombinant thrombomodulin has therapeutic potential. In an animal model for pre-eclampsia, treatment with recombinant thrombomodulin significantly improved maternal condition and fetal outcome.¹⁸⁰

Kidney problems in pre-eclampsia

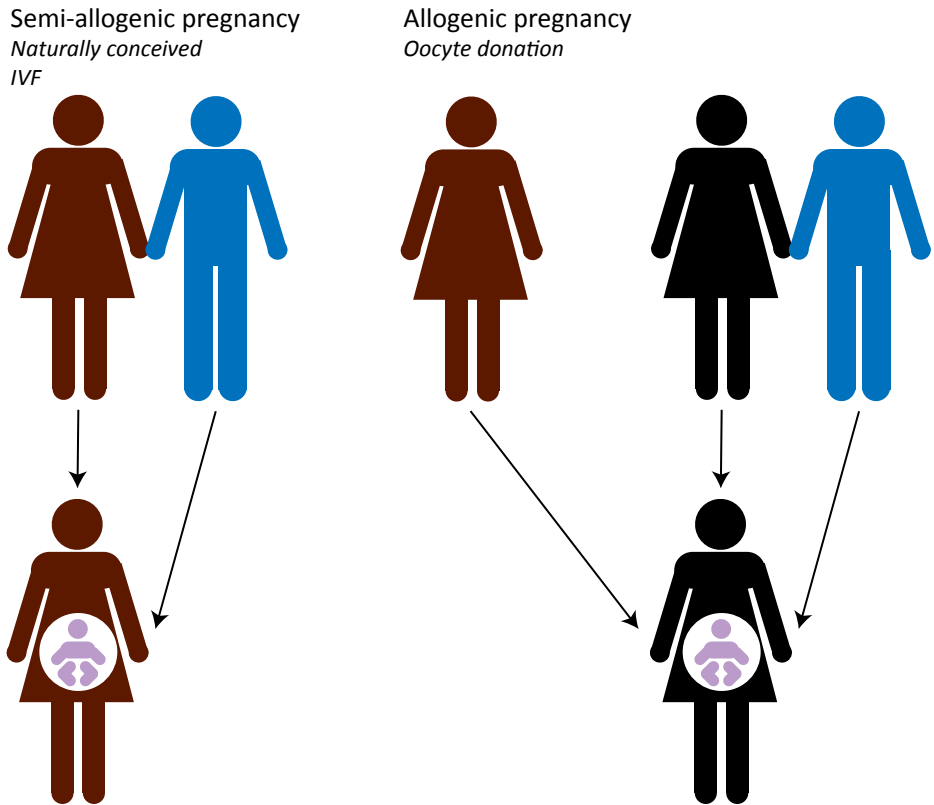
Proteinuria is one of the first symptoms in women who develop pre-eclampsia;¹¹⁴ symptoms range from mild to nephrotic proteinuria and kidney failure later in the disease process.^{181, 182} The kidneys are important for homeostasis and regulate extracellular volume, blood pressure, erythropoiesis, and calcium metabolism. The kidney contains approximately one million nephrons. These are the functional units of the kidney and consist of a glomerulus and an adjacent tubule. The glomerulus is formed by a network of capillaries and facilitates the filtration of blood for the excretion of waste products. Within the glomerulus, the blood is filtered from the capillaries, and the filtrate passes the glomerular filtration barrier. This barrier consists of fenestrated glomerular endothelial cells, the glomerular basement membrane and the slit diaphragms, which are formed by interdigitating podocyte foot processes. The glomerular filtration barrier restricts the passage of large and negatively charged molecules. Water, small proteins and electrolytes can pass through the glomerular filtration barrier and enter Bowman's space. The tubules are important for the selective reabsorption of water and essential molecules.

Throughout the course of a normal pregnancy, the glomerular filtration rate and renal blood flow increase.^{33, 183-185} Normal cardiovascular and kidney changes during pregnancy are tightly regulated via changes in vascular receptors, which leads to a reduced response to several vasopressors.¹⁸⁶

The pathophysiological changes in the kidney of women with pre-eclampsia are distinct; endothelial cells become swollen and lose their fenestrations, a manifestation known as endotheliosis, and podocyte foot process effacement and podocyte loss in urine are observed.^{187, 188} The systemic angiogenic imbalance likely causes the disruption of the glomerular filtration barrier by disturbing vascular endothelial growth factor (VEGF) signalling in the glomerulus.^{136, 189} Podocytes produce VEGF, which is the most important source of VEGF for glomerular endothelial cells and is crucial for the maintenance of the glomerular filtration barrier.¹⁹⁰ Systemic VEGF inhibition, as well as glomerular VEGF inhibition, could disrupt the glomerular filtration barrier. In animal models exposed to anti-VEGF antibodies (e.g., bevacizumab), sFlt-1 or VEGF signalling inhibition (e.g., sunitinib), which mimics systemic VEGF inhibition, pre-eclampsia-like symptoms and renal lesions are observed. This is also the case in animal models with a podocyte-specific VEGF knockout, which mimics glomerular VEGF inhibition.^{76, 135, 137, 190-192} However, VEGF inhibition due to sFlt-1 might not be the only factor that causes glomerular endothelial injury in women with pre-eclampsia. Hypertension during pre-eclampsia could damage the glomerular endothelium as well. Furthermore, VEGF inhibition results in the expression of endothelin-1 by endothelial cells.¹⁹³ Endothelin-1 can subsequently induce podocyte damage, and endothelin-1-induced hypertension is an important common pathway in pre-eclampsia.¹⁹⁴ Another pathway of kidney damage could be classical complement activation secondary to endothelial dysfunction. The activation of the classical complement pathway is seen in the kidneys of women with pre-eclampsia and in a mouse model exposed to sFlt-1.¹⁹⁵ Complement deposits in mice exposed to sFlt-1 suggest that angiogenic imbalance may play an important role in the activation of the complement system in the kidney during pre-eclampsia.¹⁴⁸

Oocyte donation

Oocyte donation is an artificial reproductive technique that enables women with diminished ovarian reserve to conceive.¹⁹⁶ Since the first successful oocyte donation procedure in 1984,¹⁹⁶ immense amounts of oocyte donation procedures have been performed worldwide.^{197, 198} At first, the indication for oocyte donation was premature ovarian failure,¹⁹⁹ which has been currently extended to other forms of infertility due to a diminished ovarian reserve.²⁰⁰⁻²⁰² In oocyte donation pregnancies, the fetus can be completely allogeneic to the mother, as it has inherited genes of the father and the oocyte donor (Figure 7). Therefore, it is presumed that the maternal immune system needs to adapt more, or differently, compared to a naturally conceived semi-allogenic pregnancy, to tolerate the allogenic fetus.²⁰³⁻²⁰⁶ Compared to naturally conceived pregnancies and *in vitro* fertilization (IVF) pregnancies, oocyte donation pregnancies are more often accompanied by early and late obstetrical problems:^{173, 203, 206} Pre-eclampsia, pregnancy-indu-

Figure 7 Oocyte donation

In a naturally conceived pregnancy or non-donor IVF pregnancy, the fetus inherits antigens from the father and the mother. In an oocyte donation pregnancy, no antigens from the mother may be present in the fetus. Figure based on: ²¹⁷.

ced hypertension, caesarean section, miscarriage, first-trimester bleeding and post-partum haemorrhage are more frequently observed.²⁰⁷⁻²¹¹ Women who become pregnant after an oocyte donation are often subject to an increased risk of several pregnancy complications due to advanced maternal age, primiparity, the cause of infertility (e.g., obesity), IVF, and multiple gestations.^{107, 115, 212-214} However, several studies have shown that oocyte donation is also an independent risk factor for hypertensive complications of pregnancy.¹⁷¹⁻¹⁷⁶ A significantly higher number of HLA matches between mother and child was shown in uncomplicated oocyte donation pregnancies than one would expect by chance.²¹⁵ More HLA alloantibodies are found in the serum of women who conceived via oocyte donation compared to naturally conceived pregnancies and autologous IVF pregnancies.²¹⁶ Furthermore, the percentage of women producing HLA alloantibodies is positively correlated with the number of HLA mismatches.²¹⁶ This further suggests that a higher number of mismatches could contribute to pregnancy complications.

The maternal adaptation to pregnancy is also different in oocyte donation pregnancies compared to naturally conceived pregnancies. In the placenta of women who became pregnant after an oocyte donation pregnancy, signs for poor placentation and maternal reactions to the fetus are found.²¹⁸ Insufficient spiral artery remodelling, a histopathological finding that is frequently found in the placenta of women with pre-eclampsia, is also frequently observed in oocyte donation pregnancies regardless of the presence of pre-eclampsia.²¹⁸ Severe chronic deciduitis with fibrin deposits is found more frequently in the placentas of oocyte donation pregnancies than in those of autologous IVF pregnancies.²¹⁹ Furthermore, a higher incidence of other placental lesions, which suggests a maternal reaction to the fetus, is found in oocyte donation cases as well.^{220, 221} Increased numbers of maternal immune cells in the decidua could suggest a maternal reaction to the fetus. Another study found that the presence of an immune infiltrate in the decidua was associated with the absence of pre-eclampsia in oocyte donation pregnancies.²²² Thus, histopathological findings in oocyte donation pregnancies show that immune modulation differs from naturally conceived pregnancies.

Functional studies on immune regulation in oocyte donation pregnancies are limited. In oocyte donation pregnancies, more intracellular interferon- γ (Th1) and interleukin-4 (Th2) CD4+ T-cells are found in the peripheral blood.²²³ Class-II HLA mismatches between the mother and fetus are associated with an increased number of CD4+ T-cells in the peripheral blood.²⁰⁴ Cytokine levels in the decidua and serum were also different between oocyte donation pregnancies and naturally conceived pregnancies.²⁰⁴ In the decidua, IL-10, IL-6, and Gal-1 levels were decreased. In the peripheral blood, IL-10 and IL-6 levels were increased.²⁰⁴ In a different study, however, no changes in cytokine levels between oocyte donation pregnancies, autologous IVF pregnancies, and naturally conceived pregnancies were found except for the expression of stromal cell-derived factor 1 alpha in the third trimester of pregnancy.²²⁴ Complement activation in the placenta of oocyte donation pregnancies has also been investigated. Placental C4d depositions are increased in the placenta of women with pre-eclampsia after a naturally conceived

pregnancy compared with an oocyte donation pregnancy.²²⁵ C4d deposition in uncomplicated oocyte donation pregnancies is comparable to the C4d depositions in autologous IVF pregnancies. This suggests that C4d deposition might be a sign of antibody-mediated fetal rejection in pre-eclampsia.²²⁵ Placental mRNAs of complement regulatory proteins are significantly decreased in uncomplicated oocyte donation pregnancies and oocyte donation pregnancies complicated by pre-eclampsia.²²⁵ In naturally conceived pregnancies complicated by pre-eclampsia, CD55 and CD59 mRNA expression is upregulated.¹⁴⁸ In conclusion, several regulating mechanisms are important for successful oocyte donation pregnancy.

Chronic intervillitis of unknown aetiology

Chronic intervillitis of unknown aetiology (CIUE) is a relatively rare condition, first described by Labarrere and Mullen in 1987.²²⁶ The incidence of CIUE in the second and third trimesters is 6 out of 10,000 pregnancies, and in miscarriage specimens, CIUE is seen in 44 out of 1000 samples in which the fetus has a normal karyotype.²²⁷ CIUE is characterized by the intervillous infiltration of mononuclear cells in the placenta, and the lesion appears to be associated with poor perinatal outcomes, such as (recurrent) miscarriage, impaired fetal growth and fetal death.^{228, 229} Furthermore, CIUE has a high chance of recurrence.²²⁷⁻²³¹

The aetiology of CIUE remains unclear, and immunological dysregulation probably plays a role. The presence of villitis^{228, 230} and C4d deposits²³² with the intervillous infiltrates are indicative of immunological disturbance. In addition, increased expression of intercellular adhesion molecule-1 in the placenta with intervillous infiltrates²³³ and the presence of CIUE-specific cell infiltrates^{229, 234-237} suggest an immunopathological component. Recently, it was suggested that the pathophysiology of CIUE might be based on HLA mismatch between the “donor” (fetal-paternal antigens) and the “recipient” (the mother).²³⁸ This hypothesis is based on the observation of mixed lymphocyte reactions and the presence of cytotoxic T-cell precursor cells.²³⁸ Furthermore, CIUE is frequently observed in fetal neonatal alloimmune thrombocytopenia, a disease characterized by maternal alloantibodies directed against fetal thrombocytes. This further suggests a process comparable to the chronic reaction in CIUE.^{239, 240} Based on these findings, several treatment strategies in the direction of influencing immunological disbalance have been proposed; treatment with corticosteroids, prednisone, aspirin, heparin, intravenous immunoglobulin or different combinations was reported to be effective in some subsequent pregnancies.^{228, 229, 241}

Outline of this thesis

Investigating uncomplicated and complicated pregnancies will contribute to the unravelling of maternal adaptation to pregnancy and will provide insights into regulating mechanisms at the fetal-maternal interface. In this thesis, the focus lies on a spectrum of immunological challenges of pregnancy ranging from uncomplicated

naturally conceived pregnancies and uncomplicated oocyte donation pregnancies to pregnancies complicated by pre-eclampsia or CIUE.

The maintenance of the syncytiotrophoblast and systemic endothelium in immunologically challenging pregnancies is studied first. Based on the allogenic nature of oocyte donation pregnancies, the role the placenta plays in the pathogenesis of pre-eclampsia in oocyte donation pregnancies could be different from that in pre-eclampsia in naturally conceived pregnancies.^{218, 225, 242} Therefore, placental thrombomodulin expression, the downstream effects of thrombomodulin, as well as the regulation of thrombomodulin in women with pre-eclampsia after oocyte donation and in women with uncomplicated pregnancies that were naturally conceived, induced by IVF, or induced by oocyte donation are investigated in **Chapter 2** of this thesis.

The kidney is frequently affected in women with pre-eclampsia, and proteinuria is usually one of the first symptoms that women with pre-eclampsia present. The pathophysiological changes in the kidneys of women with pre-eclampsia are distinct and characterized by endotheliosis, podocyte foot process effacement, complement activation and, in some cases, thrombotic microangiopathy.¹⁸⁷ Thrombomodulin-dependent activated protein C formation plays an important role in the subsistence of the glomerular filtration barrier.²⁴³ Thus, thrombomodulin could have an important protective function on the renal endothelium by regulating coagulation and complement activation in the kidney. Therefore, we investigate thrombomodulin expression in the kidneys of women with pre-eclampsia in **Chapter 3** of this thesis.

Since hypoxia, inflammation and vascular stress are accompanied by the aberrant production of reactive oxygen species, an enhanced presence of reactive oxygen species may play a central role in pre-eclampsia. The placental tissue itself is an important site of the production of reactive oxygen species and other reactive species; indeed, the pre-eclamptic placenta is characterized by the increased production of reactive species.²⁴⁴⁻²⁴⁶ This aberrant placental production of reactive oxygen species and reactive nitrogen species may conceivably contribute to systemic endothelial dysfunction in pre-eclampsia.^{130, 247-249} In allograft organ transplantation, systemic redox status predicts graft survival and mortality,²⁵⁰⁻²⁵² and reduced oxidative damage is associated with a better kidney transplant outcome.²⁵³ Based on the allogeneic conditions in oocyte donation pregnancies, we evaluate the reactive species interactome status in naturally conceived and oocyte donation pregnancies in both the absence and presence of pre-eclampsia in **Chapter 4** of this thesis.

Chronic intervillitis of unknown aetiology (CIUE) is characterized by a prominent mononuclear inflammatory cell infiltrate in the intervillous space. The aetiology of CIUE is not yet fully understood, but the histopathological lesion is accompanied by unfavourable pregnancy outcomes. The literature suggests that CIUE is some expression of cellular rejection of the mother to the fetus. In this thesis, the diagnostic criteria for CIUE are defined in **Chapter 5**, and the clinical impact of CIUE is studied in **Chapter 6** of this thesis.

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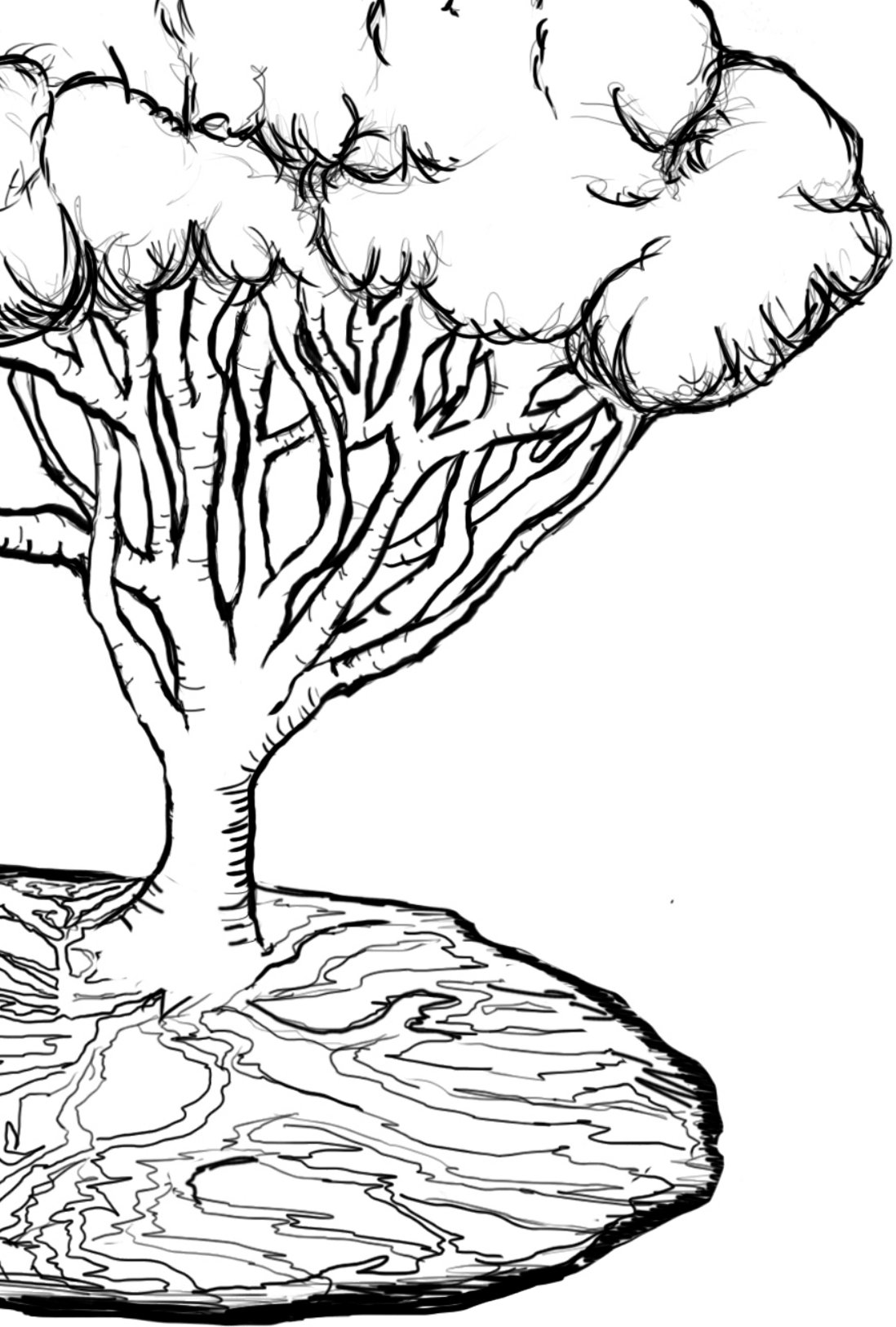
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Loss of placental thrombomodulin in oocyte donation pregnancies

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Abstract

Objective

To investigate whether thrombomodulin dysregulation is involved in the development of preeclampsia after oocyte donation (OD). Women who become pregnant after OD are prone to develop preeclampsia, a syndrome characterized by an aberrant immunologic response, hypercoagulability, and endothelial dysfunction. A mediator of inflammation and coagulation is thrombomodulin, which has a possible role to play in this syndrome.

Design

Case-control study.

Patients

Placentas from 82 women with an uncomplicated pregnancy (48 naturally conceived, 21 IVF, and 33 OD pregnancies) and 9 women with an OD pregnancy complicated by preeclampsia have been studied.

Main Outcome Measures

Abundance of thrombomodulin protein and vitamin D receptor (VDR) were determined using immunohistochemistry; mRNA expression was determined using quantitative polymerase chain reaction.

Results

Placental thrombomodulin protein abundance was lower in OD pregnancies (diffuse pattern in 45%) than in controls (diffuse pattern in 96%). Placental thrombomodulin mRNA expression was lower in OD pregnancies complicated by preeclampsia (0.72 ± 0.47) compared with uncomplicated OD pregnancies (0.43 ± 0.18). Thrombomodulin expression correlated with inflammation and coagulation. VDR expression was decreased in OD pregnancies complicated by preeclampsia and correlated with thrombomodulin mRNA.

Conclusions

Pregnancies conceived through OD lose placental thrombomodulin expression. This loss is associated with an increased coagulation and inflammation, and indicates that endothelial protection is diminished in OD pregnancies, which might be an explanation for the increased risk for preeclampsia. The vitamin D metabolism is dysregulated in OD pregnancies and might be a target for therapy.

Introduction

Oocyte donation (OD) is a technique that enables women with diminished ovarian reserve to conceive. Pregnancy is a challenging state for the mother's immune system, where a controlled environment with a regulated immune response to the semiallogeneic foetus has to be established. After OD, the foetus is completely allogeneic; this even more challenging environment for the immune system is presumed to contribute to the increased number of obstetrical complications observed after OD.^{1, 2} These complications can be explained, in large part, by the increased prevalence of pregnancy induced hypertension and preeclampsia.³⁻⁸

Preeclampsia, a hypertensive disorder during pregnancy, is a leading cause of maternal and neonatal morbidity and mortality worldwide.⁹ The pathophysiology of preeclampsia is not fully understood, but the syndrome is characterised by impaired placental development and subsequent shedding of syncytial trophoblast. This results in the release of antiangiogenic factors such as soluble Flt-1, which binds to vascular endothelial growth factor in the circulation.¹⁰ These factors contribute to a maternal intravascular systemic inflammatory response, leading to generalised endothelial dysfunction, enhanced leukocyte and complement activation, and coagulation.¹⁰

The role of the placenta in the pathogenesis of preeclampsia in OD pregnancies is presumed to be different from that in preeclampsia in naturally conceived pregnancies.¹¹⁻¹³ Although these patients are subject to an increased risk of preeclampsia because of older age,⁹ it is also known that OD is an independent risk factor for hypertensive complications of pregnancy.^{14, 15} Furthermore, the clinical presentation of these patients is different from patients with preeclampsia after a naturally conceived pregnancy: growth restriction after OD pregnancies complicated by preeclampsia is less severe.^{6, 12, 16, 17} Moreover, the pathophysiology of preeclampsia after naturally conceived pregnancies seems to be of a more vascular origin,¹⁰ whereas preeclampsia after OD presumably has a more immunological origin.^{1, 11, 12, 18}

In women with preeclampsia in naturally conceived pregnancies, serum levels of the breakdown product of thrombomodulin are higher in comparison to uncomplicated naturally conceived pregnancies,¹⁹ and placental thrombomodulin protein and mRNA expression in naturally conceived pregnancies is decreased.²⁰ Thrombomodulin is a protein essential for the maintenance of endothelium; it inhibits inflammatory pathways and apoptotic pathways in endothelial cells, and it inhibits coagulation.²¹ The pathways through which thrombomodulin is regulated in the placenta are currently not precisely known, but the angiogenic imbalance, as seen in preeclampsia, has been shown to decrease thrombomodulin expression.²⁰ Another possible regulator of placental thrombomodulin is vitamin D; decreased vitamin D levels are associated with an increased incidence of preeclampsia,²² and vitamin D increases thrombomodulin expression in endothelial aorta cells.²³

Despite the fact that the placenta is presumed to have a different role in the pathophysiology of preeclampsia after OD pregnancies and naturally conceived pregnancies; the placenta thrombomodulin expression might be altered in OD pregnancies complicated by preeclampsia as well. In both naturally conceived and OD pregnancies, preeclampsia is characterised by endothelial dysfunction, inflammation, and hypercoagulability.¹² Therefore, our objectives are to investigate placental thrombomodulin expression, downstream effects of thrombomodulin, and the regulation of thrombomodulin in women with preeclampsia after OD and in women with uncomplicated pregnancies which were either naturally conceived,²⁰ induced by IVF, or induced by OD, as control subjects. We hypothesise that thrombomodulin expression is altered in OD pregnancies complicated by preeclampsia.

Materials and methods

Patients who received OD and control groups

A case-control study with 56 placentas from women pregnant after OD in the Leiden University Medical Centre (LUMC) and teaching hospitals in the region between 2004 and 2013 was performed; 40 placentas were from pregnancies without hypertensive complications, and 16 placentas were from women who suffered from preeclampsia according to ISSHP guidelines.²⁴ Patients who had an OD with at least information on maternal age, gestational age, birth weight, highest diastolic blood pressure, and available paraffin embedded placenta samples were included. Twenty-eight uncomplicated naturally conceived pregnancies and 21 IVF-induced pregnancies were selected from available patients as controls. Using IVF pregnancies and naturally conceived pregnancies as controls for OD pregnancies has been described before in comparable studies.^{12, 18} Controls were selected on the basis of mode of delivery, because mode of delivery has a broad impact on the placenta and influences gene expression.²⁵ No other selection criteria were used. Small for gestational age was defined as birth weight below the 10th percentile for gestational age according to the Dutch reference curves for birth weight by gestational age.²⁶ Patient characteristics were obtained from the medical records. From all placentas, paraffin-embedded samples were available for immunohistochemical staining. Frozen tissue, which was used for mRNA analysis, was available for 36 uncomplicated OD placentas, 16 OD pregnancies complicated by preeclampsia, and 10 uncomplicated naturally conceived pregnancies. As previously described, the placentas of twins and triplets were treated as individual samples, since placental pathology can be different in twins. Informed consent was obtained from all patients. This study was approved by the ethics committee of LUMC (P13.084).

Thrombomodulin and maternal age

Placentas from an additional group of older women (n=20; maternal age >37) with an uncomplicated naturally conceived pregnancy were included to investigate

the effect of maternal age on placental thrombomodulin protein expression.

Histochemical staining

Histological phosphotungstic acid-hematoxylin (PTAH) staining was performed to investigate the presence of fibrin depositions. Sections were incubated in 0.25% potassium permanganate for 15 minutes followed by 5% oxalic acid for 5 minutes. Sections were then incubated in PTAH for 24 hours at room temperature.

Immunohistochemistry

Immunohistochemical staining was performed to investigate the placental protein abundance of thrombomodulin and the vitamin D receptor (VDR). We choose to determine placental VDR expression since decreased placental VDR expression has been described as a proper measure for disturbances in vitamin D signalling before.^{27, 28} Sections were deparaffinised, and antigen retrieval was performed. Sections were incubated with anti-thrombomodulin mouse monoclonal antibody (1:200; Leica Biosystems) or an anti-VDR mouse monoclonal antibody (1:1,500; Santa Cruz Biotechnology) for 1 hour at room temperature. Binding of the primary antibody was visualized with a PO-labelled anti-mouse polymer (DAKO, Glostrup, Denmark) and diaminobenzidine as a chromogen.

Scoring of staining patterns

Slides were scored by two observers blinded with respect to cases and control groups. Twenty percent of cases were scored differently between observers, and for those, consensus was obtained during a consensus meeting. Thrombomodulin protein abundance at the surface of viable syncytiotrophoblast was scored semi-quantitatively, as absent (present on <10% of viable syncytiotrophoblast), focal (present on 10-50% of viable syncytiotrophoblast), or overall (present on >50% of viable syncytiotrophoblast) as described elsewhere.²⁰ Viability of syncytiotrophoblast was confirmed using Haematoxylin-Eosin staining. The presence of fibrin depositions on the villi was scored similarly. VDR abundance at the surface of syncytiotrophoblast was scored semiquantitatively as overall (>90% of syncytiotrophoblast positive for VDR), decreased (90-50% of syncytiotrophoblast positive for VDR) and absent (<50% of syncytiotrophoblast positive for VDR).

Quantitative Polymerase Chain Reaction (PCR)

Quantitative PCR was performed to quantify placental mRNA expression of thrombomodulin, VDR, Intercellular adhesion molecule-1, tumour necrosis factor alpha, factor VIII, tissue factor, vascular endothelial growth factor, and soluble FLT-1. Primer sequences can be found in Supplemental Table 1. RNA isolation was performed with TRIzol (Lifetechnologies). Synthesis of cDNA was performed with AMV reverse transcriptase (Roche), and SYBR green quantitative PCR was performed according to the manufacturer's protocol (Bio-Rad Laboratories). Expression was measured by the comparative threshold cycle method and normalized to expression of housekeeping genes hypoxanthine phosphoribosyltransferase and GAPDH. A melting curve analysis was performed to verify the specificity of amplification.

Cell culture experiments

Cell culture experiments were performed to investigate placental thrombomodulin regulation through vitamin D signalling. The human choriocarcinoma cell line BeWo (CCL-98, ATCC) was cultured in RPMI medium supplemented with 10% fetal calf serum. Short tandem repeat analysis with GenePrint® (Promega) confirmed the identity of the cells. Cells were used within 25 passages. Forskolin (50µM, dissolved in dimethyl sulfoxide 0.1%; Sigma-Aldrich) was used to stimulate VDR expression.²⁹ Vitamin D (dissolved in ethanol 1%; Sigma-Aldrich) was added to the medium with a final concentration of 100nM.²⁹ Cells were cultured for 24 hours, and then forskolin alone or forskolin with 1,25-di-hydroxyl-vitamin D was added to refreshed medium.²⁹ Cells were cultured for 48 hours before mRNA was isolated.²⁹ Normalized mRNA expression of thrombomodulin and VDR were measured. Experiments were repeated 4 times.

Statistical analysis

For statistical analyses, placentas from OD pregnancies complicated by preeclampsia were compared to placentas from uncomplicated OD pregnancies. Placentas from uncomplicated OD pregnancies were compared to placentas from uncomplicated naturally conceived and uncomplicated IVF induced pregnancies. Factors considered potential confounders were maternal age at pregnancy, body mass index (BMI), smoking, gravidity, parity and twin or triplet pregnancy. Continuous data were compared between groups using the independent t-test for normally distributed data or a Mann-Whitney U-test for skewed distributions. Discontinuous data were analysed with the Chi-square test of Fisher's exact test. Correlations between thrombomodulin mRNA and other mRNA levels or clinical parameters were determined with the Spearman's rho test or Pearson test. $P < 0.05$ was considered statistically significant. Analyses were performed with the IBM SPSS statistics software package (ver. 21; IBM).

Results

Patient characteristics

Patient characteristics can be found in [Table 1](#). The mean age of women who had a naturally conceived pregnancy (33.7 year) or a pregnancy after IVF (35.0 year) were significantly lower than the mean age of women with an uncomplicated OD pregnancy (38.5 year; $p < 0.05$ for both). The average age of women with OD pregnancies complicated by preeclampsia was 42.9 years; this was significantly higher compared with women with uncomplicated OD pregnancies ($p < 0.05$). The mean BMI of women with a naturally conceived pregnancy was significantly higher than the mean BMI of women with an uncomplicated OD pregnancy (respectively; 25.81 and 21.37; $p < 0.05$ for both). The mean BMI of women with preeclampsia during OD pregnancies was higher than the mean BMI of women with uncomplicated OD pregnancies ($p = 0.044$). Medical histories from previous pregnancies were available for all control cases and for 46 out of 56 OD pregnancies. In none of our cases was a history of hypertension or preeclampsia reported in the medical records. Furthermore, none of the patients with an OD-induced pregnancy had diabetes mellitus, gestational diabetes or disturbances in coagulation according to the medical records. Three women with an uncomplicated OD pregnancy had proteinuria, and five had a diastolic blood pressure > 85 mmHg. Nevertheless, none of these women fulfilled the ISSHP criteria²⁴ for the diagnosis of preeclampsia nor were treated for these symptoms.

Pregnancy characteristics

Mean gestational age was significantly lower in children born after OD pregnancies complicated by preeclampsia ($p < 0.001$). In OD pregnancies, more twins and triplets were seen compared to naturally conceived pregnancies ($p < 0.01$), and women pregnant from a twin or triplet had more often preeclampsia ($p < 0.01$).

Fetal characteristics

The mean birth weight of children was lower in IVF pregnancies compared with naturally conceived pregnancies and compared with OD pregnancies complicated by preeclampsia ($p < 0.001$). When corrected for gestational age, these differences were no longer significant.

	NC N = 28	IVF N = 21	OD N = 33	OD PE N = 9
Maternal characteristics				
Mean maternal age, years (SD)	33.7 (4.0)	35.0 (3.9)	38.5 (5.8) *	42.9 (4.7) ‡
Mean maternal BMI, kg/m ² (SD)	25.81 (4.9)	24.65 (5.2)	21.37 (1.9) *	24.21 (0.9) ‡
Smoking				
Yes (%)	1 (3.6)	2 (10)	1 (6.7)	1 (16.7)
No (%)	27 (96.4)	18 (90)	14 (93.3)	5 (83.3)
Mean gravidity (range)	2.9 (1-7)	2.2 (1-5) *	2.1 (1-7) *	2.1 (1-5)
Mean parity (range)	1.4 (0-5)	0.5 (0-2) *	0.4 (0-2) *	0.2 (0-1)
Highest diastole, mmHg (SD)	75 (70-80)	80 (65-95) *	80 (60-90) *	99 (85-115) ‡
Proteinuria				
Yes (%)	0 (0)	1 (5.6)	3 (23.1)	9 (100)
No (%)	28 (100)	17 (94.4)	10 (76.9) *	0 (0) ‡
Prior history of hypertension or preeclampsia				
Yes (%)	0 (0)	0 (0)	0 (0)	0 (0)
No (%)	28 (100)	21 (100)	25 (100)	7 (100)
Pregnancy characteristics				
Gestational age at delivery, weeks + days (SD, days)	39+4 (9)	38+5 (17)	39+2 (15)	34+2 (21) ‡
Term				
A term (%)	28 (100)	15 (78.5)	30 (93.8)	2 (22.2)
Pre term (%)	0 (0)	4 (21.5) *	2 (6.2)	7 (77.8) ‡
Mode of delivery				
Caesarean section (%)	16 (57.1)	9 (45)	17 (54.8)	6 (66.7)
Vaginal delivery (%)	12 (42.9)	11 (55)	14 (45.2)	3 (33.3)
Twin or triplet				
Yes (%)	0 (0)	1 (5)	7 (21.2)	6 (66.7)
No (%)	28 (100)	19 (95)	26 (78.8) *	3 (33.3) ‡
Foetal characteristics				
	N = 28	N = 21	N = 40	N = 16
Sex of Child				
Male (%)	12 (42.9)	10 (47.6)	20 (54.1)	9 (56.3)
Female (%)	16 (57.1)	11 (52.4)	17 (45.9)	7 (43.9)
Birth weight, g (SD)	3597 (435)	3113 (701) *	3297 (717)	1952 (660) ‡
Small for gestational age				
Yes (%)	2 (7.1)	3 (14.3)	3 (8.1)	0 (0)
No (%)	26 (92.9)	18 (85.7)	34 (91.9)	14 (100)
Placenta weight, g (SD)	638 (140)	611 (149)	698 (204)	863 (238)

Table 1 Patient characteristics

*, $p < 0.05$ compared to uncomplicated naturally conceived pregnancies; †, $p < 0.05$ compared to uncomplicated IVF pregnancies; ‡, $p < 0.05$ compared to uncomplicated OD pregnancies. NC, naturally conceived; OD PE, OD pregnancies complicated by preeclampsia.

Thrombomodulin protein expression

Placental thrombomodulin staining was observed in three distinct patterns; representative examples of these patterns are depicted in [Figure 1A-C](#). Syncytiotrophoblast thrombomodulin protein abundance was decreased more often after uncomplicated OD pregnancies and after OD pregnancies complicated by preeclampsia compared with pregnancies conceived through IVF and naturally conceived pregnancies ($p < 0.001$; [Figure 1D](#)).

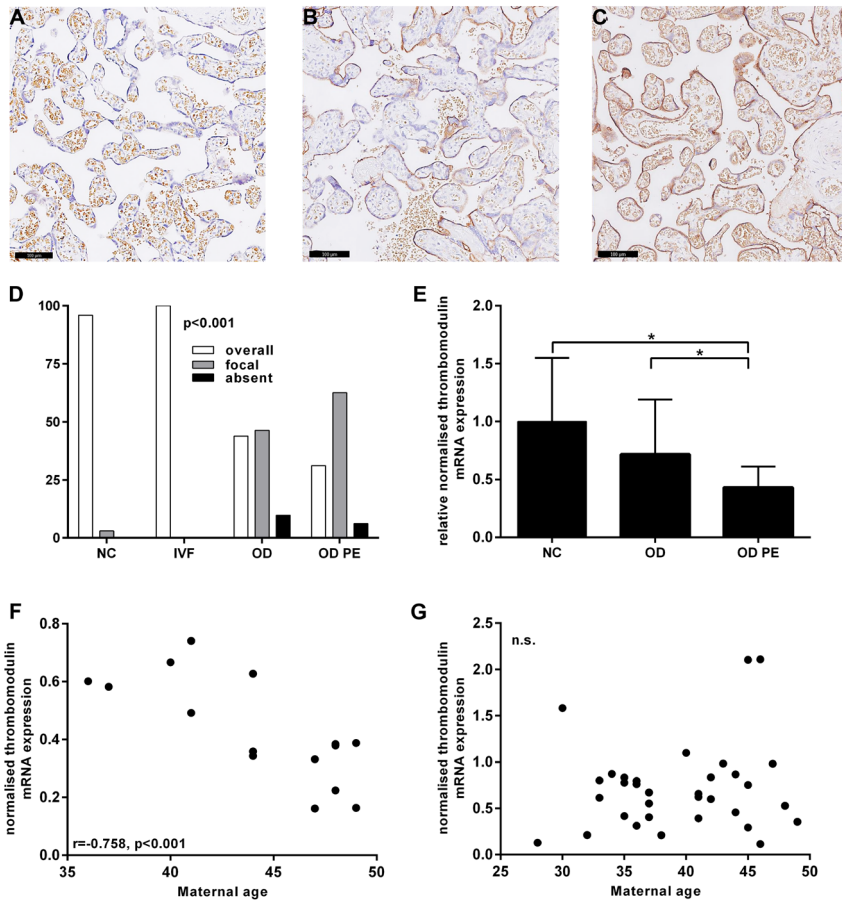
Thrombomodulin protein expression in older women

Since the maternal age of women who had an uncomplicated OD pregnancy was significantly higher than that of women who had a naturally conceived pregnancy or a pregnancy after IVF, we included an additional group of women >37 years who had an uncomplicated naturally conceived pregnancy to study the effect of maternal age on placental thrombomodulin expression. The mean maternal age of these women was 39.4 years; this was significantly higher compared with women who had an uncomplicated naturally conceived pregnancy; characteristics of this additional group are depicted in Supplemental Table 2. Placental thrombomodulin expression was diffuse in 19 out of 20 older women, this was similar as the placental thrombomodulin abundance in the group of uncomplicated naturally conceived control pregnancies in younger women.

Thrombomodulin mRNA expression

Placental thrombomodulin mRNA expression was decreased in women with preeclampsia after OD, compared with uncomplicated OD pregnancies ($p < 0.001$, [Figure 1E](#)). Placental thrombomodulin mRNA expression was similar in naturally conceived pregnancies and uncomplicated OD pregnancies. Thrombomodulin mRNA expression was slightly higher in samples with an overall thrombomodulin protein staining pattern (mean = 0.5415, SD = 0.1315) compared with samples with a focal or absent staining pattern (mean = 0.3891, SD = 0.1850) in OD pregnancies complicated by preeclampsia ($p = 0.138$). Thrombomodulin mRNA correlated inversely with maternal age in the preeclampsia OD group ($r = -0.758$, $p = 0.001$; [Figure 1F](#)). This correlation was not present in uncomplicated OD pregnancies ([Figure 1G](#)). Thrombomodulin mRNA levels were not associated with fetal characteristics nor patient characteristics. Furthermore, thrombomodulin mRNA levels were similar in multiples and singletons in uncomplicated OD pregnancies and OD pregnancies complicated by preeclampsia (Supplementary Figure 1).

Figure 1 Placental thrombomodulin expression



A Representative example of an absent (<10% of viable syncytiotrophoblast) placental thrombomodulin protein expression pattern. **B** Representative example of a focal (10-50% of viable syncytiotrophoblast) placental thrombomodulin protein expression pattern. **C** Representative example of an overall (>50% of viable syncytiotrophoblast) placental thrombomodulin protein expression pattern. **D** Distribution of placental thrombomodulin protein abundance patterns in women with an OD pregnancy complicated by preeclampsia and control groups ($p < 0.001$ for overall chi-square testing). **E** Relative normalised placental thrombomodulin mRNA expression in case and control groups (* $p < 0.05$, Mann-Whitney-U test). **F** Correlation of thrombomodulin mRNA expression and maternal age in OD pregnancies complicated by preeclampsia ($r = -0.758$, $p < 0.001$, Pearson's correlation test). **G** Correlation of thrombomodulin mRNA expression and maternal age in uncomplicated OD pregnancies ($p > 0.05$, Pearson's correlation test). NC, naturally conceived; OD PE, OD pregnancies complicated by preeclampsia; n.s., not significant.

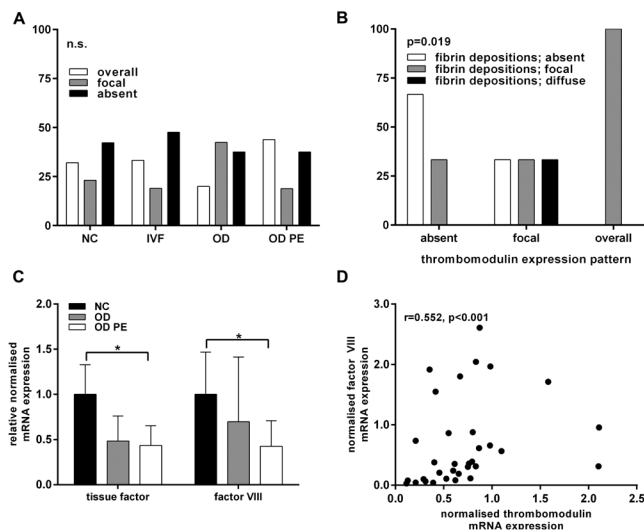
Downstream effects of thrombomodulin: Coagulation

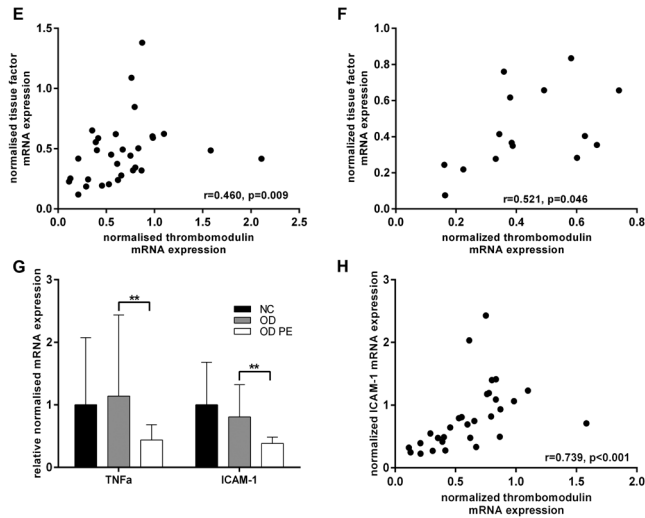
Fibrin depositions were evenly distributed over all groups (Figure 2A). Increasing thrombomodulin protein abundance was associated with decreasing presence of fibrin deposits in women with OD pregnancies complicated by preeclampsia ($p=0.019$; Figure 2B). This association was not present between thrombomodulin mRNA levels and fibrin depositions. Placental mRNA expression of tissue factor and factor VIII was slightly lower in uncomplicated OD pregnancies compared with naturally conceived pregnancies and were lower in OD pregnancies complicated by preeclampsia compared with naturally conceived pregnancies ($p<0.05$ for both; Figure 2C). However, placental mRNA levels of tissue factor and factor VIII were not different between OD pregnancies complicated by preeclampsia and uncomplicated OD pregnancies. Placental thrombomodulin mRNA expression and factor VIII mRNA expression were correlated in uncomplicated OD pregnancies ($r=0.552$, $p<0.001$; Figure 2D). Placental thrombomodulin mRNA expression correlated with tissue factor mRNA expression in uncomplicated OD pregnancies ($r=0.460$, $p=0.009$; Figure 2E) and OD pregnancies complicated by preeclampsia ($r=0.521$, $p=0.046$; Figure 2F).

Downstream effects of thrombomodulin: Inflammation

Placental tumour necrosis factor alpha and intercellular adhesion molecule-1 mRNA expression were similar in uncomplicated OD pregnancies and naturally conceived pregnancies. Remarkably, tumour necrosis factor-alpha and intercellular adhesion molecule-1 mRNA levels were lower in OD pregnancies complicated by preeclampsia compared with uncomplicated OD pregnancies ($p<0.001$ for both; Figure 2G). Placental thrombomodulin mRNA expression correlated positively with intercellular adhesion molecule-1 mRNA expression in uncomplicated OD pregnancies ($r=0.739$, $p<0.001$; Figure 2H).

Figure 2 Downstream effects of thrombomodulin: coagulation and inflammation





A Presence of fibrin deposits as detected with histological PTAH staining in placentas from OD pregnancies complicated by preeclampsia and control pregnancies (naturally conceived, IVF and OD; $P>0.05$ with overall Chi-square testing). **B** The association between placental thrombomodulin expression pattern and the amount of fibrin deposits in OD pregnancies complicated by preeclampsia ($p=0.019$, with overall Chi-square testing). **C** Relative normalised tissue factor and factor VIII mRNA expression in OD pregnancies complicated by preeclampsia and control groups ($*=p<0.05$, Mann-Whitney-U test). **D** Correlation of factor VIII and thrombomodulin mRNA expression in the uncomplicated OD control group ($r=0.552$, $p<0.001$). **E** Correlation of normalised tissue factor mRNA and thrombomodulin mRNA expression in the uncomplicated OD control group ($r=0.460$, $p=0.009$). **F** Correlation of normalised tissue factor mRNA and thrombomodulin mRNA expression in OD pregnancies complicated by preeclampsia ($r=0.521$, $p=0.046$). **G** Relative normalised tumour necrosis factor alpha and intercellular adhesion molecule 1 mRNA expression in placentas of women with preeclampsia after OD and control groups ($**p<0.001$; Mann-Whitney-U test). **H** Correlation of intercellular adhesion molecule 1 and thrombomodulin mRNA expression in uncomplicated OD control pregnancies ($r=0.739$, $p<0.001$; Pearson's correlation test). ICAM-1, intercellular adhesion molecule 1; NC, naturally conceived; OD PE, OD pregnancies complicated by preeclampsia; TNF α , tumour necrosis factor alpha; n.s., not significant.

Regulation of thrombomodulin: Angiogenic factors

Placental vascular endothelial growth factor mRNA expression was higher in uncomplicated naturally conceived pregnancies compared to uncomplicated OD pregnancies and OD pregnancies complicated by preeclampsia ($p=0.038$ and $p=0.013$; Supplementary Figure 2). Placental soluble Flt-1 mRNA expression was higher in complicated OD pregnancies compared with uncomplicated OD pregnancies ($p=0.006$; Supplementary Figure 2). Thrombomodulin mRNA expression and protein abundance were not associated with vascular endothelial growth factor or soluble Flt-1 mRNA expression.

Regulation of thrombomodulin: Vitamin D

Placental VDR protein abundance was observed in 3 distinct patterns; representative examples of these patterns can be found in Figure 3A-C. VDR abundance differed between groups, with OD pregnancies complicated by preeclampsia tending to be associated with decreased VDR abundance ($p<0.05$; Figure 3D). No seasonal changes of VDR abundance were observed in this cohort. Placental VDR mRNA expression was lower in OD pregnancies complicated by preeclampsia compared to naturally conceived pregnancies ($p=0.016$; Figure 3E). Placental thrombomodulin mRNA expression was positively correlated with placental VDR mRNA expression in uncomplicated OD pregnancies ($r=0.704$, $p<0.001$; Figure 3F) and OD pregnancies complicated by preeclampsia ($r=0.582$, $p=0.029$; Figure 3G). Placental VDR mRNA expression was significantly higher in women pregnant after OD when the child was born in the spring compared to children born in the autumn ($p<0.05$). Other associations with seasonality and VDR mRNA expression levels could not be found (Supplementary Figure 3).

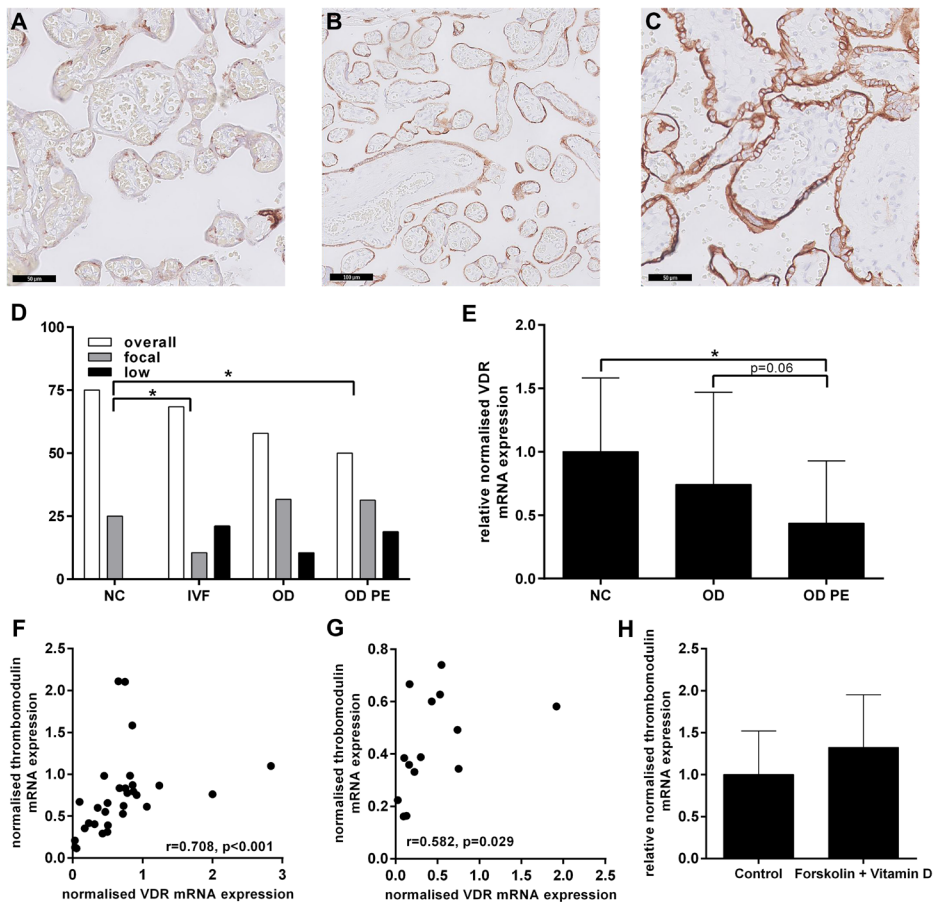
In-vitro experiments on vitamin D signalling and thrombomodulin

Adding forskolin to cell culture medium led to increased VDR mRNA expression compared to cells cultured in control medium, as described elsewhere.²⁹ Cells cultured with 50 μM forskolin and 100 nM vitamin D expressed higher levels of thrombomodulin mRNA compared with cells cultured with control medium (Figure 3H).

Discussion

Compelling evidence indicates that pregnancies after OD are subject to a vast risk of preeclampsia,¹⁻⁸ but the underlying pathophysiology of this phenomenon remains uncertain. Similar to what has been described in naturally conceived pregnancies complicated by preeclampsia,²⁰ we show that placental thrombomodulin mRNA expression is decreased in OD pregnancies complicated by preeclampsia. Furthermore, thrombomodulin protein abundance is decreased in uncomplicated OD pregnancies and OD pregnancies complicated by preeclampsia; this could indicate that loss of thrombomodulin, due to loss of endothelial protection, plays

Figure 3 Regulation of thrombomodulin: Vitamin D



a role in the development of more pregnancy complications in OD pregnancies. Decreased thrombomodulin expression is associated with changes in coagulation pathways and inflammatory pathways. Furthermore, this study demonstrates that disturbances in the vitamin D metabolism possibly contribute to placental thrombomodulin loss during OD pregnancies. We found that decreased VDR expression is associated with loss of thrombomodulin in OD pregnancies and that vitamin D increases thrombomodulin expression *in vitro*.

Loss of placental thrombomodulin in OD pregnancies calls into question whether thrombomodulin loss in OD pregnancies lies in the causal pathway of the development of preeclampsia or if this loss is merely a result of the OD and subsequent immunological disturbances. Thrombomodulin loss may be caused by specific maternal characteristics typical for endothelial dysfunction. For example, we previously showed that thrombomodulin mRNA expression correlated significantly with maternal BMI and diastolic blood pressure in preeclampsia in naturally conceived pregnancies.²⁰ Furthermore, in this study we showed that maternal age correlated significantly with thrombomodulin mRNA in OD pregnancies complicated by preeclampsia. Because of this correlation, and because increasing age is associated with loss of endothelial protection, the older age of the OD group could explain the thrombomodulin loss seen in this group. However, placental thrombomodulin protein expression was not decreased in older women who had an uncomplicated naturally conceived pregnancy. Also, no correlations between thrombomodulin expression and maternal characteristics such as BMI or hypertension were present. Therefore, it seems less likely that loss of placental thrombomodulin is part of the causal pathway of the development of preeclampsia after OD. Logically, thrombomodulin loss could be a result of OD, since thrombomodulin can be downregulated by inflammatory factors, such as matrix metalloproteinases and tumour necrosis factor alpha.²¹ An OD pregnancy is a challenging state for the mother's immune system; a regulated immune response to the allogeneic foetus has to be established. OD pregnancies are characterized by more HLA mismatches, more T-helper cells are found in the peripheral blood,^{18, 30} and the number of activated regulatory T-cells in the parietal decidua correlates with the number of HLA mismatches.³¹ Therefore, thrombomodulin loss in uncomplicated OD pregnancies could be caused by OD specific immune regulation.

On the contrary, thrombomodulin exerts cytoprotective effects through activation of anti-inflammatory pathways. Immune dysregulation is a distinct pathogenic pathway in preeclampsia. Preeclampsia is associated with placental dysregulation of intercellular adhesion molecule-1 and tumour necrosis factor-alpha.^{32,34} Indeed, we found an upregulation of tumour necrosis factor-alpha and intracellular adhesion molecule-1 in OD pregnancies complicated by preeclampsia and a significant correlation between placental thrombomodulin and intercellular adhesion molecule-1. An association between preeclampsia and hypercoagulation is also well established; it is associated with mutations in the prothrombin and factor V Leiden genes and the use of the anticoagulant drugs decreases development, mortality and morbidity of preeclampsia.^{35,37} Loss of thrombomodulin

protein abundance in OD pregnancies could result in reduced anticoagulation and thereby contribute to symptoms of preeclampsia. In this study, thrombomodulin protein abundance was inversely associated with fibrin deposits in women with an OD pregnancy complicated by preeclampsia. Furthermore, significant correlations of mRNA expression of tissue factor and factor VIII with thrombomodulin mRNA expression were found in OD pregnancies. Together these results indicate that loss of thrombomodulin could contribute to the pathogenesis of preeclampsia after OD through its distinct effects on inflammation and coagulation.

In this study, lower VDR expression on the syncytiotrophoblast of women with OD pregnancies complicated by preeclampsia compared with naturally conceived pregnancies was found. Decreased VDR expression is known to be associated with disturbances in vitamin D signalling, subsequent placental disorders, and growth restriction.^{27, 28} In our cohort, a decrease in VDR mRNA expression was associated with a decrease in thrombomodulin mRNA expression. Moreover, addition of vitamin D to cell culture medium increased thrombomodulin mRNA expression *in vitro*. These results are suggestive of vitamin D as a regulator of thrombomodulin in placental cells. Perhaps increasing levels of thrombomodulin through increasing vitamin D serum levels could restore syncytiotrophoblast maintenance in OD pregnancies. Vitamin D serum levels can be increased via vitamin D supplementation.³⁸ Since thrombomodulin protein expression decreases in uncomplicated OD pregnancies, and thrombomodulin mRNA is not upregulated in this group, loss of thrombomodulin on the syncytiotrophoblast is not compensated by an increased production of thrombomodulin mRNA in uncomplicated OD pregnancies. This is suggestive for a role of a diminished vitamin D metabolism in the development of preeclampsia after OD. An interesting point for further research would be to investigate the effect of maternal vitamin D status on placental cytoprotection through thrombomodulin.

It is difficult to define a proper control group for OD pregnancies. Conception through assisted reproductive techniques on its own is associated with distinct maternal characteristics as older age and premature ovarian failure¹⁴ and an increased risk of pregnancy complications solely due to OD.¹⁵ In our cohort, uncomplicated OD pregnancies were significantly different from uncomplicated naturally conceived pregnancies with respect to maternal age, maternal BMI, gravidity, parity and the number of twin or triplet pregnancies. However, IVF-induced pregnancies were only significantly different from uncomplicated OD pregnancies regarding maternal age and could therefore serve as a proper control group. Additionally, placentas from older women with an uncomplicated naturally conceived pregnancy were included to investigate the effect of maternal age on thrombomodulin protein expression. Thrombomodulin expression was mainly diffuse in these samples. Furthermore, no correlation was found between maternal age and thrombomodulin mRNA in uncomplicated OD pregnancies. Therefore, maternal age can probably not elucidate thrombomodulin downregulation in uncomplicated OD pregnancies. Multiplets occur more often in pregnancies induced by OD and IVF induced pregnancies compared with naturally conceived pregnancies.^{39, 40} This might

theoretically influence thrombomodulin expression. To our knowledge, no current literature on the influence of multiplet pregnancies on placental thrombomodulin expression exists. In our study, thrombomodulin levels were similar in placentas from singleton and multiplet pregnancies; thrombomodulin mRNA appears not to be effected by twin pregnancy.

A limitation of this study is that only nine patients are included in the group of OD pregnancies complicated by preeclampsia. In the Netherlands anonymous and commercial oocyte donation is not allowed.⁴¹ Hence, the number of women who receive oocyte donation is relatively low in the Netherlands.

In summary, in this study we showed that placental thrombomodulin protein expression is lower after uncomplicated OD pregnancies and OD pregnancies complicated by preeclampsia, which might contribute to a higher susceptibility for the development of preeclampsia after OD. Thrombomodulin expression is associated with parameters of inflammation and coagulation. Furthermore, downregulation of the VDR in OD pregnancies complicated by preeclampsia might contribute to loss of thrombomodulin on the syncytiotrophoblast. More research is needed to understand thrombomodulin regulation and other pathways contributing to maintenance of endothelium in women with preeclampsia to find possible targets to treat endothelial dysfunction in women susceptible to preeclampsia. Although, a very specific patient group was studied, our results provide new insights into the pathogenesis of preeclampsia, but also set the stage for further research into the role of endothelium in the regulation of inflammation and coagulation.

Acknowledgments

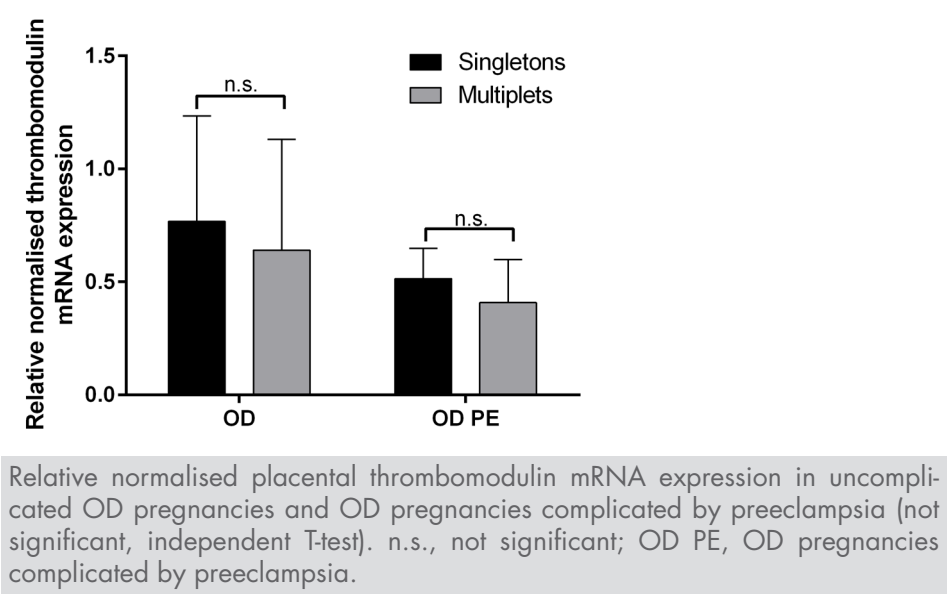
We thank G.M.J.S. Swings and C. van der Keur and F.H.J. Claas from the laboratory Reproductive Immunology of the Department of Immunohematology and Blood Transfusion, LUMC, Leiden for their help with collecting and storing data and patient materials.

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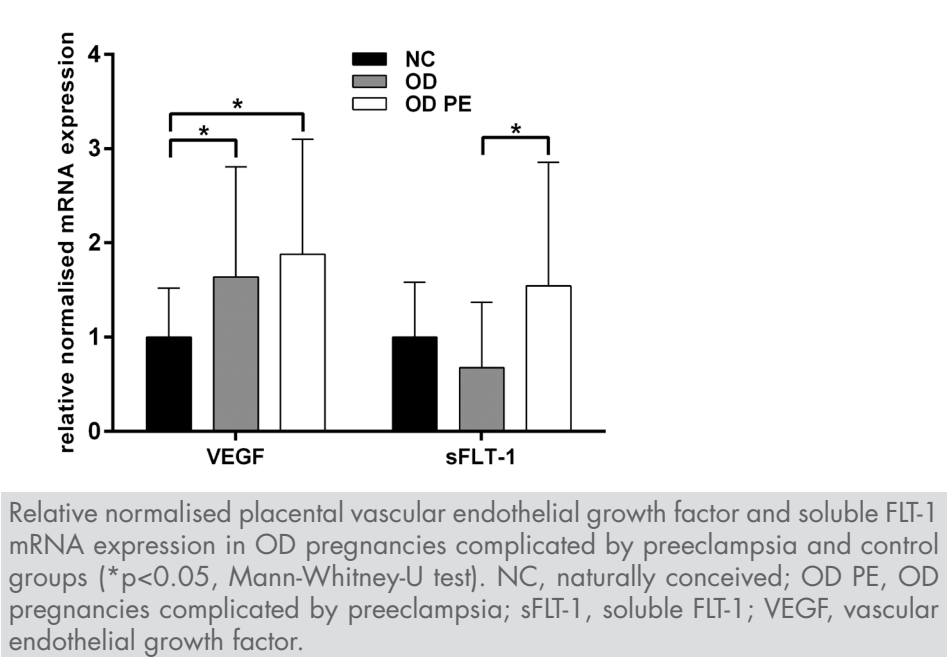
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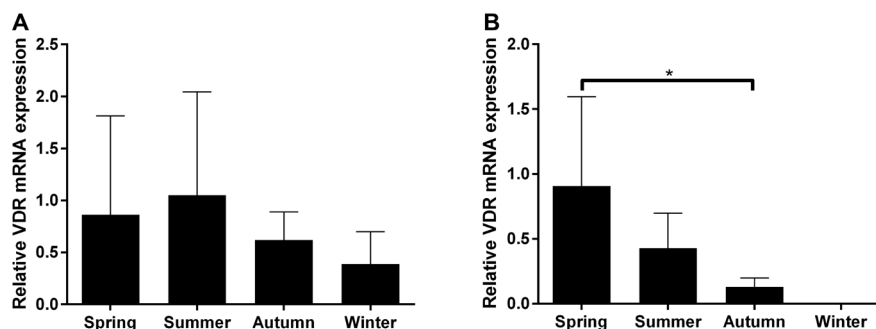
Supplementary Figure 1 Thrombomodulin mRNA expression in singletons and multiplets



Supplementary Figure 2 Regulation of thrombomodulin: Angiogenic factors



Supplementary Figure 3 Seasonality of placental VDR mRNA expression



A Relative placental VDR mRNA expression of children born in spring (21 March – 20 June), summer (21 June – 20 September), autumn (21 September – 20 December) or winter (21 December – 20 March) after an uncomplicated OD pregnancy. **B** Relative placental VDR mRNA expression of children born in spring, summer or autumn after OD pregnancies complicated by preeclampsia. Placental VDR mRNA expression is significantly lower in children born in autumn compared to children born in spring (* $p < 0.05$, Mann-Whitney-U test).

Gene	Primer sequence (F: Forward, R: Reverse)	
Factor VIII	F	GCTTCCCATCCTGTCACTCT
	R	CAGAGGCCATTGGACCACTCT
GAPDH	F	TTCCAGGAGCGAGATCCCT
	R	CACCCATGACGAACATGGG
HPRT	F	TGACACTGGCAAAACAATGCA
	R	GGTCCTTTTACCAGCAAGCT
ICAM-1	F	ACCATCTACAGCTTCCGGC
	R	TCAGCGTCACCTTGGCTCTA
sFLT-1	F	CATTGAGGCCGAGGGGGCTG
	R	TGCACCCCTGGGGCCCATTT
Tissue factor	F	GGGAACCCAAACCCGTCAAT
	R	GTCGGTGAGGTCACACTCTG
Thrombomodulin	F	ACATCCTGGACGACGGTTTC
	R	CGCAGATGCACTCGAAGGTA
TNF α	F	CCCGAGTGACAAGCCTGTAG
	R	TGAGGTACAGGCCCTCTGAT
VDR	F	GCCCAACTCCAGACACACTC
	R	GGGTCACAGAAGGGTCATCT
VEGF	F	TGTGCCCTGATGCGATGCG
	R	TCCTTCTCTGCCCCGGCTC

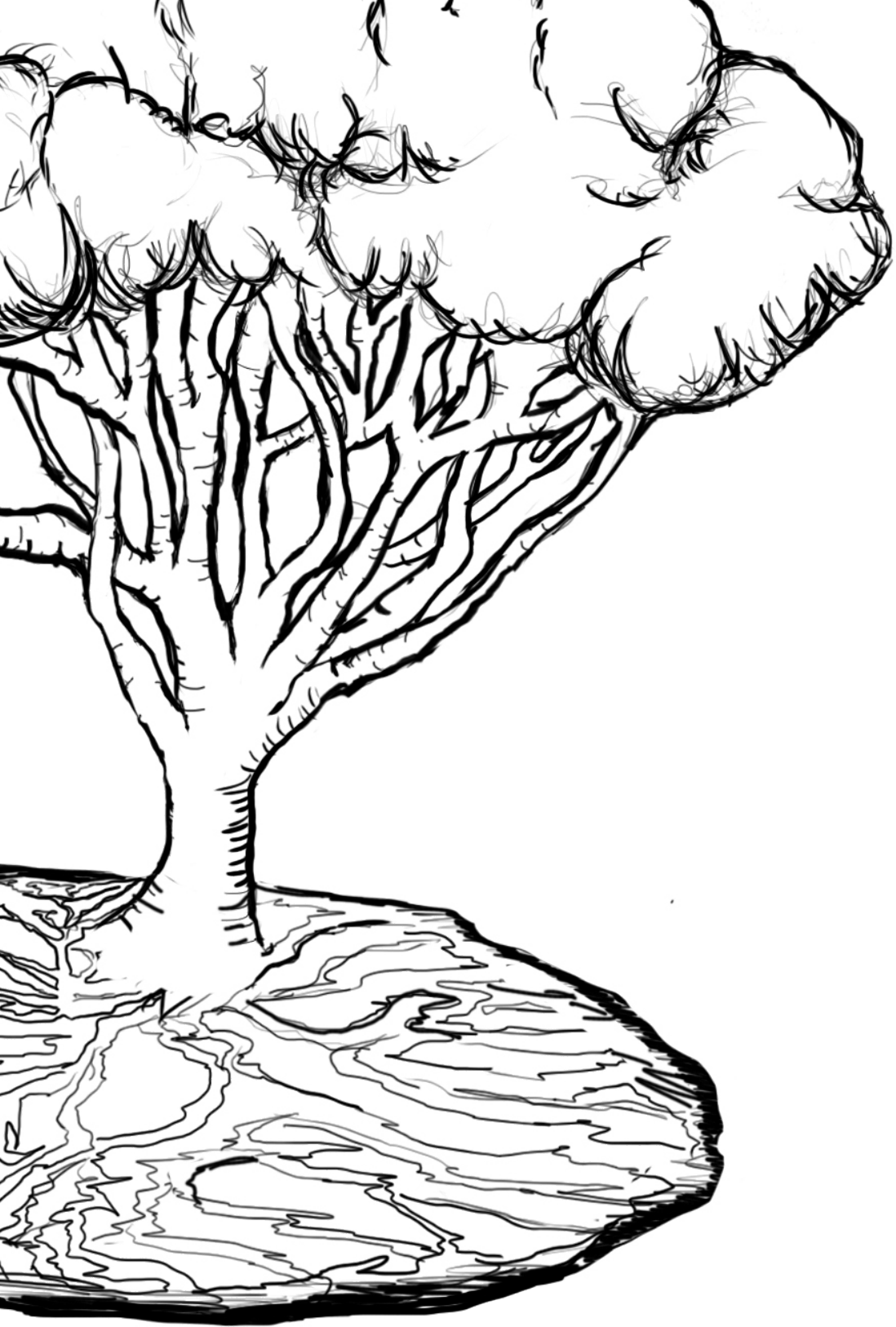
Supplementary Table 1 Primer sequences used for quantitative PCR

HPRT, hypoxanthine phosphoribosyltransferase; ICAM-1, intercellular adhesion molecule 1; sFLT-1, soluble Flt-1; TNF α , tumour necrosis factor alpha; VDR, vitamin D receptor; VEGF, vascular endothelial growth factor.

	NC N = 28	Older age N = 20
Maternal characteristics		
Mean maternal age, years (SD)	33.7 (4.0)	39.4 (1.5)*
Mean maternal BMI, kg/m ² (SD)	25.81 (4.9)	25.67 (5.6)
Smoking		
Yes (%)	1 (3.6)	2 (12.6)
No (%)	27 (96.4)	14 (87.6)
Mean gravidity (range)	2.9 (1-7)	3.6 (1-8)
Mean parity (range)	1.4 (0-5)	1.9 (0-5)
Highest diastole, mmHg (SD)	75 (70-80)	78 (70-85)
Proteinuria		
Yes (%)	0 (0)	0 (0)
No (%)	28 (100)	7 (100)
Prior history of hypertension or preeclampsia		
Yes (%)	0 (0)	0 (0)
No (%)	28 (100)	20 (100)
Pregnancy characteristics		
Gestational age at delivery, weeks + days (SD, days)	39+4 (9)	39+1 (9)
Term		
A term (%)	28 (100)	20 (100)
Pre term (%)	0 (0)	0 (0)
Mode of delivery		
Caesarean section (%)	16 (63.2)	12 (63.2)
Vaginal delivery (%)	12 (36.8)	12 (36.8)
Twin or triplet		
Yes (%)	0 (0)	0 (0)
No (%)	28 (100)	20 (100)
Foetal characteristics		
	N = 28	N = 20
Sex of Child		
Male (%)	12 (42.9)	13 (65)
Female (%)	16 (57.1)	7 (35.0)
Birthweight, g (SD)	3597 (435)	3563 (564)
Small for gestational age		
Yes (%)	2 (7.1)	1 (5)
No (%)	26 (92.9)	19 (95)
Placenta weight, g (SD)	638 (140)	611 (119)

Supplementary Table 2 Patient characteristics of older age group

*, p<0.05 compared to uncomplicated naturally conceived pregnancies.
NC, naturally conceived.



Thrombomodulin expression is increased in the kidney of women with pre-eclampsia

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Submitted for publication.

Abstract

Background

Pre-eclampsia is a pregnancy-related syndrome characterised by systemic angiogenic imbalance, which results in symptoms such as hypertension and proteinuria. Similar symptoms are observed in patients exposed to drugs that inhibit angiogenic signalling. Increased endothelin signalling is an important pathway for the development of hypertension and renal failure in PE. Thrombomodulin is essential for the maintenance of the glomerular filtration barrier and in women with PE soluble thrombomodulin levels are increased. Here, we investigated thrombomodulin expression in kidneys of women with PE and rats exposed to sunitinib and endothelin receptor (ETR) antagonists.

Methods

Renal tissue was collected from 34 pregnant women (11 pre-eclampsia, 23 controls) and 14 hypertensive non-pregnant women. Furthermore, kidneys were collected from male WKY rats treated with vehicle, sunitinib (7, 14 or 26.7 mg/kg/day) or sunitinib in combination with sitaxentan (ETR type A (ET_A) antagonist, 30 or 100 mg/kg/day) or macitentan (dual $ET_{A/B}$ antagonist, 30 mg/kg/day) for 8 days. Thrombomodulin expression was investigated with immunohistochemistry and qPCR.

Results

Glomerular thrombomodulin protein expression was increased in the kidneys of women with PE and rats exposed to sunitinib as compared to controls. Glomerular thrombomodulin expression was non-linearly associated with sunitinib dose. Sitaxentan, but not macitentan, normalised the sunitinib-induced increase in thrombomodulin mRNA expression and albuminuria. Both ETR antagonists normalized sunitinib-induced hypertension.

Conclusions

Renal thrombomodulin expression was increased in women with pre-eclampsia and in rats exposed to sunitinib. Upregulation of thrombomodulin is likely mediated via ET_A signalling and may represent a reno-protective mechanism in response to damage induced by diminished VEGF-signalling.

Introduction

Pre-eclampsia affects 3-5% of pregnant women and is an important cause of maternal and neonatal morbidity and mortality.¹ The pathophysiology of pre-eclampsia is not fully understood, but is characterised by placenta dysfunction and placental production of several anti-angiogenic and pro-inflammatory factors (e.g. soluble Flt-1, soluble endoglin and TNF α).^{2,4} These factors contribute to systemic endothelial dysfunction, an increase in vascular resistance and problems in multiple organs.^{2,5-9} The kidney is frequently affected in women with pre-eclampsia, with symptoms ranging from mild proteinuria to nephrotic-range proteinuria and kidney failure in later stages of the disease.¹⁰⁻¹² The pathological changes in the kidneys of women with pre-eclampsia are characterised by endotheliosis, podocyte foot process effacement and podocyte loss.^{13, 14} What drives these pathological changes is incompletely understood.

Renal dysfunction in pre-eclampsia is caused by disruption of signalling pathways involved in maintaining the glomerular filtration barrier.¹⁵ For instance, increased levels of circulating anti-angiogenic factors lead to decreased availability of vascular endothelial growth factor (VEGF) in the glomerulus.¹⁶ Several studies have shown that impaired glomerular VEGF signalling leads to disruption of the glomerular filtration barrier, resulting in proteinuria and renal lesions which are comparable to those observed in pre-eclampsia.^{2, 17-21} Moreover, treatment with angiogenesis inhibitors such as sunitinib and anti-VEGF antibodies produce a pre-eclampsia-like kidney phenotype.²²⁻²⁵ Together, these findings indicate that low levels of circulating VEGF result in an angiogenic imbalance that contributes to renal pathology in pre-eclampsia. Another important mediator of renal injury in pre-eclampsia is the endothelin system. Endothelin is a potent vasoconstrictor which is produced by endothelial cells. Activation of the endothelin system is implicated in the pathogenesis of pre-eclampsia and VEGF blockade-induced hypertension and renal injury.^{26, 27} Stimulation of endothelin type A receptor (ET_AR) on vascular smooth muscle cells results in vasoconstriction.²⁸ Furthermore, stimulation of the endothelin type B receptor (ET_BR) results in production of nitric oxide and prostaglandin which promotes vasodilatation.^{28, 29} In hypertension, endothelial ET_BR-induced vasodilatation can be lost.³⁰ In preclinical studies, ETR blockade, and in particular selective ET_AR antagonism, is effective in lowering blood pressure and proteinuria in rodent models of pre-eclampsia.^{27, 31-33} Similarly, we recently demonstrated that selective ET_AR antagonism is sufficient to prevent VEGF-inhibition-induced hypertension and albuminuria in rats.³⁴

Thrombomodulin is a transmembrane glycoprotein which is essential for the maintenance of the endothelium.³⁵ Thrombomodulin mediates coagulation, complement activation and cell survival in endothelial cells.^{35, 36} In women with pre-eclampsia, increased serum level of soluble thrombomodulin have been reported.³⁷ The increased serum level of soluble thrombomodulin reflects increased cleavage of thrombomodulin from the endothelium.³⁸ Thrombomodulin is essential in the maintenance of the glomerular filtration barrier; decreased thrombomodulin

signalling leads to disrupted crosstalk between glomerular endothelial cells and podocytes, resulting in aggravated proteinuria and histological lesions in diabetic mice.³⁹ Thus, thrombomodulin has important cytoprotective effects on the glomerular filtration barrier by regulating coagulation and complement activation, and may also be involved in kidney disease in pre-eclampsia. In this study, we investigated renal thrombomodulin expression in kidneys of women with pre-eclampsia and in a rat model of VEGF-inhibition with the receptor tyrosine kinase inhibitor sunitinib. Furthermore, we investigated the role of the endothelin system on renal thrombomodulin expression in this rat model by using a selective ET_AR antagonist and a dual ET_{A/B}R antagonist.

Materials and methods

Patient Materials

A nationwide search of the Dutch Pathology Registry (PALGA) was conducted to collect renal tissue from women who died from the consequences of pre-eclampsia and control subjects. PALGA is a registration network of all pathology laboratories in The Netherlands. The pathology data were linked with the records of the National Maternal Mortality Committee of the Dutch Society of Obstetrics. Pre-eclampsia was defined based on the diagnostic criteria of the International Society for the Study of Hypertension in Pregnancy (ISSHP).⁴⁰ Two control groups were included; the first group consisted of pregnant women without a hypertensive disorder prior to or during pregnancy, who died from a cause unrelated to hypertension, and the second group consisted of non-pregnant women with a history of chronic hypertension. Paraffin-embedded kidney samples from 11 women with pre-eclampsia, 22 normotensive pregnant controls and 11 non-pregnant hypertensive controls were available for this study. The patient characteristics of these cases have been described before (see: ⁴¹). This study was approved by the Medical Ethics Committee of the Leiden University Medical Center (P12.107).

Sunitinib exposed rats

Experiments were performed in accordance with the guidelines from Directive 2010/63/EU of the European Parliament and the Netherlands Experiments on Animals Act, after obtaining approval from the Erasmus Medical Center Animal Ethics Committee. Male Wistar Kyoto rats (WKY, 280-300 gram) were obtained at 10 weeks of age. The animals were housed in an experimental room with temperature maintained at 21-22 °C and a 12-hour light/dark cycle. Animals had access to standard laboratory rat chow and water ad libitum. Two different experiments were used in this study, with detailed methods and the phenotype of these animals published previously.^{34, 42} In brief, rats were treated with different doses of sunitinib with or without ET R antagonists for 8 days. Aortic blood pressure was measured using radiotelemetry. Before and after administration of the treatment(s), rats were housed in metabolic cages for 48 hours. The first day was used for acclimatization and the second day for the collection of 24-hour

urine samples. In the first study, rats were randomly assigned to receive a low, intermediate or high dose of sunitinib (7, 14 or 26.7 mg/kg/day p.o. sunitinib-L-malate (Sutent, Pfizer), respectively) or vehicle for 8 days. At the end of the experiment, rats were euthanised with 60mg/kg pentobarbital intraperitoneal and exsanguination via abdominal vein puncture.⁴² In the second study, rats were administered vehicle or the intermediate dose of sunitinib (14 mg/kg/day p.o.) alone or in combination with macitentan (dual $ET_{A/B}$ antagonist, 100 mg/kg/day p.o.) or sitaxentan (selective ET_A antagonist, 30 or 100 mg/kg/day p.o.). At the end of the experiment, rats were euthanised by via Forane (isoflurane) anaesthesia overdose and exsanguination via abdominal vein puncture.³⁴ In both studies, following euthanasia, the kidneys were rapidly excised for subsequent analyses.

Renal histology

Kidney sections were blindly evaluated by a pathologist for the presence or absence of endothelial cell swelling, epithelial cell swelling, ischemia and intra-epithelial protein. For electron microscopy two glomeruli were examined, the presence of glomerular endotheliosis and podocyte morphology was studied. Histopathological findings in human subjects and animals exposed to different concentrations of sunitinib have been published before.^{41, 42}

Immunohistochemistry

For immunohistochemistry, sections were deparaffinised. Antigen retrieval was performed with citrate (for the mouse-anti human thrombomodulin antibody) or with Tris/EDTA (for the rabbit-anti mouse/rat thrombomodulin antibody) treatment. Peroxidase was blocked by incubating the sections in a hydrogen peroxide solution for 20 minutes. Kidney samples from humans were incubated with a mouse monoclonal thrombomodulin antibody (1:200, Leica Biosystems, Danvers) for one hour, and kidney samples from rats were incubated with a rabbit monoclonal thrombomodulin antibody (1:2000, Abcam) for one hour at room temperature. Binding of the primary antibody was visualised with labelled anti-mouse or labelled anti-rabbit polymer (DAKO, Belgium) and diaminobenzidine as a chromogen. As positive and negative controls, human placenta and kidney tissue of an experimental chronic serum sickness rat model were used.

Quantification of immunohistochemistry

Thrombomodulin protein expression was scored in 25 glomeruli per sample by two independent observers blinded with respect to clinical diagnosis or treatment (MB and CCLA). Individual glomeruli were scored semi-quantitatively in five different scores; 0; thrombomodulin absent in the glomerulus, 1; <10% of glomerulus thrombomodulin positive, 2; 10-50% of glomerulus thrombomodulin positive, 3; 50-90% of glomerulus thrombomodulin positive or 4; >90% of the glomerulus thrombomodulin positive. Representative examples of the different scores are shown in Supplemental Figures 1 and 2. When observers scored selected glomeruli differently, consensus was obtained during a consensus meeting. The average score of 25 glomeruli resulted in a semi-quantitative thrombomodulin protein expression score per sample. Interobserver agreement was substantial in human specimens

($\kappa=0.647$) and sufficient in rats ($\kappa=0.446$).

qPCR

Quantitative PCR was performed to quantify mRNA expression of thrombomodulin and ETAR. RNA was isolated with TRIzol® (Lifetechnologies, San Francisco, CA, USA). Synthesis of cDNA was performed with AMV reverse transcriptase (Roche, Basel, Switzerland), and SYBR Green quantitative PCR was performed according to the manufacturer's protocol (Bio-Rad Laboratories Inc, Hercules, CA, USA). Primer sequences are described in Supplementary Table 1. Expression was measured by the comparative threshold cycle method and normalised to hypoxanthine phosphoribosyltransferase expression. A melting curve analysis was performed to verify the specificity of amplification.

Statistical analyses

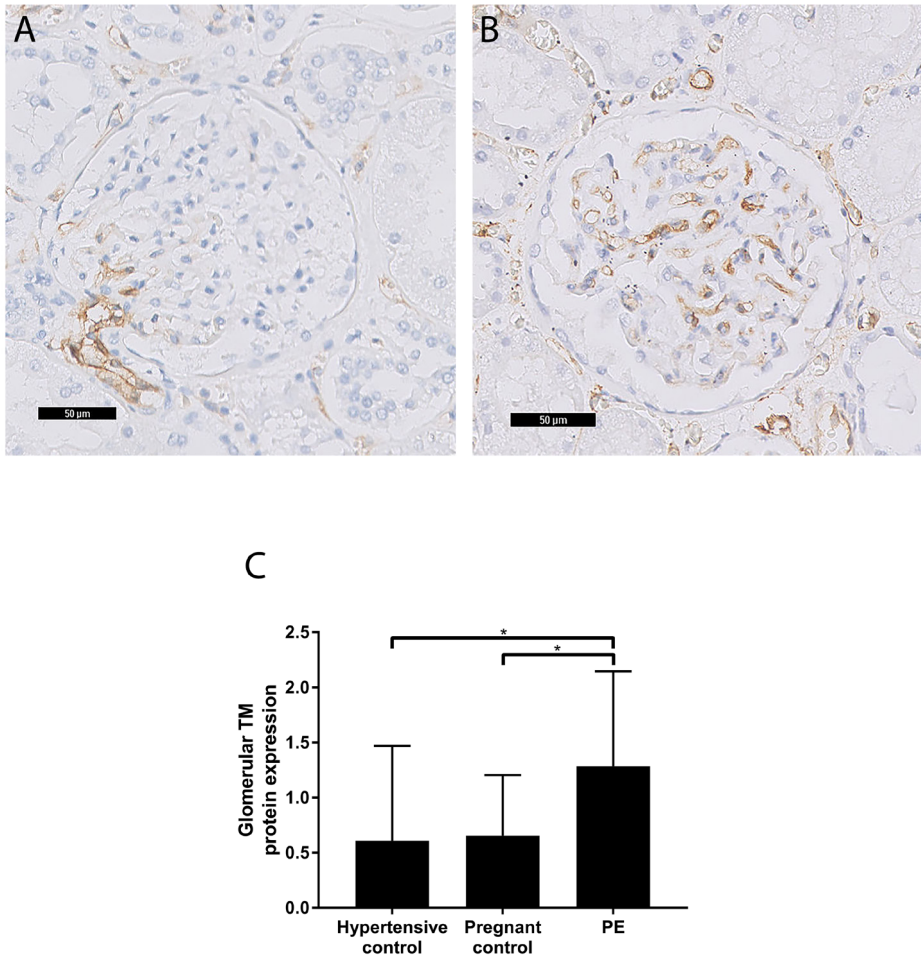
Interobserver variation was determined using the kappa statistic. Normally distributed continuous data were analysed using ANOVAs followed by least significant difference post-hoc test for the planned pairwise group comparisons. For correlations Pearson's correlation was used. The shape of the thrombomodulin response curve over the sunitinib dose range was also examined by means of regression lines. $P \leq 0.05$ was considered statistically significant. All analyses were performed using the SPSS statistics software (version 23.0, Armonk, NY: IBM Corp).

Results

Glomerular thrombomodulin expression is increased in the kidney of women with pre-eclampsia

Thrombomodulin expression was studied in kidney samples from 11 women with pre-eclampsia, 22 normotensive pregnant controls and 11 non-pregnant hypertensive controls. Non-pregnant hypertensive controls were older than women with pre-eclampsia and normotensive pregnant controls, and blood pressure was higher in women with pre-eclampsia as compared to normotensive pregnant controls.⁴¹ Glomerular thrombomodulin expression seemed to originate from the glomerular vascular pole (Figure 1 A). Regardless of the presence of thrombomodulin in the glomerulus, thrombomodulin was present in the peritubular capillaries in all samples (Figure 1 A and B). Glomerular thrombomodulin protein expression was higher in women with pre-eclampsia compared to non-pregnant hypertensive controls and pregnant normotensive controls (Figure 1 C, $p < 0.05$). Furthermore, glomerular thrombomodulin protein expression was negatively associated with the size of the glomerular tuft in hypertensive controls ($r = -0.789$, $p < 0.01$).

Figure 1 Glomerular thrombomodulin expression is increased in the kidneys of women with pre-eclampsia



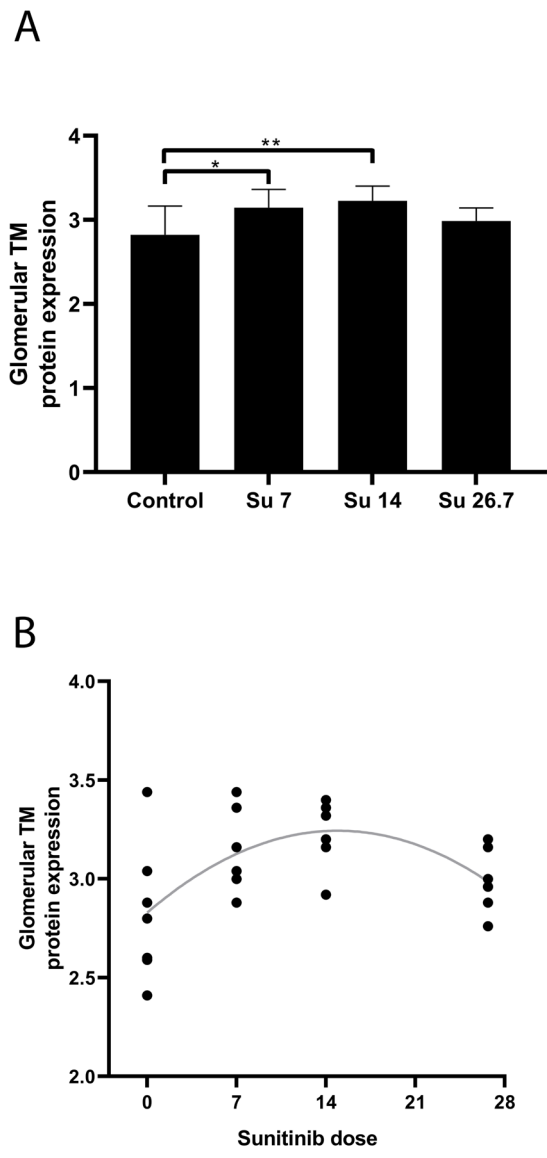
A Representative example of a glomerulus in which 10-50% of the glomerulus was positive for thrombomodulin in a pregnant control. **B** Representative example of a glomerulus in which more than 90% of the glomerulus was positive for thrombomodulin in a pre-eclampsia case. **C** Glomerular thrombomodulin protein expression was higher in women with pre-eclampsia compared to non-pregnant hypertensive controls, and pregnant normotensive controls.

* $p < 0.05$. PE; pre-eclampsia, TM; thrombomodulin.

Glomerular thrombomodulin expression in rats exposed to sunitinib

To better understand our findings in human specimens, glomerular thrombomodulin expression was studied in rats exposed to different doses of sunitinib. Administration of sunitinib resulted in an increase in blood pressure and proteinuria in a dose-dependent manner.⁴² Endothelial cell swelling was observed in the kidneys of rats exposed to the intermediate and high doses of sunitinib. Fibrin deposits were only seen in the glomerular capillaries of animals exposed to a high dose of sunitinib.⁴² None of the scored glomeruli in rats was negative for thrombomodulin. Glomerular thrombomodulin protein expression was increased in the kidneys of rats exposed to the low and intermediate doses of sunitinib ($P < 0.05$ as compared to control animals, **Figure 2A**), but not in rats exposed to the highest dose of sunitinib. In support of these findings, a regression model showed that the sunitinib dose is non-linearly related to the glomerular thrombomodulin protein expression as a statistically significant curvilinear (quadratic) relation (**Figure 2 B**, $Y = 2.829 + 0.056 * X - 0.002 * X^2$, $p < 0.009$).

Figure 2 Thrombomodulin expression is increased in rats exposed to a low and intermediate dose of sunitinib



A Glomerular thrombomodulin protein expression was increased in the kidneys of rats exposed to a low and intermediate dose of sunitinib compared to controls, but not in kidneys of rats exposed to a high dose of sunitinib. **B** Glomerular thrombomodulin protein expression was associated with sunitinib dose.

* $p < 0.05$, ** $p < 0.01$. Su; sunitinib, TM; thrombomodulin.

Treatment with sitaxentan normalises thrombomodulin expression.

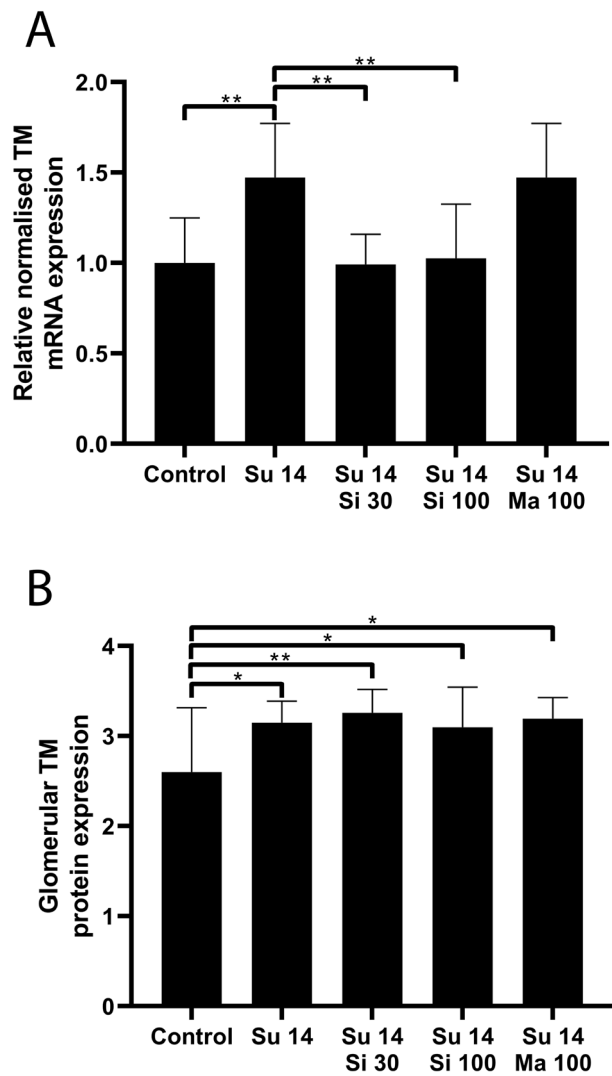
To study whether thrombomodulin expression is regulated by endothelin signalling, rats exposed to an intermediate dose of sunitinib were treated with ETR blocking agents. Treatment with a selective ET_A R antagonist (sitaxentan) normalised blood pressure and albuminuria in sunitinib-treated rats.³⁴ Conversely, treatment with a dual $ET_{A/B}$ R antagonist (macitentan) resulted in normalisation of blood pressure but not in the normalisation of sunitinib-induced albuminuria.³⁴ Thrombomodulin mRNA expression was increased in the kidneys of rats exposed to sunitinib compared to controls (Figure 3A, $P < 0.01$). Administration of sitaxentan, at both concentrations, on top of sunitinib normalised thrombomodulin mRNA expression to control levels ($p < 0.01$), but macitentan did not affect thrombomodulin mRNA levels. Relative thrombomodulin mRNA expression was positively associated with relative ET_A R mRNA expression in the control group ($r = 0.936$, $p < 0.01$). Albuminuria was positively associated with renal thrombomodulin mRNA expression in animals exposed to sunitinib alone ($r = 0.876$, $p < 0.01$). Overall, renal thrombomodulin mRNA expression was positively associated with the change in mean arterial pressure ($r = 0.511$, $p < 0.001$). At the protein level, sunitinib exposure resulted in higher glomerular thrombomodulin expression as compared to controls (Figure 3B, $p < 0.05$). Neither co-administration of sunitinib with sitaxentan (30mg/kg/day) or macitentan resulted in the normalisation of the sunitinib-induced increase in glomerular thrombomodulin protein expression (Figure 3B). Glomerular thrombomodulin protein expression was not associated with albuminuria or the change in mean arterial pressure.

Discussion

In this study, we investigated the expression of thrombomodulin in the kidney of women with pre-eclampsia and rats exposed to the receptor tyrosine kinase blocker sunitinib, which induced a pre-eclampsia-like phenotype. We found that glomerular thrombomodulin protein expression was increased in the kidney of women with pre-eclampsia and in rats exposed to a low and intermediate dose of sunitinib. Furthermore, sunitinib increased glomerular thrombomodulin expression in rats in a non-linear dose-dependent manner, which was normalised by the ET_A R antagonist sitaxentan.

In our study, anti-angiogenic conditions resulted in marked upregulation of renal thrombomodulin expression. In pre-eclampsia and in sunitinib-treated rats, both of which are characterised by impaired VEGF signalling, we found an increased glomerular thrombomodulin protein. Furthermore, in rats exposed to an intermediate dose of sunitinib, we found an upregulation of renal thrombomodulin mRNA expression, indicating that systemic VEGF inhibition increases renal transcription levels of thrombomodulin. In line with our findings, several studies have reported elevated serum levels of soluble thrombomodulin in women with pre-eclampsia,³⁷ which reflect increased production and/or cleavage of thrombomodulin from the

Figure 3 Renal thrombomodulin expression in rats exposed to sunitinib with or without sitaxentan or macitentan



A Exposure to sunitinib results in a higher renal thrombomodulin mRNA expression, and sitaxentan (ET_AR antagonist) at both dosages normalised this increase. **B** Sunitinib exposure resulted in a higher glomerular thrombomodulin protein expression compared to controls. Administration of sitaxentan (ET_AR antagonist) nor macitentan (dual ET_{A/B}R antagonist) resulted in a normalisation of the increased glomerular thrombomodulin protein expression.

*p<0.05, **p<0.01

vascular endothelium.³⁸ As we previously showed that placental thrombomodulin is downregulated both at the mRNA and protein level in pre-eclampsia,⁴³ the renal endothelium may be the source of increased soluble thrombomodulin in the circulation. Increased renal thrombomodulin expression under anti-angiogenic conditions such as pre-eclampsia or exposure to an angiogenesis inhibitor seems counterintuitive as *in vitro* experiments have identified VEGF as a strong up-regulator of thrombomodulin in endothelial cells.⁴⁴ Thrombomodulin expression can be also regulated via thrombin levels, shear stress and TNF α signalling.^{35, 45, 46} The precise regulation of thrombomodulin expression in glomerular endothelial cells remains to be elucidated.

Sunitinib dose was non-linearly related to the glomerular thrombomodulin protein expression as a statistically significant curvilinear (quadratic) relation. Therewith, increased thrombomodulin expression was present before the onset of renal histopathologic damage in our anti-angiogenic rat model. Pathologic changes were not seen in the kidneys of rats exposed to low-dose sunitinib,⁴² whereas glomerular thrombomodulin protein expression was increased in these rats. This finding suggests that the early increase in thrombomodulin expression serves as a mechanism to protect the glomerular filtration barrier. Further, the renal phenotype in our human samples was relatively mild compared to the renal phenotype observed in rats exposed to sunitinib. Endotheliosis was observed in 6 women with pre-eclampsia and microthrombi was seen in just one case,⁴¹ whereas endotheliosis was observed in the kidneys of rats exposed to an intermediate dose of sunitinib and endotheliosis in combination with fibrin deposits was seen in the glomeruli of rats exposed to a high dose of sunitinib.⁴² This may explain why glomerular thrombomodulin expression was increased in our human pre-eclampsia samples. Renal thrombomodulin mRNA expression was positively associated with albuminuria in the rats in our study. From a study in diabetic mice, it is known that thrombomodulin is essential in maintenance of the glomerular filtration barrier as diminished thrombomodulin expression is directly involved in the development of glomerular damage and proteinuria.⁴⁷ Hence, we speculate that renal thrombomodulin protein levels increase at an early stage of glomerular damage as a protective mechanism. When the glomerular endothelial cells can no longer be maintained, the cytoprotective thrombomodulin may be lost from glomerular endothelial cells by cleavage, thereby contributing to renal histopathological damage. Recent studies have provided evidence for a proinflammatory role of thrombomodulin. Thrombomodulin can bind to the leukocyte integrins LFA-1 and Mac1, thereby promoting leukocyte adhesion to the endothelium *in vitro*.⁴⁸ The marked upregulation of glomerular thrombomodulin in pre-eclampsia may therefore also directly contribute to increased glomerular inflammation and damage. However, no signs of increased inflammation were observed in the kidney of women with pre-eclampsia.⁴¹ In a model of anti-glomerular basement membrane glomerulonephritis, an anti-angiogenic milieu induced by sFlt-1 accelerated the progression of glomerulosclerosis and renal dysfunction.⁴⁹ In these rats, glomerular thrombomodulin protein expression was decreased.⁴⁹ More research is warranted to delineate the function of thrombomodulin in the glomerular filtration barrier.

In the present study, we were also able to look into the interaction between the endothelin system and glomerular thrombomodulin expression under anti-angiogenic conditions. Increased endothelin signalling is an important pathway for the development of hypertension and renal failure in pre-eclampsia.^{27, 50-52} Multiple studies have found elevated plasma endothelin levels in women with pre-eclampsia,⁵³ and this is associated with systemic sFlt-1 levels.^{54, 55} Furthermore, treatment with ET_AR antagonists is effective in lowering blood pressure in pre-eclampsia-like animal models.^{32, 33} Treatment with sitaxentan (ET_AR antagonist) and macitentan (dual ET_{A/B}R antagonist) resulted in normalisation of sunitinib induced hypertension in our rats.³⁴ Interestingly, only treatment with sitaxentan (ET_AR antagonist) decreased sunitinib-induced albuminuria³⁴ and normalised renal thrombomodulin mRNA expression. This suggests that thrombomodulin expression in the renal endothelium is ET_AR-mediated and independent of hypertension-mediated endothelial damage. This notion is also supported by the finding that there was no evidence for increased thrombomodulin expression in our hypertensive control patients. Interestingly, thrombomodulin expression was only normalised at the mRNA level after ET_AR blockade in sunitinib-treated rats, but not at the protein level. This discrepancy may be explained by post-translational effects, half-life of the protein and factors that influence the cleavage of thrombomodulin from the endothelium.

We must acknowledge the limitations of our study. Firstly, the women with pre-eclampsia in whom we investigated glomerular thrombomodulin expression all died because of the consequences of pre-eclampsia. This makes these women a specific subgroup of pre-eclampsia patients and therefore the results of this study may not be applicable to all women who develop pre-eclampsia. Secondly, sunitinib leads to inhibition of diverse receptor tyrosine kinases and is not a specific inhibitor of VEGF. However, exposure to sunitinib does result in a pre-eclampsia-like phenotype in our rat model, namely the combination of hypertension and proteinuria.⁴²

In conclusion, renal thrombomodulin expression was increased both in pre-eclampsia and in a sunitinib-induced model of angiogenic inhibition. Upregulation of glomerular thrombomodulin levels may therefore be a reno-protective mechanism in response to diminished VEGF signalling. Furthermore, treatment with an ET_AR antagonist normalised renal thrombomodulin mRNA expression concomitantly with normalising blood pressure and proteinuria in sunitinib-treated rats. Thrombomodulin upregulation appears to be ET_AR-mediated and ET_BR-signalling counterbalances this effect in a blood pressure-independent manner. More research is warranted on the role of thrombomodulin in the glomerular filtration barrier.

Sources of funding

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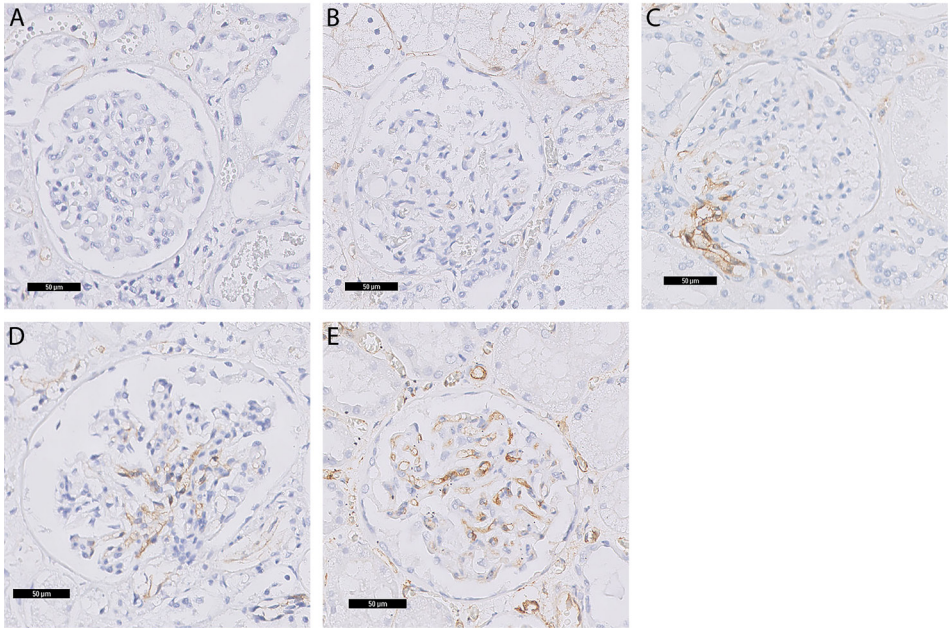
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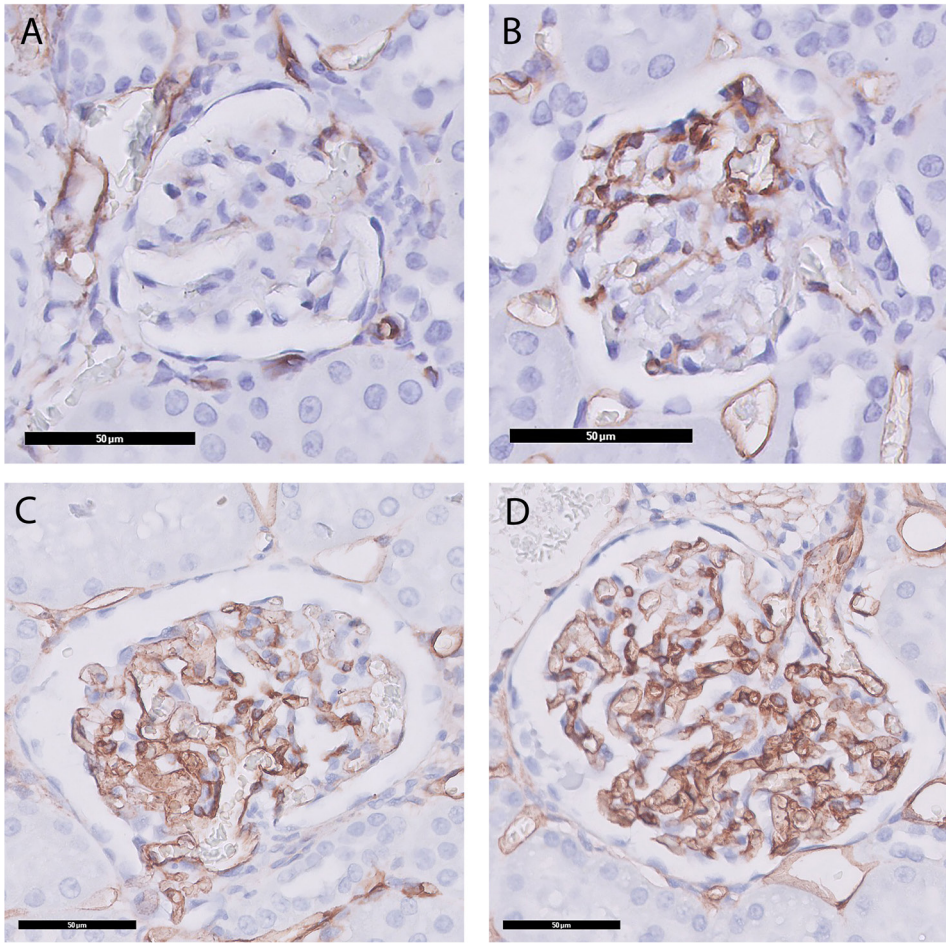
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Supplementary Figure 1 Glomerular thrombomodulin expression in humans

A Representative example of a glomerulus in which thrombomodulin was absent. In the peritubular capillaries, thrombomodulin was present. **B** Representative example of a glomerulus in which less than 10% of the glomerular capillaries were positive for thrombomodulin. **C** Representative example of a glomerulus in which 10-50% of the glomerulus was positive for thrombomodulin. **D** Representative example of a glomerulus in which 50-90% of the glomerulus was positive for thrombomodulin. **E** Representative example of a glomerulus in which more than 90% of the glomerulus was positive for thrombomodulin.

Supplementary Figure 2 Glomerular thrombomodulin expression in rats

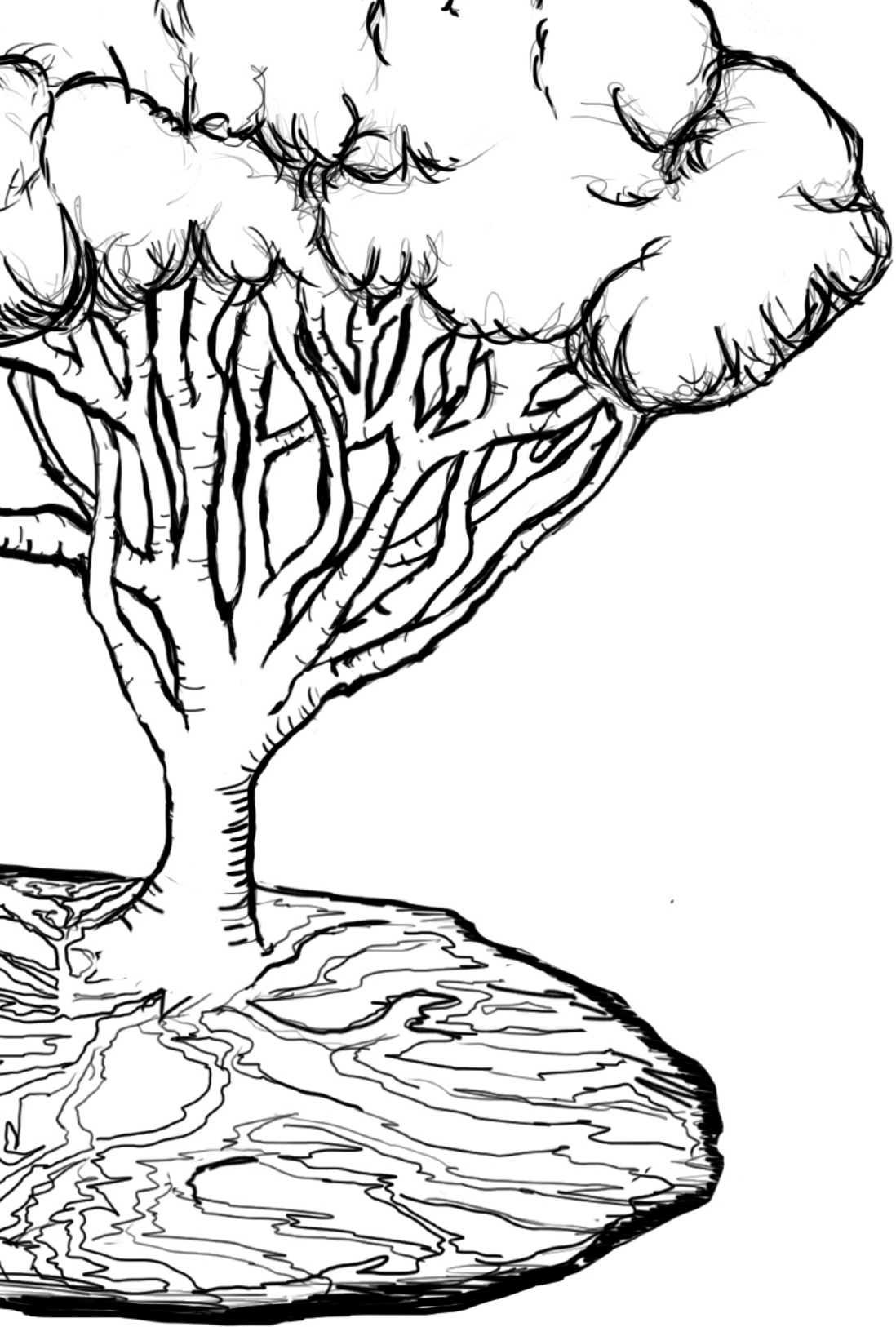


A Representative example of a glomerulus in which less than 10% of the glomerular capillaries were positive for thrombomodulin. **B** Representative example of a glomerulus in which 10-50% of the glomerulus was positive for thrombomodulin. **C** Representative example of a glomerulus in which 50-90% of the glomerulus was positive for thrombomodulin. **D** Representative example of a glomerulus in which more than 90% of the glomerulus was positive for thrombomodulin.

Gene	Primer sequence (F: Forward, R: Reverse)	
HPRT	F	GGC TAT AAG TTC TTT GCT GAC CTG
	R	AAC TTT TAT GTC CCC CGT TGA
ETAR	F	AGG GGA TCC CGA TTC CTT GA
	R	GTG GGC ATC ACT GTC CTG AA
Thrombomodulin	F	CCT TTG TCT TTC CGG GCT CT
	R	TCA AGT CCT CCC TAC CCT CG

HPRT, hypoxanthine phosphoribosyltransferase.

Supplementary table 1 Primer sequences used for quantitative PCR



Reactive species interactome alterations in oocyte donation pregnancies in the absence and presence of pre-eclampsia

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Abstract

In pregnancy, maternal physiology is subject to considerable adaptations, including alterations in cardiovascular and metabolic function as well as development of immunological tolerance towards the fetus. In an oocyte donation pregnancy, the fetus is fully allogeneic towards the mother, since it carries both oocyte donor antigens and paternal antigens. Therefore, oocyte donation pregnancies result in an immunologically challenging pregnancy, which is reflected by a higher-than-normal risk to develop pre-eclampsia. Based on the allogeneic conditions in oocyte donation pregnancies, we hypothesized that this situation may translate into alterations in concentration of stable readouts of constituents of the reactive species interactome (RSI) compared to normal pregnancies, especially serum free thiols, nitric oxide (NO) and hydrogen sulfide (H₂S) related metabolites. Indeed, total free thiol levels and nitrite (NO₂⁻) concentrations were significantly lower whereas protein-bound NO and sulfate (SO₄²⁻) concentrations were significantly higher in both oocyte donation and naturally conceived pregnancies complicated by pre-eclampsia. The increased concentrations of nitrite observed in uncomplicated oocyte donation pregnancies suggest that endothelial NO production is compensatorily enhanced to lower vascular tone. More research is warranted on the role of the RSI and bioenergetic status in uncomplicated oocyte donation pregnancies and oocyte donation pregnancies complicated by pre-eclampsia.

Introduction

In pregnancy, maternal physiology is subject to considerable adaptation including alterations in cardiovascular and metabolic function as well as development of immunological tolerance towards the fetus.¹⁻⁴ In a naturally conceived pregnancy, the fetus can be considered semi-allogeneic since it carries both paternal and maternal antigens.⁵ Although the semi-allogeneic conceptus is recognized by the maternal immune system, as reflected by the presence of allo-antibodies directed against paternal antigens in the maternal circulation, the conceptus is tolerated rather than rejected.^{6,9} Furthermore, fetal cells are in direct contact with maternal cells at the fetal maternal interface.^{7, 10, 11}

Oocyte donation is an artificial reproductive technique that enables women without ovarian activity or diminished ovarian reserve to conceive.¹² Furthermore, women with a serious genetic disease can choose to use oocytes of a healthy donor. In an oocyte donation pregnancy, the fetus is fully allogeneic towards the mother, since it carries both oocyte donor antigens and paternal antigens; this antigenic dissimilarity in unmatched oocyte donation pregnancies is comparable to the antigenic dissimilarity in unmatched organ transplantation.⁵ Due to the enhanced allogeneic nature of oocyte donation, these pregnancies presumably need more or different maternal immune adaptations, compared to naturally conceived pregnancies.^{5, 13-15} The increased adaptive load on the immune system between naturally conceived and oocyte donation pregnancies may explain why the latter are more often accompanied by immune-disturbance related obstetrical complications, such as pregnancy induced hypertension and pre-eclampsia.^{13, 15, 16} Oocyte donation pregnancies more often coincide with other risk factors for pregnancy complications, such as the self-evident need for artificial reproductive techniques, advanced maternal age, primiparity, cause of infertility, and multiple gestations.¹⁷⁻²¹ However, several studies have shown that the allogeneic nature of oocyte donation itself is an independent risk factor for pre-eclampsia.^{16, 22-26}

Pre-eclampsia is a syndrome of pregnancy that is characterised by hypertension and problems in multiple organ systems.^{27, 28} It is caused by a hypoxic placenta which results in an aberrant placental production of pro-inflammatory cytokines and anti-angiogenic factors. This translates into an enhanced systemic inflammatory status, systemic angiogenic imbalance and subsequently, in generalised endothelial dysfunction eventually resulting in the manifestation of clinical symptoms of pre-eclampsia.^{17, 28} Since hypoxia, inflammation and vascular stress are accompanied by an aberrant production of reactive oxygen species (ROS), as is the case in many other disease processes,²⁹ an enhanced presence of ROS may play a central role in pre-eclampsia. The placental tissue itself is an important site of production of ROS and other reactive species; indeed, the pre-eclamptic placenta is characterised by an increased production of reactive species.³⁰⁻³² This aberrant placental production of ROS and reactive nitrogen species (RNS) may conceivably contribute to systemic endothelial dysfunction in pre-eclampsia³³⁻³⁶ and is likely to be accompanied by corresponding changes in the production of reactive sulfur species (RSS).

The gasotransmitters nitric oxide (NO), carbon monoxide (CO) and hydrogen sulfide (H₂S) are important for vascular adaptations in pregnancy.³⁷ Gasotransmitters are known to regulate angiogenesis and vascular tone and their production is linked to the regulation of antioxidant status; the abnormal production of these small molecules is associated with pre-eclampsia.^{34, 38-40} Many of these reactive small molecules (some of which belong to the group of gasotransmitters) are known to chemically interact with each other.⁴¹ Furthermore, ROS, RNS and RSS also react with other biological targets such as thiol (SH) groups of enzymes, transcription factors and ion channels, which enables sensing and adaptation processes of cells and tissues to changes in environmental conditions and/or metabolic demand. This interaction of reactive species with other small molecules and biological targets has been defined as the “reactive species interactome” (RSI).⁴¹

In allograft organ transplantation, systemic redox status predicts graft survival and mortality,⁴²⁻⁴⁴ and reduced oxidative damage is associated with a better kidney transplant outcome.⁴⁵ Based on the allogeneic conditions in oocyte donation pregnancies, we hypothesized that the RSI status is affected in oocyte donation pregnancies. Therefore, we evaluated the RSI status, measured by quantifying circulating total free thiols, total 8-iso-prostaglandin F_{2α}, and metabolites of the NO and H₂S pathway in naturally conceived and oocyte donation pregnancies, in the absence and presence of concomitant pre-eclampsia.

Materials and Methods

Study participants and biospecimen collection

A retrospective case-control study was performed in women pregnant after oocyte donation or natural conception, and delivered in the Leiden University Medical Center (LUMC) and peripheral hospitals in the Leiden region between 2012 and 2016. Maternal age, gestational age, highest diastolic blood pressure, and presence of pre-eclampsia were documented. Different groups were clinically matched for BMI, smoking habits, gravidity, parity, and mode of delivery. This study was carried out following the rules of the Declaration of Helsinki and collection of samples was approved by the ethics committee of the Leiden University Medical Center (P13.084, 17 June 2013). All subjects gave their informed consent for inclusion before they participated in the study.

Clinical definitions

Miscarriage was defined as the spontaneous loss of pregnancy within the first 24 weeks of gestation.^{46, 47} Termination of pregnancy was defined as a termination on a medical fetal or maternal indication within the first 24 weeks of pregnancy resulting in fetal demise (Dutch guideline on termination of pregnancy until 24 weeks of gestational age;⁴⁸). Abortion was when pregnancy loss was induced within the first 24 weeks of gestation for social reasons.⁴⁹ IUFD was defined as fetal loss after 24 weeks of gestation. Gestational hypertension was defined as de novo hypertension (systolic blood pressure ≥ 140 mmHg and/or diastolic blood

pressure ≥ 90 mmHg) after gestational week 20. Pre-eclampsia was defined as de novo hypertension after gestational week 20 and new onset of proteinuria (≥ 300 mg/24h or a spot urine protein creatinine ratio ≥ 30 mg/mmol), renal insufficiency, liver disease, neurological problems, haematological disturbances or fetal growth restriction.⁵⁰ A term pregnancy was defined as childbirth after 37 weeks of pregnancy, and preterm birth was defined as birth between 24-37 weeks of gestation. Small for gestational age was defined as a birth weight below the 10th percentile for gestational age according to the Dutch reference curves for birth weight by gestational age.⁵¹ Gestational diabetes is defined as onset or first recognition of abnormal glucose tolerance during pregnancy.

Measurements

Serum samples were collected in the third trimester and stored at -80°C until measurements. The concentrations of the following analytes of the RSI were measured in serum: Total free thiols, protein bound NO (RxNO), nitrite (NO_2^-), nitrate (NO_3^-), 8-iso-prostaglandin $\text{F}_{2\alpha}$ (isoprostanes), sulfate (SO_4^{2-}) and thiosulfate ($\text{S}_2\text{O}_3^{2-}$).

Colorimetric detection of total free thiol groups

Free thiols were detected using Ellman's reagent as described previously.^{52,53} Briefly, serum samples were diluted 1:3 with Tris buffer (0.1M, pH 8.2), and background absorbance was read at 412 nm using a microplate reader with reference at 630 nm. Then, 10 μL 5,5'-Dithio-bis(2-nitrobenzoic acid) (DTNB; 3.8mM, Ellman's Reagent) diluted in phosphate buffer (0.1M, pH 7) was added. The samples were incubated with DTNB for 20 min at room temperature, sample absorbance was measured again at 412 nm with 630 nm reference and compared to a calibration curve constructed for L-cysteine under identical conditions.

Assessment of nitroso species via gas phase chemiluminescence detection

Protein bound nitric oxide (RxNO) concentrations in the serum were quantified using reductive denitrosation by iodine-iodide with subsequent detection of liberated NO by gas-phase chemiluminescence, as described elsewhere.⁵⁴ Samples were thawed for 30 min at room temperature (RT) in the presence of N-ethylmaleimide (NEM; 10 mM final concentration), and nitrite was removed by reaction with acified sulphanilamide before injection into the septum-sealed reaction chamber containing triiodide in glacial acetic acid as detailed previously.⁵⁴

Determination of nitrite and nitrate

The HPLC method used employs ion chromatography with on-line reduction of nitrate to nitrite and subsequent post-column derivatization with the Griess reagent (ENO-20, Eicom, Kyoto, Japan).⁵⁴ Samples were subjected to deproteinization using ice-cold methanol (1:1 v/v) followed by centrifugation. A total volume of 20 μL of sample was loaded onto the column, resulting in a detection limit of 10 nM for either anion.

Total free 8-iso-prostaglandin F_{2a} determination

Free 8-iso-prostaglandin F_{2a} was measured by competitive ELISA using a commercial assay kit (Cayman Chemical, Ann Arbor, USA). The concentration of total (free+bound) 8-isoprostanes was determined after subjecting serum to alkaline hydrolysis and a final dilution of 20-fold with assay buffer before measurement.

Sulfate determination

Serum sulfate concentrations were quantified by means of ion-exchange chromatography after protein precipitation with methanol and a subsequent 10-fold dilution with ultrapure water.

Thiosulfate determination

Thiosulfate was determined by HPLC following monobromobimane (MBB) derivatization, as previously described.⁵⁵⁻⁵⁷ Briefly, 25 µl of urine was derivatized with 5 µl of 46 mM MBB, 25 µl of acetonitrile, and 25 µl of 160 mM HEPES/16 mM EDTA pH 8 buffer for 30 min in the dark. The derivatization reaction was stopped by addition of 50 µl of 65 mM methanesulfonic acid and proteins were removed by centrifugation.⁵⁷

Statistical analysis

Normally distributed continuous data were analysed using ANOVA and the least significant differences as a post-hoc test. A log transformation of the data was performed based on the Levene's test and/or the distribution of the residuals. When a log transformation did not improve our analysis the Tamhane correction for unequal variances was used. Categorical data were analysed using a Pearson chi-square test, and when applicable a Fisher's exact test for subgroup analysis. For correlations between RSI metabolites clinical outcomes, Spearmans rho was used. A p-value below 0.05 was considered statistically significant. All analyses were performed using the SPSS statistics software (IBM, version 23).

Results

Study participants, pregnancy characteristics and fetal characteristics

Seventy-nine pregnant women were included in this case-control study; 23 women experienced an uncomplicated naturally conceived pregnancy, 24 women had a naturally conceived pregnancy complicated by pre-eclampsia, 27 women experienced an uncomplicated oocyte donation pregnancy, and 5 women had an oocyte donation pregnancy complicated by pre-eclampsia. Characteristics of study participants are summarized in [Table 1](#).

Maternal age was significantly lower in uncomplicated naturally conceived pregnancies and naturally conceived pregnancies complicated by pre-eclampsia compared to uncomplicated oocyte donation pregnancies and oocyte donation pregnancies complicated by pre-eclampsia. BMI, smoking habits, gravidity, and parity were comparable among groups. Moreover, obstetrical history was comparable among groups. Only the number of previous miscarriages was significantly different across groups (overall test, $p < 0.05$). Women experiencing an uncomplicated pregnancy were more likely to have had uncomplicated pregnancies before. Of the uncomplicated pregnancies, three women developed gestational hypertension after a naturally conceived pregnancy and four women who were pregnant after oocyte donation had hypertension. None of these women fulfilled the International Society for the study of Hypertension in Pregnancy (ISSHP) criteria for the diagnosis of pre-eclampsia; the women with gestational hypertension had no other obstetric problems, and their fetal growth was adequate for the gestational age.²⁸ Diastolic blood pressure in uncomplicated oocyte donation pregnancies was significantly higher compared to naturally conceived uncomplicated pregnancies. None of the women in our study had gestational diabetes or a history of gestational diabetes. Fetal sex was comparable among groups. Birthweight of fetuses born after a pregnancy complicated by pre-eclampsia was significantly lower compared to uncomplicated pregnancies.

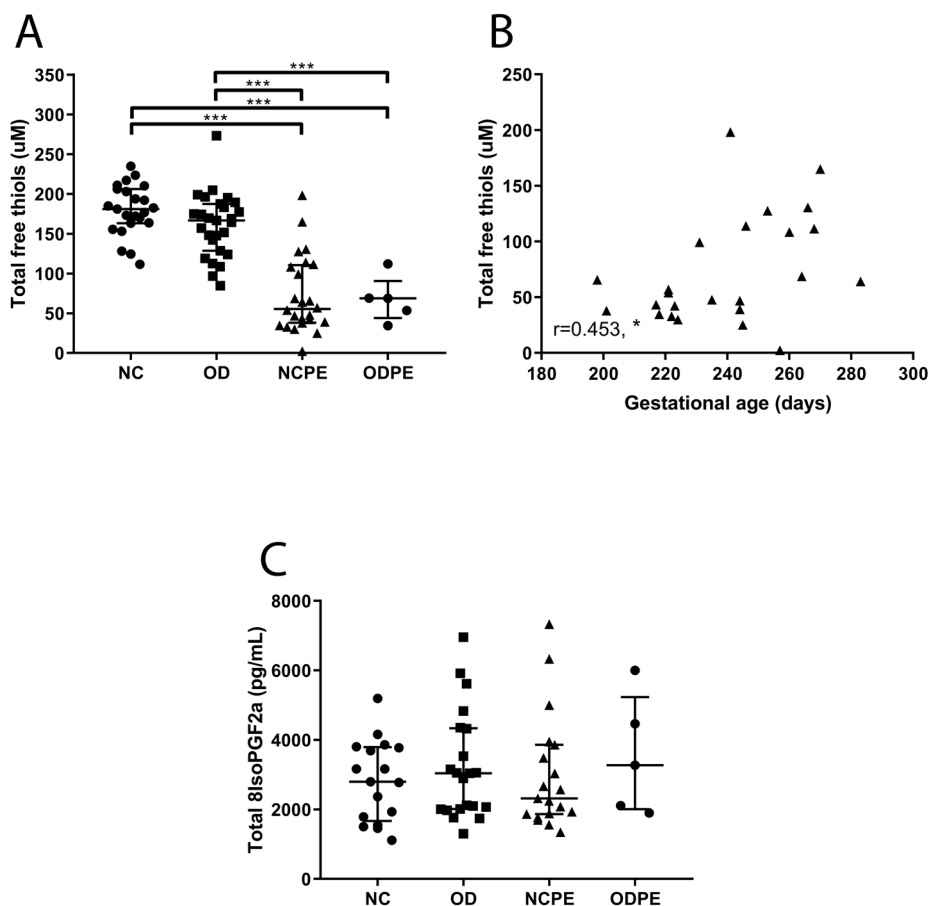
ART	Naturally conceived	Oocyte donation	Naturally conceived	Oocyte donation	
Complication	Uncomplicated	Uncomplicated	Pre-eclampsia	Pre-eclampsia	
Maternal characteristics	n=23	n=27	n=24	n=5	Overall sig.
Maternal Age (years), mean (SD)	31.8 (5.2)	41.1 (6.4)*	30.8 (5.8)	40.0 (7.2)*	p<0.001a
BMI (kg/m ²), median [range]	25.1 [18.8-37.8]	24.3 [16.9-31.6]	24.6 [19.3-36.8]	25.3 [20.7-27.2]	nsa
Smoking, number (%)	2 (9.5)	1 (5)	6 (26.1)	1 (20.0)	nsc
Gravidity, median [range]	1 [1-8]	2 [1-8]	1 [1-5]	1 [1-10]	nsa
Gravidity 1, number (%)	12 (52.2)	9 (36.0)	14 (58.3)	3 (60.0)	nsc
Parity, median [range]	0 [0-3]	0 [0-1]	0 [0-4]	0 [0-0]	nsa
Parity 0, number (%)	13 (56.5)	15 (60.0)	19 (79.2)	5 (100)	nsc
Obstetrical history	n=11	n=15	n=10	n=2	
Previous pregnancies	n=26	n=39	n=15	n=10	
Miscarriage, number (%)	8 (30.8)	27 (69.2)	7 (46.7)	8 (80.0)	p<0.05c
Abortion, number (%)	1 (3.8)	0	1 (6.7)	0	nsc
EUG, number (%)	0	3 (7.7)	0	2 (20)	nsc
TOP, number (%)	0	0	1 (6.7)	0	nsc
Pre-term birth, number (%)	0	2 (5.1)	1 (6.7)	0	nsc
IUFD, number (%)	0	0	1 (6.7)	0	nsc
Gestational hypertension, number (%)	0	2 (5.1)	1 (6.7)	0	nsc
Pre-eclampsia, number (%)	0	0	1 (6.7)	0	nsc
Gestational diabetes, number (%)	0	0	0	0	
Pregnancies without complications, number (%)	17 (65.4)	5 (12.8)*	3 (20)*	0	p<0.05c
Pregnancy characteristics					
ART, number (%)	0	27 (100)*	0	5 (100)#	p<0.001c
Hypertension, number (%)	3 (13.6)	4 (17.4)	24 (100)*	5 (100)&	p<0.001c
Highest diastolic BP, (mmHg), mean (SD)	74 (10)	82 (10)*	101 (9)*	101 (11)&	p<0.001a
Proteinuria, number (%)	0	0	23 (95.8)*	5 (100)&	p<0.001c
Pre-eclampsia, number (%)	0	0	24 (100)*	5 (100)&	p<0.001c
HELLP-syndrome, number (%)	0	0	4 (16.7)*	0	p=0.022c
Gestational age (days), median [range (days)]	275 [269-290]	279 [231-290]	243 [198-283]*	217 [204-270]&	p<0.001a
Preterm birth, number (%)	0	1 (4.2)	18 (75.0)*	4 (80.0)&	p<0.001c
Gestational diabetes, number (%)	0	0	0	0	
Delivery, vaginal, number (%)	6 (26.1)	11 (45.8)	12 (50.0)	1 (25.0)	
Delivery, CS, number (%)	17 (73.9)	13 (54.2)	12 (50.0)	3 (75.0)	nsc
Twin, number (%)	0	2 (7.4)	0	1 (20.0)	nsc
Fetal characteristics	n=23	n=29	n=24	n=6	
Sex, male/female (%male)	16/7 (69.6)	10/18 (35.7)*	14/10 (58.3)	3/3 (50.0)	nsc
Birthweight (gram), median [range]	3455 [2445-4415]	3500 [1611-4500]	2372 [705-4030]*	1319 [1100-3855]&	p<0.001a
Small for gestational age, number (%)	1 (4.3)	5 (19.2)	10 (41.7)*	3 (50.0)	p<0.01c

Table 1 Patient characteristics, obstetrical history, pregnancy characteristics and fetal characteristics

ART, artificial reproductive technique; BP, blood pressure; CS, caesarean section; EUG, extra uterine gravidity; IUFD, intra uterine fetal demise; ns, not significant; TOP, termination of pregnancy. *significantly different compared to uncomplicated naturally conceived pregnancies, p<0.05. #significantly different compared to naturally conceived pregnancies complicated with pre-eclampsia, p<0.05. &significantly different compared to uncomplicated oocyte donation pregnancies, p<0.05. Statistical tests; °ANOVA, Post-Hoc test LSD or Tamhane when applicable, °Pearson Chi-Square, when applicable Fisher's Exact Test for subgroup analysis.

Presence of oxidative stress

The concentrations of both total free thiols and total 8-iso-prostaglandin F_{2a} in blood are a measure of redox stress. Protein-bound free thiols may function as a buffer system for a number of reactive species and may also fulfil specific transport functions. Total (free and bound) 8-iso-prostaglandin F_{2a} is a product of lipid oxidation and indicative of systemic oxidative stress. Total free thiol concentrations were lower in pregnancies complicated by pre-eclampsia, both after naturally conceived pregnancies and oocyte donation pregnancies, compared to uncomplicated pregnancies of either type (Figure 1A, $p < 0.001$). Furthermore, total free thiol concentrations revealed a weak positive association with gestational age in naturally conceived pregnancies complicated by pre-eclampsia (Figure 1B; $r = 0.453$, $p < 0.05$). Serum levels of total 8-iso-prostaglandin F_{2a} did not differ between groups (Figure 1C).

Figure 1 Indicators of oxidative stress

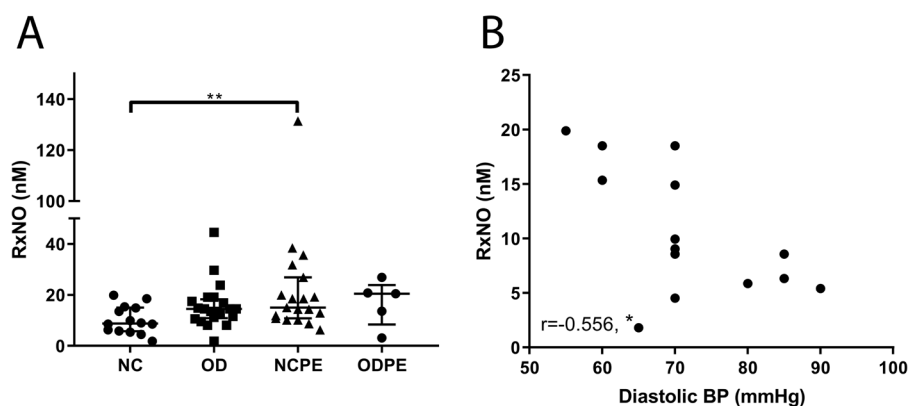
A Total free thiol concentrations were lower in pregnancies complicated by pre-eclampsia compared to controls and uncomplicated oocyte donation pregnancies (all, $p<0.001$). **B** Total free thiol concentrations were positively correlated with gestational age in naturally conceived pregnancies complicated by pre-eclampsia ($r=0.453$, $*p<0.05$). **C** Total 8-iso-prostaglandin F_{2a} concentrations in serum did not differ among groups.

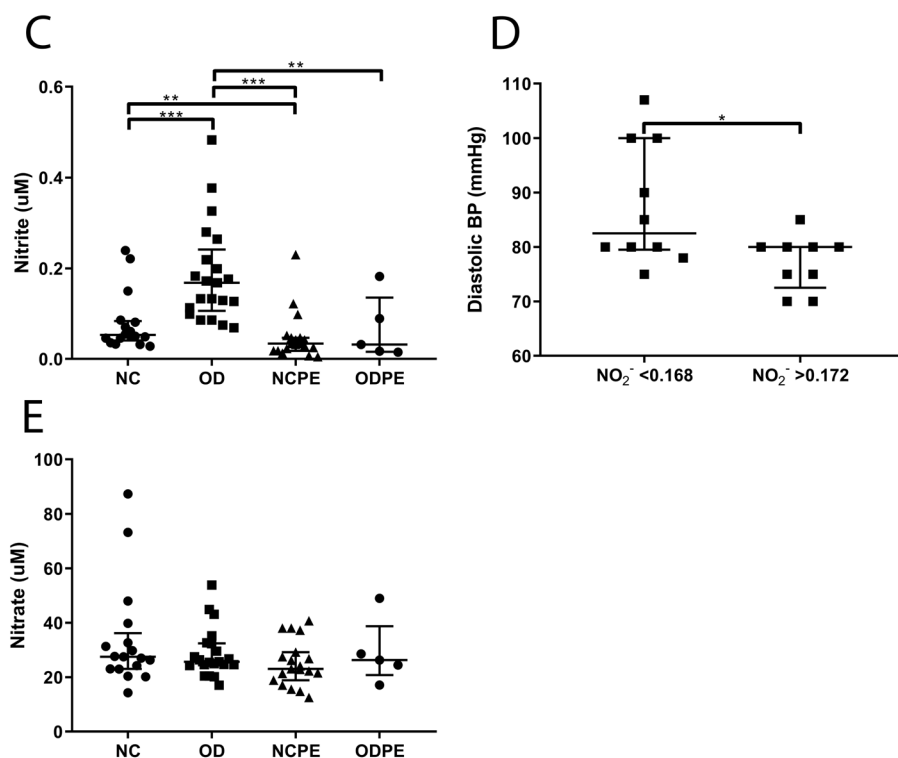
NC, uncomplicated naturally conceived pregnancies; OD, uncomplicated oocyte donation pregnancies; NCPE, naturally conceived pregnancies complicated by pre-eclampsia; ODPE, oocyte donation pregnancies complicated by pre-eclampsia. $*p<0.05$. $***p<0.001$.

Nitric oxide pathway

Nitric oxide (NO) is an important cellular messenger produced by the oxidation of L-arginine by nitric oxide synthases (NOS). In addition to its acute local production, NO bound to circulating proteins, such as serum albumin, can function as a storage and transport system of bioactive NO;^{54, 58} nitrite (NO_2^-) and nitrate (NO_3^-) are the major oxidation products of NO, but are also contained in food. The concentration of protein bound NO (RxNO) was higher in pregnancies complicated by pre-eclampsia (Figure 2A, $p < 0.01$). Interestingly, diastolic blood pressure correlated inversely with RxNO concentrations in women with an uncomplicated naturally conceived pregnancy (Figure 2B). Nitrite concentrations were lower in naturally conceived pregnancies complicated by pre-eclampsia and oocyte donation pregnancies complicated by pre-eclampsia compared to uncomplicated naturally conceived pregnancies and uncomplicated oocyte donation pregnancies, respectively. Also, circulating nitrite was higher in uncomplicated oocyte donation pregnancies compared to uncomplicated naturally conceived pregnancies (Figure 2C). Moreover, diastolic blood pressures were higher in women with an uncomplicated oocyte donation pregnancy and serum nitrite levels below the median of $0.168 \mu\text{M}$ compared to women with a serum nitrite level above the median (Figure 2D, $p < 0.05$). Nitrate concentrations were not significantly different across groups (Figure 2E).

Figure 2 Readouts of the nitric oxide (NO) pathway





A The concentrations of protein bound NO (RxNO) were higher in pregnancies complicated by pre-eclampsia (**p<0.01). **B** Diastolic blood pressure was inversely associated with RxNO in uncomplicated naturally conceived pregnancies ($r=-0.556$, *p<0.05). **C** Nitrite (NO_2^-) concentrations were lower in naturally conceived pregnancies complicated by pre-eclampsia and oocyte donation pregnancies complicated by pre-eclampsia compared to uncomplicated naturally conceived pregnancies and uncomplicated oocyte donation pregnancies, respectively (**p<0.01 and ***p<0.001). Nitrite levels were higher in uncomplicated oocyte donation pregnancies compared to uncomplicated naturally conceived pregnancies (***p<0.001). **D** Patients in the uncomplicated oocyte donation group were divided into two groups based on the nitrite serum levels: One group contained serum nitrite values below the median of 0.168 μM and the other group contained serum nitrite values above the median. Diastolic blood pressure was found to be higher in women with serum nitrite values below the median compared to women with a serum nitrite values above the median (*p<0.05). **E** Nitrate (NO_3^-) concentrations did not differ between groups. NC, uncomplicated naturally conceived pregnancies; OD, uncomplicated oocyte donation pregnancies; NCPE, naturally conceived pregnancies complicated by pre-eclampsia; ODPE, oocyte donation pregnancies complicated by pre-eclampsia. *p<0.05. **p<0.01. ***p<0.001.

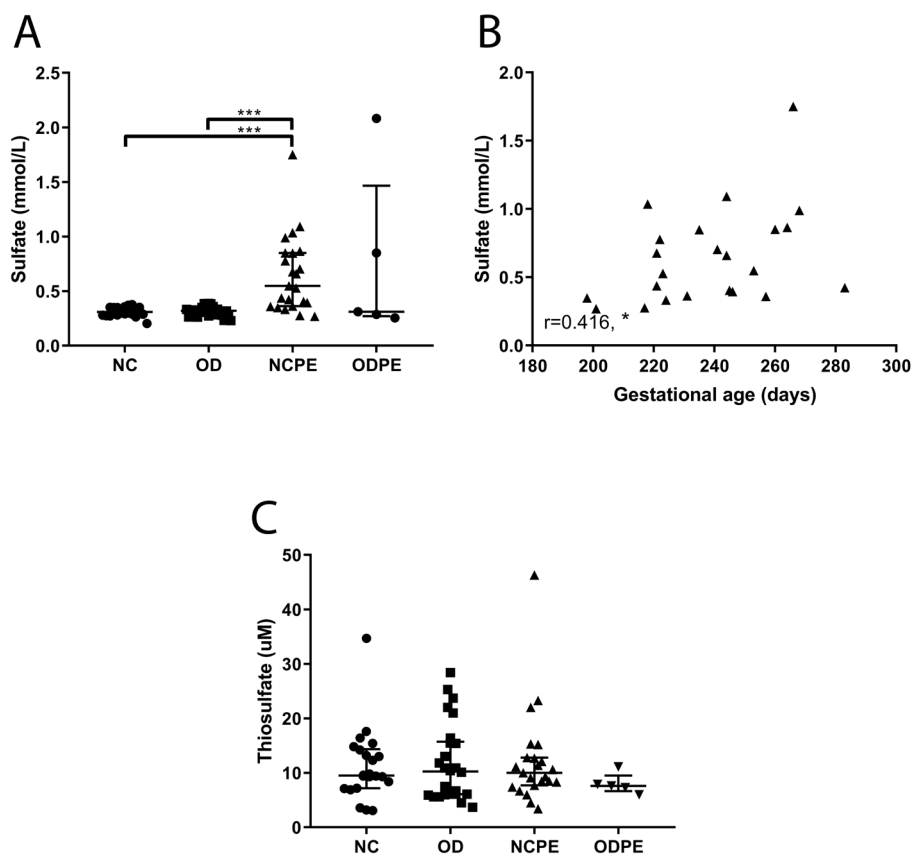
Hydrogen sulfide pathway

Thiosulfate ($S_2O_3^{2-}$) and sulfate (SO_4^{2-}) are oxidative metabolites of hydrogen sulfide (H_2S) and dietary constituents. Serum sulfate concentrations were higher in both naturally conceived pregnancies and oocyte donation pregnancies complicated by pre-eclampsia compared to uncomplicated naturally conceived pregnancies and uncomplicated oocyte donation pregnancies (Figure 3A; both, $p < 0.001$). Sulfate (SO_4^{2-}) concentrations correlated positively with gestational age in naturally conceived pregnancies complicated by pre-eclampsia (Figure 3B; $r = 0.416$, $p < 0.05$). By contrast, thiosulfate ($S_2O_3^{2-}$) concentrations did not differ across groups (Figure 3C).

Discussion

In this observational study, we evaluated the RSI status in naturally conceived and oocyte donation pregnancies in the absence and presence of pre-eclampsia. Naturally conceived and oocyte donation pregnancies complicated by pre-eclampsia appear to be characterized by systemic redox stress; total free thiol levels and nitrite concentrations were significantly lower, whereas protein bound NO and sulfate concentrations were significantly higher in pregnancies complicated by pre-eclampsia compared to uncomplicated pregnancies. Some of these changes have been described before in women with pre-eclampsia.⁵⁹⁻

⁶¹ However, the literature is inconsistent.^{62, 63} The hypoxic placenta is known to produce higher fluxes of ROS³⁰⁻³² and the same is true for the endothelium of women with pre-eclampsia.³⁶ Since superoxide anions (O_2^-), an important ROS, directly react with NO to produce peroxynitrite ($ONOO^-$), enhanced oxidative stress typically translates into lower NO bioavailability. Consistent with this paradigm, a reduced bioavailability of NO is known to be involved in the development of generalised endothelial dysfunction and hypertension in women with pre-eclampsia.³⁵ More recently, oxidative stress-induced S-glutathionylation of endothelial nitric oxide synthase has been suggested to account for impaired NO production in the placenta.⁶⁴ We have shown that the concentrations of free thiols and sulfate appear to positively correlate with gestational age in naturally conceived pregnancies complicated by pre-eclampsia. While these associations are modest, they would seem to be consistent with the notion that perturbations in systemic redox status are tightly linked to the development of pre-eclampsia; a shorter gestational age in women with pre-eclampsia is mainly due to iatrogenic birth (caesarean section or induction of labour) based on the fetal or maternal condition and is therefore always directly associated with the severity of the disease. Both source and utilization of sulfate deserve further study in this context, as this anion is not only the final oxidation product of H_2S but also an important nutrient for human growth and development.⁶⁵ This is particularly important in view of the limited capacity of fetal tissues to produce sulfate, thereby relying almost entirely on its supply via the maternal circulation.

Figure 3 Readouts of the hydrogen sulfide (H_2S) pathway

A Sulfate (SO_4^{2-}) values were higher in naturally conceived pregnancies complicated by pre-eclampsia compared to uncomplicated naturally conceived pregnancies and uncomplicated oocyte donation pregnancies (** $p < 0.001$). **B** Sulfate (SO_4^{2-}) values correlated positively with gestational age in naturally conceived pregnancies complicated by pre-eclampsia ($r = 0.416$, $*p < 0.05$).

C Thiosulfate ($\text{S}_2\text{O}_3^{2-}$) values did not differ between groups.

NC, uncomplicated naturally conceived pregnancies; OD, uncomplicated oocyte donation pregnancies; NCPE, naturally conceived pregnancies complicated by pre-eclampsia; ODPE, oocyte donation pregnancies complicated by pre-eclampsia. $*p < 0.05$. $***p < 0.001$.

In oocyte donation pregnancies, the fetus could be completely allogeneic to the gestational carrier.⁵ This condition is thus comparable with the situation of antigenic dissimilarity present in organ transplantation.^{5, 13} Based on the immunogenic dissimilarity in oocyte donation, and what is known from the redox status in transplantation, one might expect more oxidative stress in oocyte donation pregnancies. However, total free thiol and total 8-iso-prostaglandin F_{2a} concentrations were not changed in uncomplicated oocyte donation pregnancies compared to uncomplicated naturally conceived pregnancies. Curiously, increased nitrite concentrations were observed in uncomplicated oocyte donation pregnancies compared to naturally conceived pregnancies. Circulating nitrite levels are a reliable biomarker of endothelial NO production.^{66, 67} NO is a potent vasodilator and an important regulator of vascular tone, and upregulated NO production could reduce blood pressure and the development of hypertension.^{37, 43, 68} However, this would be expected to be accompanied by a concomitant increase in circulating nitrate (NO_3^-) concentrations. Diastolic blood pressure was slightly higher in uncomplicated oocyte donation pregnancies compared to uncomplicated naturally conceived pregnancies (Table 1, $p < 0.05$). Upregulation of NO/nitrite in oocyte donation could be a compensatory mechanism to limit further increases in blood pressure in these women. This is supported by the inverse association between diastolic blood pressure and protein bound NO (RxNO) concentrations in uncomplicated naturally conceived pregnancies. RxNO functions as a NO storage pool,⁵⁸ and lower RxNO levels may reflect higher utilization of this alternative source of NO. Furthermore, diastolic blood pressure is higher in women with an uncomplicated oocyte donation pregnancy with nitrite serum concentrations below the median compared to women with concentrations above the median (Figure 2 D). The importance of NO in the regulation of blood pressure in pregnancy is shown in an animal experiment where NO production was blocked in virgin and pregnant rats. The blockage of NO synthases resulted in the development of hypertension in pregnant rats as well as in virgin rats. Moreover, pregnant animals are more sensitive for NO synthesis blockade than non-pregnant animals.⁶⁹ Thus, higher serum nitrite concentrations in women with uncomplicated oocyte donation pregnancies could indicate a role of this NO metabolite in the regulation of vascular tone, which might function as a compensatory mechanism in uncomplicated oocyte donation pregnancies to prevent a further increase in blood pressure caused by the genetic incompatibility between mother and fetus in oocyte donation pregnancies. However, we cannot exclude other possible explanations, such as the increased maternal age in women pregnant after oocyte donation.

Study limitations include the small group size of women with oocyte donation complicated by pre-eclampsia, which was a direct consequence of the definition of case selection criteria. We were concerned that the results could be influenced by the duration of sample storage and therefore opted to select samples from a defined study period to avoid this potential influence. Another limitation of our study is that maternal age and gestational age were different between study groups. Maternal age is an important risk factor for pre-eclampsia and could influence the rate of production and metabolism of reactive species.^{27, 41, 70} Since women

pregnant after oocyte donation are usually older, it is difficult to match maternal age between naturally conceived and oocyte donation pregnancies. Nevertheless, none of the measured molecules was associated with maternal age. Likewise, due to the presence of pre-eclampsia (and the consequent preterm delivery), matching for gestational age is difficult. A preterm control group does not exist due to the underlying factors leading to preterm delivery. Lastly, the dietary intake of nitrite, nitrate and sulfate by the study participants, which could affect the RSI status^{41, 71} and constitute an analytical confounding factor, was not determined.

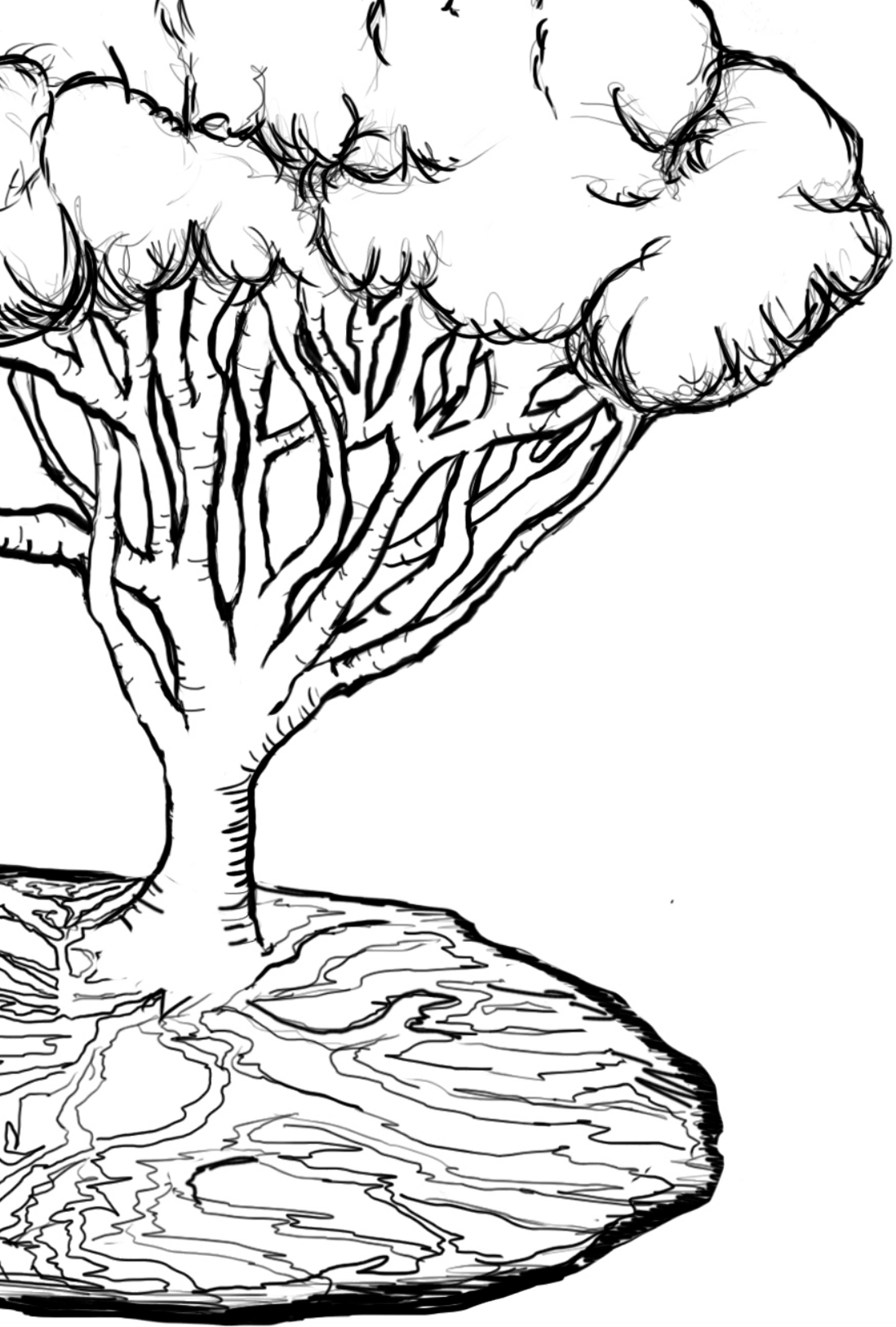
In conclusion, alterations in the RSI status are present in naturally conceived pregnancies as well as in oocyte donation pregnancies complicated by pre-eclampsia, and the concentrations of both total free thiols and sulfate are associated with the severity of pre-eclampsia in naturally conceived pregnancies. Furthermore, changes in the RSI status are also seen in uncomplicated oocyte donation pregnancies compared to uncomplicated naturally conceived pregnancies. In this study, a possible compensatory mechanism via the NO-pathway was found in uncomplicated oocyte donation pregnancies. This exploratory study is the first that elucidated possible changes in select readouts of the RSI in oocyte donation pregnancies. More research is warranted on the role of the RSI in uncomplicated oocyte donation pregnancies and oocyte donation pregnancies complicated by pre-eclampsia. Given that mitochondrial function and cellular energetics are intimately interconnected to the extracellular redox status and modulated by several RSI constituents at multiple levels,⁴¹ it is possible that the latter plays an important role in fetal development and the prevention of complications in pregnancy.

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Towards standardized criteria for diagnosing chronic intervillitis of unknown etiology: a systematic review

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Abstract

Chronic intervillitis of unknown etiology (CIUE) is a poorly understood, relatively rare condition characterized histologically by the intervillous infiltration of mononuclear cells in the placenta. Clinically, CIUE is associated with poor pregnancy outcome (e.g., impaired fetal growth, preterm birth, fetal death) and high risk of recurrence in subsequent pregnancies. Because CIUE is not defined consistently, it is essential to clearly define this condition. We therefore review the published definitions of CIUE. In addition, we provide an overview of the reviewed histopathological and maternal characteristics, obstetric features, and pregnancy outcomes. Medical publication databases were searched for articles published through February 2017. Eighteen studies were included in our systematic review. The sole inclusion criterion used in all studies was the presence of intervillous infiltrates. Overall, CIUE was characterized by adverse pregnancy outcome. Miscarriage occurred in 24% of cases, with approximately half of these miscarriages defined as late. Impaired growth was commonly observed, 32.4% of pregnancies reached term, and the live birth rate was 54.9%. The high recurrence rate (25.1%) of the intervillous infiltrates in subsequent pregnancies underscores the clinical relevance of CIUE, the need for increased awareness among pathologists and clinicians, and the need for further research. Criteria for the diagnosis of CIUE are proposed and a Delphi study could be used to resolve any controversy regarding these criteria. Future studies should be designed to characterize the full clinical spectrum of CIUE.

Introduction

Chronic intervillitis of unknown etiology (CIUE) is a poorly understood, relatively rare condition first described in 1987 by Labarrere and Mullen as massive chronic intervillitis. They defined massive chronic intervillitis as a histopathological finding characterized by the intervillous infiltration of mononuclear cells in the placenta, fibrin deposits and trophoblast necrosis.¹ CIUE appears to be associated with poor perinatal outcome, including miscarriage, reduced fetal growth, and fetal death.^{2, 3} In addition, CIUE has a 4-100% chance of recurrence in a subsequent pregnancy.²⁻⁶ The incidence of CIUE in the second and third trimester is 6 out of 10,000 pregnancies, and CIUE-related miscarriage occurs in 44 out of 1000 pregnancies in which the fetus has a normal karyotype.⁴

Nomenclature

In this review is referred to a condition that encompasses the presence of chronic intervillitis accompanied by pregnancy complications, a high recurrence risk, and the absence of a known (infectious) cause. Since these chronic intervillous infiltrates were first described as massive chronic intervillitis by Labarrere and Mullen, a variety of terms have been used to describe this condition, including "chronic intervillitis of unknown etiology", "chronic intervillitis", "chronic histiocytic intervillitis of unknown etiology", "chronic histiocytic intervillitis", "massive histiocytic chronic intervillitis", "massive perivillous histiocytosis", "intervillitis", and "massive chronic intervillitis".⁸ It is important to distinguish between chronic intervillitis referring to a histologic placenta lesion irrespective of the cause, and the specific condition we define. The term "chronic intervillitis of unknown etiology (CIUE)" should be used in further research on the condition we define in this systematic review.

Immunological and/or coagulation disturbances may play a role in the pathophysiology of CIUE. For example, the occurrence of intervillous infiltrates with focal villitis^{2, 5} and the presence of C4d deposits in CIUE are indicative of an immunological disturbance.⁷ In addition, increased placental expression of intercellular adhesion molecule-1⁸ and the presence of CIUE-specific cell infiltrates⁹ suggest an immunopathological component. Moreover, Reus *et al.* recently suggested that the pathophysiology of CIUE might be based on a HLA mismatch between the "donor" (fetal-paternal antigens) and the "recipient" (the mother); this suggestion was based on observations of mixed lymphocyte reactions and the prevalence of cytotoxic T lymphocyte precursors cells.¹⁰ Furthermore, the presence of CIUE in cases with neonatal alloimmune thrombocytopenia, which is caused by maternal antibodies against paternally derived human platelet antigens, may suggest a process comparable to chronic rejection.^{11, 12} The presence of perivillous fibrin deposits in CIUE suggest coagulative disturbances, this likely is due to an immune-mediated process.^{4, 13} Interestingly, chronic intervillitis is also observed in

the placentas of women with malaria and/or acute cytomegalovirus infection.^{14, 15} Although reduced fetal growth and preterm birth are also observed in pregnancies complicated by malaria, perinatal mortality is not frequently observed in these pregnancies.¹⁴ The co-occurrence of chronic intervillitis and malaria has given rise to the hypothesis that an underlying, not yet identified, infection may be associated with CIUE.

Given that CIUE is associated with a high risk of recurrence and with adverse pregnancy outcome,^{2,6} prevention is the best approach. A few studies reported positive effects of treatment with aspirin, heparin, prednisolone, and/or corticosteroids in various dosages and combinations.^{2,4, 16, 17} A meta-analysis by Contro *et al.* revealed that the reported live birth rate does not significantly improve with treatment.⁵ However, more recent studies suggest a different combination therapy for CIUE, which was beneficial in few cases.^{18, 19} This combination treatment was not reviewed in the meta-analysis.⁵ Extensive national or international studies including as many patients as possible are needed to elucidate the etiology of CIUE and to investigate therapeutic approaches.

Since different terms are used over the time to describe CIUE,⁸ it is likely that different definitions, inclusion criteria and exclusion criteria are used in various studies. Therefore, developing a clear definition of CIUE is an essential first step towards comparable study results and understanding the etiology of CIUE.

Our primary objective is to review the published definitions of CIUE, as well as the inclusion and/or exclusion criteria used in all studies regarding CIUE published from 1987 through February 2017. In addition, we provide an overview of the investigated histopathological parameters and immunological characteristics of the cellular infiltrates, and we review the clinical features, obstetric characteristics, and outcomes in the published cases. Based on these results, we propose standardized criteria for diagnosing CIUE.

Methods

Search methods

The following databases were searched for articles regarding CIUE published from 1987 through February 2017: PubMed, Embase, Web of Science, Emcare, Academic Search Premier, ScienceDirect, Wiley-Blackwell, LLW, Highwire, and Google Scholar. Each database was searched using the following terms: "massive chronic intervillitis", "chronic histiocytic intervillitis", "intervillitis", "perivillous histiocytosis", and "intervillitis". The literature search was performed by authors MB and JS. Complete details regarding the search strategy details are available in Appendix A.

Selection of studies

Potentially relevant studies were reviewed independently by authors MB and RT by scanning the article's title and abstract. All peer-reviewed publications regarding histologically confirmed cases of CIUE were included. We excluded non-observational studies, reports of an intervillous infiltrate due to documented infection, reports of other forms of villitis, case reports, and studies with no full text available. Any disagreement regarding including a study was resolved by consensus between authors MB and RT. The references were checked in the included studies, and citation tracking was performed.

Patient characteristics

Maternal age and gestational age were obtained from the patient data provided in each study and are reported as the mean, standard deviation, and range. Gravidity and parity were also obtained and were reported as median and range. In the event that data were not published per patient, we used the reported outcomes.

Pregnancy outcome

We collected the following data: the number of early and/or late miscarriage, growth restriction, preterm births, stillbirths, perinatal mortality, and the recurrence of CIUE in subsequent pregnancies. Miscarriage was defined as the spontaneous loss of pregnancy within the first 22 weeks of gestation.²⁰ Early and late miscarriages were defined as miscarriage that occurred at ≤ 12 weeks of gestation and 12-22 weeks of gestation, respectively. Intrauterine growth restriction was defined as an estimated fetal weight in the bottom 10th percentile for gestational age, and small for gestational age was defined as a birth weight in the bottom 10th percentile for the corresponding gestational age.²¹ One study in a Hispanic population did not determine growth based on gestational age; we therefore determined these data using the appropriate reference curves for this population.²² A term pregnancy was defined as birth ≥ 37 weeks, and preterm birth was defined as birth < 37 weeks of gestation. In approximately 50% of early miscarriages, the fetus has a chromosomal abnormality.²³ However, cases of CIUE in a study group of early miscarriages have been reported to be karyotypically normal.²⁴ Maternal factors, such as coagulation abnormalities, are more predominant in late miscarriages.²⁵ Since both, early and late miscarriages, might have a different etiology, also in the

context of CIUE, the proportions of term births were calculated with and without early miscarriages.²⁶ Worldwide, the definition used for stillbirth varies.²⁷ For our purposes, stillbirth was defined as pregnancy loss after 22 weeks of gestation.²⁰ A stillbirth event at term was considered as a pregnancy that reached term. Perinatal mortality refers to both stillbirths and neonatal deaths within the first postnatal week. We defined pregnancies resulting in a living child as the total number of pregnancies with gestational age ≥ 12 weeks minus the number of pregnancies that resulted in late miscarriage, stillbirth, or perinatal death. Cases with missing data were excluded separately for each outcome.

Results

Selected studies

Our literature search revealed 361 unique publications. Figure 1 shows a flowchart of the inclusion and exclusion of these publications. First, 312 publications were excluded based on the title and/or abstract. Thirty-one of the remaining 49 publications were excluded for the following reasons: 16 were meeting abstracts, 13 were case reports that included fewer than three cases, and the full text was not available for one publication. Furthermore, one additional publication²⁸ was excluded because these cases were already included in two other publications.^{29, 30} Reference checking and citation tracking did not yield any new publications. Thus, a total of 18 publications met our criteria and were included in our systematic review.^{1-3, 6-10, 16-18, 29-35} These 18 publications reported a total of 291 women, including 350 pregnancies with either an intervillous infiltrate in the placenta diagnosed as CIUE or comparable lesions that were given a different name, e.g. massive chronic intervillitis, chronic intervillitis, chronic histiocytic intervillitis, chronic intervillitis or chronic histiocytic intervillitis of unknown etiology. The publications included in this review are summarized in Table 1.

Diagnostic criteria for CIUE

The inclusion and exclusion criteria for the collection of cases in the included studies are summarized in Table 2. Some groups did not clearly state their inclusion and/or exclusion criteria for selecting cases.

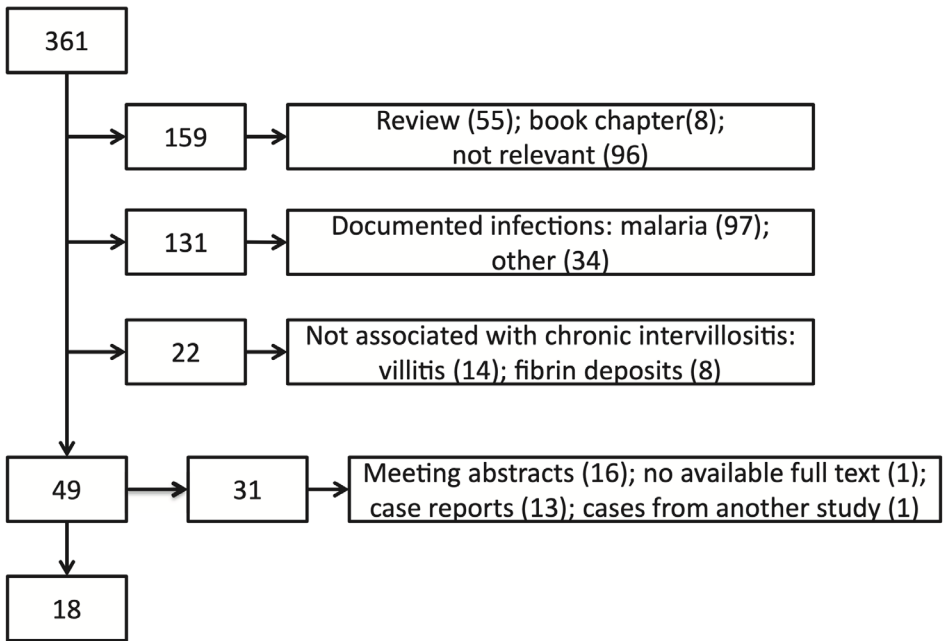
Inclusion criteria for the selection of cases

The only inclusion criterion used in all 18 studies was the presence of an infiltrate in the intervillous space. In 67% of the studies (12 publications) CIUE was defined explicitly as the presence of mononuclear cells in the intervillous space.^{1, 2, 6, 7, 9, 10, 16-18, 31, 33-35} Nine groups (50%) mentioned that the infiltrate should contain histiocytes.^{3, 6-8, 18, 29, 31, 32, 34, 35} Seven groups (39%) stated that only patients with a massive, diffuse, or widespread infiltrate in the intervillous space should be included.^{1, 3, 8, 18, 30, 33, 34} Four and three groups used the presence of fibrin deposits and trophoblastic necrosis, respectively, as an inclusion criterion.^{1, 2, 9, 16, 17} Finally, three groups stated that the infiltrate should be maternal in origin to be defined as CIUE.^{10, 34, 35}

Exclusion criteria for the selection of cases

Eleven groups (61%) specifically excluded patients with an infectious cause of CIUE;^{1, 2, 6, 8, 9, 17, 18, 30, 33, 34} four groups excluded patients with malaria or cytomegalovirus infection,^{2, 18, 30, 35} three groups excluded patients with chorioamnionitis,^{8, 17, 33} and four groups excluded patients with other infectious diseases.^{1, 6, 9, 34} The largest discrepancy with respect to exclusion criteria was regarding the co-occurrence of chronic villitis with intervillitis; five groups excluded cases with chronic villitis,^{3, 7, 9, 16, 29} whereas one group explicitly stated that they included only cases with focal chronic villitis.² Marchaudon *et al.* reported that they excluded cases with intervillitis associated with other placental lesions.³¹

Figure 1 Selection of publications



Flowchart of the selection of publications included in this review.

	Study design	Term for intervillous infiltrates	Number of pregnancies with intervillous infiltrates	Number of women with intervillous infiltrates	Number of control pregnancies
Labarrere and Mullen, 1987.	Re CC	Massive chronic intervillitis	6	6	12
Jacques and Qureshi, 1993.	Re CS	Chronic intervillitis	6	6	
Boyd and Redline, 2000.	Re CS	Chronic histiocytic intervillitis	31	21	
Rota et al, 2006.	Re CS	Chronic intervillitis	28	25	
Parant et al, 2009.	Re CS	Chronic intervillitis of unknown etiology	20	14	
Traeder et al, 2010.	Re CS	Chronic histiocytic intervillitis	4	4	
Marchaudon et al, 2011.	Re CS	Chronic histiocytic intervillitis of unknown etiology	69	50	
Heller, 2012.	Re CC	Chronic histiocytic intervillitis	9	8	11
Capuani et al, 2013.	Re CS	Chronic histiocytic intervillitis of unknown etiology	20	16	
Freitag et al, 2013.	Re CC	Chronic histiocytic intervillitis	2	2	11
Reus et al, 2013.	Re CS	Chronic histiocytic intervillitis	27	22	
Labarrere et al, 2014.	Re CC	Massive chronic intervillitis	7	7	14
Bendon et al, 2015.	Re CC	Chronic intervillitis	32	28	32
Labarrere et al, 2015.	Re CC	Massive chronic intervillitis	17	17	34
Mekinian et al, 2015.	Pr CS	Chronic histiocytic intervillitis	24	24	
Revaux et al, 2015.	Re CS	Chronic intervillitis	18	12	
Nowak C et al, 2016.	Re CC	Chronic intervillitis of unknown etiology	24	23	154
Sabra et al, 2016.	Re CS	Chronic histiocytic intervillitis	6	6	
			350	291	

Table 1 Included studies

18 studies were included in this systematic review. One study had a prospective study design. These studies describe 350 pregnancies with CIUE in 291 women. Several control groups were used in case-control studies. Data per patient were provided in eight studies. Abbreviations; Re, Retrospective; CC, Case-control study; CS, Case series; Pr, Prospective.

Number of control patients	Type of controls	Total	Comment
12	Chronic villitis (12)	18	
		6	
		45	
		28	
		20	
		4	
		69	
11	second-trimester cases with chromosomal abnormalities or multiple severe anomalies (11)	20	
		20	
11	Villitis of unknown etiology (4), normal placenta (7)	16	case 1, 3 and 4 from Traeder et al. (2010)
		30	3 cases without an intervillous infiltrate
14	villitis (7), without villitis or massive chronic intervillitis (7)	21	
31	Placentas of complicated and uncomplicated pregnancies with villitis of varying degrees.	64	We defined cases as placentas with massive chronic intervillitis (18) as well as placentas with few intervillous monocytes (14).
34	Chronic villitis of unknown etiology (17), without chronic villitis of unknown etiology or massive chronic intervillitis (17)	51	
		24	
		38	
143	Villitis of unknown etiology (78), villitis and intervillitis (76)	178	
		6	

Histopathological findings

Supplemental Tables 1 and 2 provide an overview of the histological findings and the composition of the cell infiltrates. One group found fibrin deposits in 18% of cases;¹⁷ another group found fibrin deposits in 100% of cases.¹ Four groups used the presence of perivillous fibrin deposits as an inclusion criterion;^{1, 2, 9, 16} in contrast, groups that also mentioned the presence of fibrin deposits did not use this as an inclusion criterion.^{3, 17, 29, 30} The presence of fibrin was reported in different grades from low to diffuse.^{3, 16, 31} Trophoblast necrosis and erosion were noted by Capuani *et al.*,⁹ and Labarrere *et al.* reported a prevalence of 100% in their original paper on massive chronic intervillitis.¹ The co-occurrence of villitis was observed in 25-76% of cases with CIUE;^{1, 2, 6, 17, 18, 29, 33} however, the presence of villitis was also used as an exclusion criterion by six groups.^{2, 3, 7, 9, 16, 29} Two groups studied combined lesions with CIUE and villitis.^{6, 7} Five groups extensively studied the composition of the intervillous cell infiltrates.^{3, 9, 29, 30, 33} The majority of cells (approximately 80%) were positive for CD68; moreover, Boyd *et al.*³ found that approximately 30-40% of these CD68+ cells expressed MRP14. The marker MRP14 is also known as S100A936, this marker is expressed by an activated immature monocyte/macrophage subset.³⁷ T cells (5-24%) and subtypes of T cells were also observed in the infiltrate, including (CD8+) cytotoxic T cells (7.7-17.1% of cells), (CD4+) T helper cells (5.1-14.4% of cells),^{9, 30, 33} and regulatory T cells (approximately 5% of cells).⁹ Finally, one group reported that 4% of the cells in the infiltrate were B cells (see Supplemental Table 2).⁹

Maternal characteristics and obstetrical features

Eight of the publications reported the maternal characteristics, obstetrical features, and pregnancy outcome per patient.^{1, 2, 7, 9, 16, 29, 30, 35} The maternal characteristics and obstetrical features varied widely among the studies (Table 3). Maternal age ranged from 18 years to 45 years, gravidity ranged from 1 to 11, and parity ranged from 0 to 8. The presence of CIUE was reported in placenta samples from all three trimesters. Several conditions and factors appeared to be associated with CIUE, including autoimmune disease, preeclampsia, assisted reproduction, and smoking (Supplemental Table 3).^{3, 8, 10, 16-18, 29-31, 33, 34}

Pregnancy outcome

An overview of the documented pregnancy outcomes in patients with CIUE is provided in Supplemental Table 4. The overall incidence rates of each pregnancy outcome are shown in Table 4. Miscarriage was reported in 62 out of 256 (24.2%) pregnancies. This might be underestimated, as some groups included only pregnancies that resulted in a live birth.^{8, 33} Approximately 50% of miscarriages were late miscarriages (24 out of 52); in seven publications the majority of documented miscarriages were late miscarriages.^{2, 6, 7, 9, 16, 30, 32} However, in two studies with a relatively high incidence of miscarriage, 70-82% of documented miscarriages were early miscarriages.^{3, 31} Impaired fetal growth was reported for nearly 65% of pregnancies. Specifically, 63 out of 88 fetuses had intrauterine growth restriction, and 40 out of 66 fetuses were small for their gestational age. The number of fetuses with impaired growth was slightly underestimated, as some

Inclusion criteria		
Intervillitis / infiltrate in intervillous space	+	100
Mononuclear infiltrate	+	67
Histiocytic monomorphic infiltrate	+	50
Infiltrate is massive / wide spread / diffuse	+	39
Fibrin deposits	+	22
Trophoblastic necrosis	+	17
Maternal origin of infiltrate	+	17
Exclusion criteria		
Signs of infection	+	61
Villitis	+	28
Chorioamnionitis	+	17
Cytomegalovirus infection	+	17
The presence of "other obvious placental lesions"	+	17
Malaria	+	11
Polymorphic infiltrate	+	11
Infiltration/ destruction of placental tissue	+	6
Lymphocytic vasculitis	+	6
Congenital malformations	+	6

Table 2 Inclusion and exclusion criteria used for the diagnosis of CIUE

Several inclusion criteria and exclusion criteria were used for the selection of patients. "+" in the table indicates that the study used this particular inclusion criterion or exclusion criterion. The only uniform inclusion criterion used was the presence of an intervillous infiltrate (100%). Most of the studies excluded pregnancies with signs of infections (61%).

groups defined impaired fetal growth as either an estimated fetal weight or birth weight in the bottom 3rd percentile of reference curves.^{6, 31} Only 59 out of 182 pregnancies (32.4%), which provided sufficient information on gestational age at birth, resulted in a term birth; this percentage increased slightly to 38.1% when early miscarriages were excluded. Fifty-five out of 190 documented pregnancies ended in stillbirth, and six neonatal deaths were documented.¹⁶ Among the pregnancies with CIUE, 135 resulted in live-born infants (54.9%); this percentage increased to 59.4% when early miscarriages were excluded. The rate of recurrence among 199 women was 25.1% and ranged from 4.2% to 100%. One prospective study reported a recurrence rate of 33.3% among 24 women with CIUE.¹⁸

Discussion

Our objective was to investigate the definitions, inclusion criteria, and exclusion criteria in studies regarding CIUE published since 1987. In addition, we provided an overview of the histological findings, maternal characteristics, obstetric features, and reported outcome of cases in the included studies. We found that studies regarding CIUE use different inclusion criteria and exclusion criteria for selecting cases with CIUE, or they used different definitions. Indeed, the only criterion used by all groups was the presence of an intervillous infiltrate in the placenta. Despite this wide variation among publications, all groups agree that CIUE is a serious condition characterized by adverse fetal outcome, including high rates of miscarriage, impaired growth, reduced term births, and a reduced live birth rate. In addition, the relatively high rate of recurrence (25.1% of patients) underscores the high clinical relevance of CIUE.

Strengths and limitations of this study

This study provides an accurate overview of publications describing the lesion CIUE. Furthermore, this review is the first step towards standardized criteria for diagnosing CIUE. Limitations of this study are the limited number of included patients and incomplete patient characteristics and obstetric characteristics, caused by the study design and reporting of included studies. For instance, some groups provided limited information regarding pregnancy outcome, used different criteria for pregnancy outcome, or reported the outcome of pregnancies with an intervillous infiltrate together with pregnancies without CIUE.

Limitations of the included studies

We found that the definitions used differed among publications; patient characteristics, histopathological findings, and pregnancy outcomes differed between studies, giving rise to the question of whether CIUE is a self-contained entity. With one exception,¹⁸ all the studies included in our review were retrospective studies. A retrospective study may favour the selection of patients with a severe and/or suspicious case history, thereby leading to selection bias. Furthermore, most of these cases were selected based on a previous diagnosis by a pathologist.

	Study provided data per patient	Mean maternal age, years (SD, years) [range, years]	Gravidity, median [range]	Parity, median [range]	Mean gestational age, days (SD, days) [range, days]
Labarrere and Mullen, 1987.	+	30.5 (7.4) [21-42]	3.5 [1-5]	2 [1-5]	265 (14) [238-280]
Jacques and Qureshi, 1993.	+	29.0 (4.6) [23-37]	3.5 [3-11]	1 [1-7]	191 (41) [140-266]
Boyd and Redline, 2000.		29.8 (6.2) [20-43]	5 [1-9]		
Rota et al, 2006.		30.1			
Parant et al, 2009.	+	30 [24-39]	2.5 [1-10]	1 [0-6]	199 (65) [56-284]
Traeder et al, 2010.	+	34.0 (4.2) [30-40]	1.5 [1-4]	1 [1-2]	218 (22) [189-249]
Marchaudon et al, 2011.		31.2 (6.1) [16-43]			
Heller, 2012.					
Capuani et al, 2013.	+	30 (5.4)	3 [1-9]	0 [0-4]	188 (54) [77-273]
Freitag et al, 2013.	+	[25-35]	[1-3]	[0-1]	[134-225]
Reus et al, 2013.		31.8 (4.9) [22-45]	mean (SD), 4.5 (3.1) [1-13]	mean (SD), 2.3 (1.9) [0-8]	181 (72) [56-283]
Labarrere et al, 2014.		median 28 [21-42]	1 [0-4]	1 [0-4]	median 266 [238-280]
Bendon et al, 2015.	+		2.5 [1-11]		224 (50) [91-280]
Labarrere et al, 2015.		median 29 [18-42]	2 [1-6]	1 [0-4]	median 266 [238-287]
Mekinian et al, 2015.		34 (5)			
Revaux et al, 2015.		median 30 [22-40]			
Nowak C et al, 2016.					
Sabra et al, 2016.	+	34.8 (2.4) [32-39]	3 [2-6]	1 [0-1]	94 (62) [56-231]

Table 3 Maternal characteristics and obstetrical features

The maternal characteristics of the included patients with an intervillous infiltrate and the obstetrical features of pregnancies complicated with CIUE varied widely among the studies. Two publications did not provide information regarding maternal characteristics. Abbreviations; SD, standard deviation.

	Miscarriage	Late miscarriage	IUGR	SGA	Term births	Pregnancies reaching term after 12 weeks of gestation	Stillbirth	Neonatal mortality	Live birth	Pregnancies resulting in a living child	Recurrence
Number of studies	15	7	7	7	11	11	13	2	13	11	10
Total number of pregnancies or patients	256	52	88	66	182	155	190	9	246	180	199
Number of pregnancies or patients with outcome	62	24	63	40	59	59	55	6	135	107	50
%	24.2	46.2	71.6	60.6	32.4	38.1	28.9	60.0	54.9	59.4	25.1

Table 4 Summary of pregnancy outcomes

Number of studies represents the number of publications that reported the respective outcome; number of pregnancies or patients represents the number of patients included in those studies; number of pregnancies or patients with outcome represents the number of patients with the indicated outcome; % represents the of number of pregnancies or patients with the indicated outcome divided by the number of pregnancies or patients, expressed as a percentage. Abbreviations; IUGR, intrauterine growth restriction; SGA, small for gestational age.

Pathologists who do not specialize in examining placentas can experience difficulties in properly recognizing the lesion and therefore tend to diagnose the more severe cases.³² On the other hand, pathologists who are familiar with CIUE may diagnose less suspicious cases. Kramer *et al.* reported high levels of both intra-observer and inter-observer agreement with respect to the histological features of acute placental inflammation;³⁸ however, such results can be generalized only to experienced placental pathologists.³⁸ Moreover, a diagnosis of CIUE is based on a process of elimination, most groups attempted to exclude an underlying infection. However, due to the retrospective study design, adequate patient materials (e.g., blood samples, frozen placental specimens, etc.) may not be available. This may lead to incorrectly diagnosing a case as well.

On the other hand, several groups reported a substantial risk of recurrence of CIUE after a reported lesion,^{3, 7, 9, 18} and the outcome in patients with CIUE is generally less favourable compared with patients with placentas without any lesions, with villitis of unknown etiology, or with a combined lesion of villitis and intervillitis.^{1, 6} The recurrence of the infiltrates and adverse outcomes in patients with CIUE is in favour of CIUE being a self-contained entity. Furthermore, there appears to be an association between CIUE and autoimmune disease and it is striking that ten of the 18 publications mentioned that some of the included patients had preeclampsia (see Supplemental Table 3). Preeclampsia is a pregnancy-related syndrome characterized by impaired placental function, immune-dysregulation and subsequent maternal endothelial dysfunction.³⁹ These associations might provide some insights in a possible immunological etiology of CIUE. However, due to case selection, it is not possible to draw definitive conclusions. To determine whether CIUE is truly a self-contained entity and to reveal the etiology of CIUE, the presence of selection bias should be resolved in further studies and prospective studies should be performed.

Towards standardized criteria for the diagnosis of chronic intervillitis of unknown etiology

Based on our thorough analysis of articles on CIUE published from 1987 through February 2017, we propose standardized criteria for diagnosing CIUE.

Characteristics of the intervillous infiltrates

The most important diagnostic criterion is the presence of an intervillous infiltrate containing predominantly mononuclear cells (Criterion I). Several groups suggested that these mononuclear cells in the intervillous space have a histiocytic phenotype.^{3, 9, 13, 18, 29-32, 35} The presence of histiocytes in the intervillous space can be confirmed with immunohistochemistry.³² In five publications is suggested that approximately 80% of the cells in the intervillous infiltrates should be CD68+ cells (supplemental table 2)^{3, 9, 29, 30, 33} (Criterion II), and the number of CD68+ cells in the intervillous space should be four-times higher than in normal placentas.³² 5% or more of the placental intervillous space should be occupied by intervillous infiltrates²⁹ (Criterion III). The intervillous infiltrates should be present in at least two out of three full-thickness sections of macroscopic normal-appearing placenta

parenchyma in third trimester placentas.⁴⁰ The required number of sections might not be available in first and second trimester samples, in these cases at least 5% or more of the placental intervillous space of the sampled specimen should contain intervillous infiltrates. The presence of a widespread infiltrate was used as an inclusion criterion in seven studies in our review and the severity of the infiltrate may be correlated with pregnancy outcome.² Severe intrauterine growth restriction and intrauterine fetal death were observed in cases with a severe infiltrate, and the perinatal prognosis was better in cases with a moderate infiltrate.² However, some groups reported that the intervillous infiltrate is less severe in cases complicated with intrauterine fetal death.³¹ The severity of the infiltrate in relation to clinical outcomes is an interesting topic for more in-depth research. In three studies it is suggested that the intervillous infiltrate should be of maternal origin,^{10, 34, 35} and this was confirmed in one study.²⁹ The maternal origin of the infiltrate is plausible, based on its location, but it is trivial for diagnosis criteria.

Co-occurring histopathological findings

The co-occurrence of different histopathological findings is not consistent in reported placentas with CIUE. This could be the result of chosen inclusion criteria in the various studies. The biggest discrepancy between studies is in the inclusion of cases with chronic villitis. Three different approaches were observed: placentas with co-occurring villitis were excluded,^{3, 7, 9, 16, 29} only placentas with focal villitis were included² or placentas with CIUE and villitis were studied.^{6, 7} Limited evidence is available to exclude cases with villitis. Therefore, cases with CIUE and co-occurring villitis should not be excluded. Presence of fibrin, trophoblastic necrosis, and/or atherosclerosis should also not be required for a diagnosis of CIUE, nor should it rule out the presence of CIUE. Nevertheless, co-occurring histopathological findings in CIUE could provide important insight into the pathophysiology of CIUE and further studies should comprehensively describe the co-occurring histopathological lesions in cases with CIUE.

Excluding infectious causes

Cases with clinical or histopathological signs of infection should be excluded (Criterion IV). In the event of an unfavorable pregnancy outcome (e.g., miscarriage, restricted fetal growth, preterm birth, or stillbirth), most women are screened for the presence of a TORCH infection (toxoplasmosis, treponema pallidum, rubella, cytomegalovirus, herpes virus, and other infections, including varicella, parvovirus B19, HIV, and enteroviruses).⁴¹ TORCH infections are a major contributor to prenatal, perinatal, and postnatal morbidity and mortality, and should therefore be excluded in the case of CIUE.⁴¹ Furthermore, one should discriminate between a polymorphic infiltrate and a monomorphic infiltrate; a polymorphic infiltrate containing neutrophils and leukocytes is indicative for acute inflammation. Cases with a predominant polymorphic infiltrate should be excluded. A pathologists' finding of evidence of infection is usually scarce; therefore, infectious causes should preferably be excluded based on clinical findings.

Maternal characteristics and obstetric features

In this review, we found that CIUE is reported in mothers of various ages and with a variety of obstetrical histories. Thus, neither the maternal characteristics nor the medical history should necessarily serve as either an inclusion or exclusion criterion. A prospective study should be conducted in order to demonstrate a clear association between the intervillous infiltrate and pregnancy outcome before an adverse pregnancy outcome can be used as an inclusion criterion. Since, associations found between intervillous infiltrates and pregnancy outcome might have been caused by selection bias. Associations between increased levels of maternal serum alkaline phosphatase and CIUE might provide an interesting new diagnostic approach, which should be investigated further.^{31, 42, 43}

Future validation

Although consensus does not yet exist regarding these diagnostic criteria, our results set the stage for establishing standardized criteria for diagnosing CIUE. The next step towards understanding the etiology of CIUE is to determine the precise criteria by performing a Delphi study.⁴⁴ Thereafter, these criteria should be validated in several patient populations; in a first step toward validation, the pathologists who contributed to the Amsterdam Placental Workshop Group Consensus Statement⁴⁰ could assess our proposed diagnostic criteria and examine the cases included in this review. Future studies regarding CIUE should attempt to determine the full spectrum of the syndrome.

Conclusions

Here, we propose a set of standardized diagnostic criteria for CIUE, a serious pregnancy complication frequently associated with miscarriage, impaired fetal growth, preterm birth, and a live birth rate of only 54.9%. The relatively high rate of recurrence in subsequent pregnancies and the accompanying pregnancy complications underscore the high clinical relevance of CIUE and the need for further in-depth research.

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	Fibrin deposits, %	Necrosis / trophoblast erosion, %	Atherosclerosis, %	Villitis, %	Infarcts, %
Labarrere and Mullen, 1987.	100	100	50	66	17
	100			17 focal, Excluded	
Jacques and Qureshi, 1993.	(50; patchy, 50; diffuse)	100	33	diffuse	33
	"In some specimens"				
Boyd and Redline, 2000.				Excluded	
Rota C et al, 2006.	18			25	
Parant O et al, 2009.				30	
	"not predominant"				
Traeder J et al, 2010.	100			25, focal	
	(27; low, 31; moderate, 11; elevated)				
Marchaudon V et al, 2011.					
	"varying degrees"	"varying degrees"		Excluded	
Capuani C et al, 2013.					
Labarrere CA et al, 2015.			58.8	47.1	
Nowak C et al, 2016.				76	

Supplementary Table 1 Co-occurring histopathological findings

Ten publications reported histopathological findings that might accompany CIUE. "Excluded" indicates that placentas with CIUE accompanied by villitis were excluded in this publication.

	Monocytes/ macrophages, % (SD) [range]	Activated immature monocytes, %	T-cells, % (SD) [range]	T helper- cells, % (SD) [range]	Type two helper- cells	cytotoxic T-cells, % (SD) [range]	Regulator v T-cells, % (SD)	B-cells, % (SD) [range]	NK-cells	Activated T- cells or B- cells
Boyd and Redline, 2000.	>90	30-40	<5		Not detected			Not detected	Not detected	
Traeder et al, 2010.	>90		few	14.4 (3.8)		17.1 (3.1)	5.3 (3.7)	4 (2.6)		
Capuani et al, 2013.	80 (6.9)		24 (5.7)							
Freitag et al, 2013.	"typical accumulation"		Not detected	few		few		Not detected	Not detected	few
Labarre et al, 2015.	83.5 [73.9- 89.2]		12.9 [8.2- 22.4]	5.1 [3.5- 7.1]		7.7 [4.7- 15.3]		0.7 [0- 2.4]		

Supplementary Table 2 Composition of the cell infiltrates

Five publications provided an overview of the composition of the cell infiltrate. Most of cells were monocytes/macrophages (e.g., CD68+ cells). Abbreviations; SD, standard deviation.

	Auto-immune disease, %	Coagulation disturbance, %	Allergy, %	Preeclampsia, %	Hypertension, %	Assisted reproduction, %	Obesity, %	Diabetes, %	miscarriage in medical history, %	Smoking, %	Other
Labarrere and Mullen, 1987.				17 (moderate)	33 (pregnancy induced hypertension)						
Jacques and Oureshi, 1993.	33 (systemic lupus erythematosus)			17 (severe), 17 (previous pregnancy)	17 (at delivery)			17	50		heroin abuse (17%), cocaine/alcohol abuse (17%), limited/no prenatal care (50%)
Boyd and Redline, 2000.	14		38; 24 (asthma), 14 (drugs)	5 (medical history)					38 (recurrent)		
Rota et al, 2006.	36	40 (4 out of 10)		12				4			
Parant et al, 2009.	Excluded			Excluded					14 (recurrent)		
Traeder et al, 2010.		25 (thrombophilia)		25 (HELLP-syndrome)		50			25		Anti-epileptic drugs (25%).
Marchaudon et al, 2011.	6	4; 2 (factor V Leiden)		7.7					24 (>3 before 22 weeks)	17.4	
Heller, 2012.											
Capuani et al, 2013.											
Freitag et al, 2013.			50				50		50 (recurrent)		
Reus et al, 2013.		9 (1 out of 11, factor V Leiden)	18	4.5	4.5 (pre-existing)			4.5		18	
Labarrere et al, 2014.				42.9			Excluded (BMI >30)			14.3	
Bendon et al, 2015.											
Labarrere et al, 2015.				52.9			Excluded (BMI >30)			11.8	
Mekinian et al, 2015.	29	4 (thrombosis)		0	8 (pre-existing)			0	16.6	12.5	
Revaux et al, 2015.	58; 33 (clinical), 58 (immunological screening)										
Novak C et al, 2016.	0			17						17	
Sabra et al, 2016.									66.3 (recurrent)	66.7	

Supplementary Table 3 Associated conditions

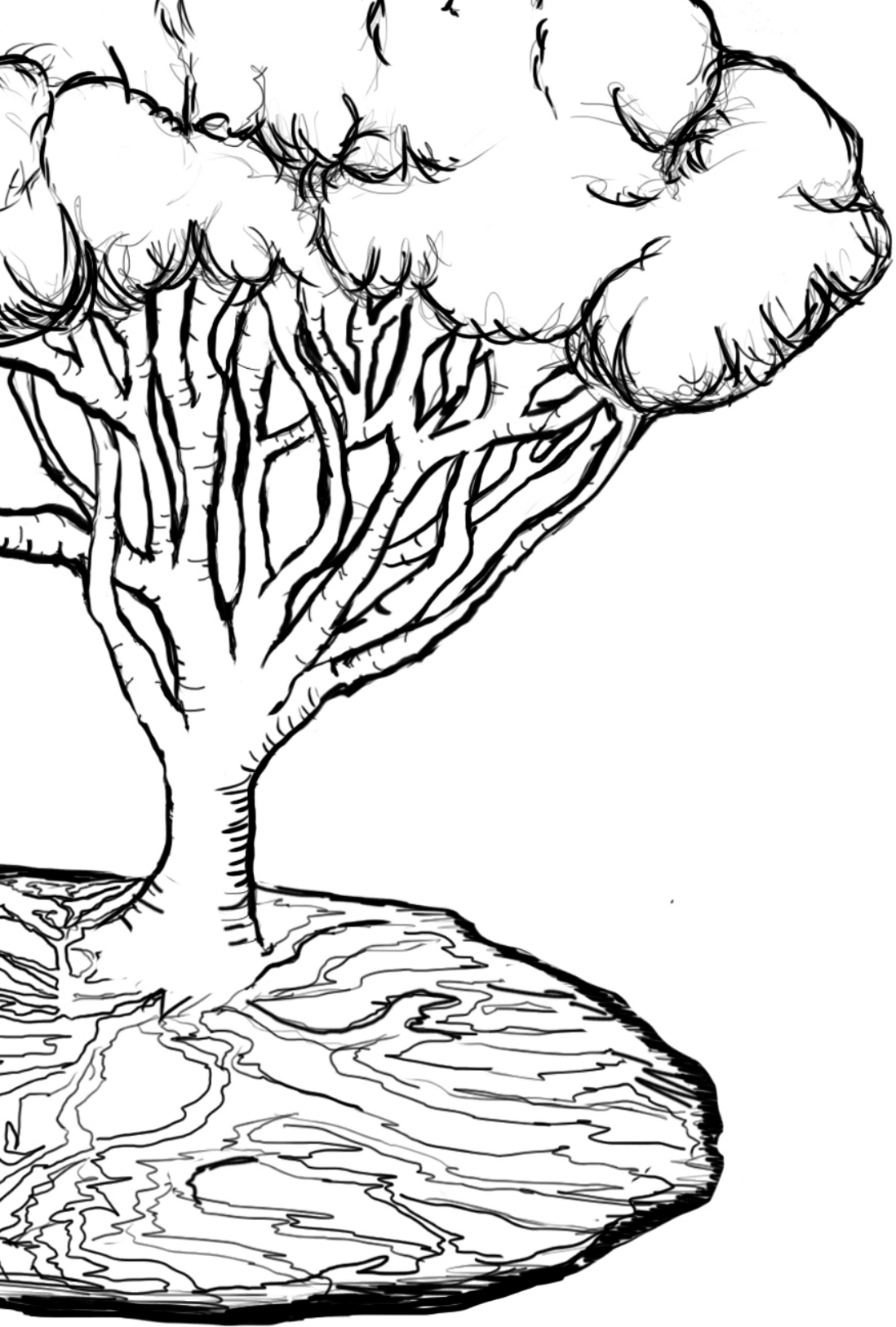
Several conditions and factors appeared to be associated with CIUE, including autoimmune disease, preeclampsia, and smoking. "Excluded" indicates that patients with the respective condition were excluded. Abbreviations; HELLP, hemolysis elevated liver enzymes and low platelets; BMI, body mass index.

	Pregnancies with an intervillous infiltrate	Patients with an intervillous infiltrate	Miscarriage, % (N+/Nt)	Late miscarriage, % (N+/N+ miscarriage)	IUGR, % (N+/Nt)	SGA, % (N+/Nt)	Term births, % (N+/Nt)	Pregnancies reaching term after 12 weeks of gestation, % (N+/Nt)
Labarrere and Mullen, 1987.	6	6	0.0 (0/6)		83.3 (5/6)	100.0 (6/6)	83.3 (5/6)	83.3 (5/6)
Jacques and Qureshi, 1993.	6	6	16.7 (1/6)	* 11.5 (3/26), GA 12-23 weeks	62.5 (5/8)	50.0 (3/6)	16.7 (1/6)	16.7 (1/6)
Boyd and Redline, 2000.	31	21	GA < 23 weeks				* 18	
Rota et al, 2006.	28	25				* 77		
Parant et al, 2009.	20	14	30.0 (4/20), * 25.0 (1/4) TOP	66.7 (2/4), * 50.0 (1/2) TOP	82.4 (14/17)		35.0 (7/20)	38.9 (7/18)
Traeder et al, 2010.	4	4	0.0 (0/4)			75.0 (3/4)	0.0 (0/4)	0.0 (0/4)
Marchaudon et al, 2011.	69	50	43.5 (30/69)	30.0 (9/30)	* 61.5 (24/39), <3th percentile		14.5 (10/69)	20.8 (10/48)
Heller, 2012.	9	8	100.0 (5/5)	100.0 (5/5)				
Capuani et al, 2013.	20	16	31.6 (6/19), * 50.0 (1/2), * 100.0 (1/1) TOP	83.3 (5/6)	64.7 (11/17)		5.9 (1/17)	6.3 (1/16)
Freitag et al, 2013.	2	2		* 100.0 (1/1), TOP		100.0 (2/2)	0.0 (0/2)	0.0 (0/2)
Reus et al, 2013.	27	22						
Labarrere et al, 2014.	7	7	0.0 (0/7)		85.7 (6/7)		85.7 (6/7)	85.7 (6/7)
Bendon et al, 2015.	32	28	12.9 (4/31)	100.0 (3/3), GA unknown in 1 miscarriage		43.3 (13/30)	44.8 (13/29)	44.8 (13/29)
Labarrere et al, 2015.	17	17	0.0 (0/17)			70.6 (12/17)	94.1 (16/17)	94.1 (16/17)
Mekinian et al, 2015.	24	24						
Revaux et al, 2015.	18	12	25.0 (3/12)		41.7 (5/12)			
Nowak C et al, 2016.	24	23	12.5 (3/24)	* 100.0 (3/3), GA 14-22 weeks	81.0 (17/21)	* 0.0 (0/21), <3th percentile		
Sabra et al, 2016.	6	6	66.6 (4/6)	0.0 (0/4)		100.0 (1/1)	0.0 (0/5)	0.0 (0/2)
Total number of pregnancies or patients			256	52	88	66	182	155
Number of pregnancies or patients with outcome			62	24	63	40	59	59
%			24.2	46.2	71.6	60.6	32.4	38.1

Supplementary Table 4 Overview of pregnancy outcome

Summary of the number of miscarriages, growth restriction, preterm births, stillbirths, neonatal mortality, and recurrence reported in each study. An asterisk ("*") indicates that the study used a definition of a specific outcome that was different than our own or did not provide adequate data for use in our overall analysis. Symbols; N+, number of pregnancies or patients with the indicated outcome; Nt, total number of pregnancies or patients. Abbreviations; IUGR, intrauterine growth restriction; SGA, small for gestational age; GA, gestational age; TOP, termination of pregnancy.

Stillbirth, % (N+/Nt)	Neonatal mortality, % (N+/Nt)	Live birth, % (N+/Nt)	Pregnancies resulting in a living child, % (N+/Nt)	Recurrence, % (N+/Nt)	Comment
16.7 (1/6)		83.3 (5/6)	83.3 (5/6)		
20.0 (1/5)	80.0 (4/5)	66.7 (4/6)	16.7 (1/6)		
	* 77			66.7 (6/9)	Term births and perinatal mortality are depicted for the total of 97 pregnancies these 21 women had.
16.0 (4/25)		75.0 (21/28)	* 32	8.0 (2/25)	Scarce information on clinical outcomes, one twin pregnancy
37.5 (6/16), 33.3 (2/6) TOP		50.0 (10/20)	56.0 (10/18)	* 100	
0.0 (0/4)	50.0 (2/4)	100.0 (4/4)	50.0 (2/4)		
46.2 (18/39)		30.4 (21/69)	43.8 (21/48)	18.0 (9/50)	
				20.0 (1/5)	Scarce history mentioned of 5 cases.
30.7 (4/13)		47.4 (9/19)	50.0 (9/18)	43.5 (7/16)	GA not depicted in 2 cases, no information depicted on outcomes and pregnancy characteristics in 1 case
0.0 (0/1)		50.0 (1/2)	50.0 (1/2)		case 1, 3 and 4 from Traeder et al.
					MCI was absent in 3 of 30 included placentas, it was not depicted in which one.
0.0 (0/7)		100.0 (7/7)	100.0 (7/7)		
37.0 (10/27)		53.1 (17/32)	56.7 (17/30)	17.9 (5/28)	GA not depicted 3 cases, stillbirth not depicted in 3 cases, SGA not depicted in two cases.
0.0 (0/17)		100.0 (17/17)	100.0 (17/17)		
				33.3 (8/24)	Clinical outcomes are not depicted for cases with MCI only.
77.8 (7/9)		16.7 (2/12)		50.0 (6/12)	Clinical outcomes of 12 index pregnancies.
19.0 (4/21), 50.0 (2/4) TOP		70.8 (17/24)	70.8 (17/24)	4.2 (1/24)	
				83.3 (5/6)	Mistake in table 1, it is unlikely that the gestational age of case 4 is 8 weeks with a birth weight of 2100 gram, we did not use this data to calculate term birth rate and pregnancies reaching term after 12 weeks of gestation.
190	9	246	180	199	
55	6	135	107	50	
28.9	60.0	54.9	59.4	25.1	



Clinical outcomes in chronic intervillitis of unknown etiology

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Abstract

Introduction

Chronic intervillitis of unknown etiology (CIUE) is a histopathological lesion of the placenta that is frequently accompanied by unfavourable pregnancy outcomes, e.g. miscarriage, fetal growth restriction (FGR) and intrauterine fetal death. Earlier described case series and cohorts have been based on diverse diagnostic criteria of CIUE. To improve our understanding of clinical outcomes associated with CIUE, we report the obstetric and perinatal outcomes in a cohort based on the recently described diagnostic criteria.

Methods

CIUE is defined as an infiltrate occupying 5% or more of the intervillous space with approximately 80% of mononuclear cells positive for CD68 in the absence of an infection. Thirty-eight cases were included. Also previous and subsequent pregnancies were described.

Results

Pregnancies accompanied by CIUE frequently resulted in FGR (51.6%) and pre-term birth (55.3%). Twenty-nine out of 38 pregnancies (76.3%) with CIUE resulted in a living baby. Women with CIUE frequently have had a miscarriage (16/38; 42%). Four-teen subsequent pregnancies in 8 women resulted in 2 miscarriages, 2 terminations of pregnancy for FGR, 1 early neonatal death and 9 living babies (9/14; 64.3%). Histopathologically confirmed CIUE recurred in 5 out of 10 subsequent pregnancies. Two pregnancies with recurrent CIUE were terminated, one pregnancy ended in a late miscarriage and another resulted in term birth complicated by FGR. Recurrent CIUE can also be accompanied by an uncomplicated pregnancy (1/5; 20%).

Conclusion

This study provides additional insight into the clinical phenotype of CIUE and emphasises the need for further research to understand the pathophysiology behind different pregnancy outcomes in CIUE.

Introduction

Chronic intervillitis of unknown etiology (CIUE) is a rare, poorly understood histopathological lesion which was first described by Labarrere and Mullen in 1987 as massive chronic intervillitis.¹ An intervillous infiltrate may occur in every trimester² and it is estimated that the lesion is found in approximately 1:10000 placentas.³ A variety of terms have been used to describe CIUE, including chronic intervillitis, chronic histiocytic intervillitis of unknown etiology, chronic histiocytic intervillitis, massive histiocytic chronic intervillitis, massive perivillous histiocytosis, intervillitis and massive chronic intervillitis.⁴ Although different criteria have been used to define CIUE,⁵ it has repeatedly been shown that an intervillous infiltrate of histiocytes is associated with adverse pregnancy outcome.⁵⁻⁷ Miscarriages, fetal growth restriction (FGR) and intrauterine fetal death (IUFD) are frequently associated with CIUE.⁵ Furthermore, a high recurrence rate of the intervillous infiltrate and associated adverse pregnancy outcomes, emphasises the clinical importance of CIUE.^{5, 7-9}

Previous studies of CIUE relied on diverse selection criteria and do not extensively report on pregnancy outcomes.⁵ In this study CIUE was defined as an infiltrate occupying 5% or more of the intervillous space of approximately 80% of mononuclear cells positive for CD68 in the absence of clinical or histopathological signs of an infection.⁵ Our objective was to report the obstetric and perinatal outcomes in a cohort of pregnancies affected by CIUE.

Methods

Patient selection

In this retrospective descriptive study, patient samples were selected from the pathology department of the University Medical Center Utrecht (UMCU) between 2000 and 2015 using the hospitals' pathology registry. In this time period, there were approximately 33200 (1700-2300/year) clinical deliveries at the UMCU and approximately 13300 (600-1150/year) placentas were studied at the pathology department. Placentas were sent to the pathology department when the pregnancy was complicated by pre-term birth, fetal growth restriction, pre-eclampsia, pregnancy induced hypertension, gestational diabetes, pre-term pre-mature rupture of membranes, asphyxia of the new born or intra uterine fetal demise (IUFD). Placentas were also studied from twin pregnancies, fetuses with (suspected) congenital defects and when the pregnancy was terminated. Between 2006 and 2007, placentas from uneventful pregnancies were also examined as part of a separate study by Houben *et al.*¹⁰ All placental samples in the UMCU were assessed according to a standardized protocol. From each placenta, samples were taken from the umbilical cord, fetal membranes and 3 full-thickness samples of normal-appearing placenta parenchyma. Samples reported as showing 'chronic intervillitis' were selected. Cases were reviewed by an experienced

pathologist and the diagnosis for CIUE was based on our previously described criteria.⁵ We defined CIUE as the presence of an infiltrate occupying 5% or more of the intervillous space with approximately 80% of mononuclear cells positive for CD68.⁵ Furthermore, clinical or histopathological signs of infection should be absent.⁵ Patient characteristics and pregnancy outcomes were obtained from the medical records. This study was approved by the UMCU biobank committee (TC-BIO number: 16-434). The pregnancy in which the diagnosis CIUE was made for the first time, was defined as the index pregnancy.

Clinical definitions

Miscarriage was defined as a spontaneous fetal loss within the first 24 weeks of pregnancy.^{11, 12} Early and late miscarriages were defined as a miscarriage ≤ 10 weeks and 10-24 weeks of gestation, respectively.^{11, 13} Recurrent miscarriage was defined as two or more pregnancy losses, either consecutive or not, before 24 weeks of gestation.¹¹ Termination of pregnancy (TOP) was an induced pregnancy loss within 24 weeks of gestation for medical reasons, for example fetal congenital anomalies, severe fetal growth restriction (FGR) or severe early-onset pre-eclampsia.¹⁴ Abortion was defined as an induced pregnancy loss within 24 weeks of gestation for psychosocial reasons.¹⁵ IUFD was defined as spontaneous fetal loss after 24 weeks of gestation. Early neonatal death refers to death during birth and within the first 7 days after birth. Term pregnancy was defined as birth from 37 weeks of pregnancy onwards, and preterm birth was defined as birth between 24-37 weeks of gestation. Fetal growth restriction (FGR) was defined as a birthweight below the 3rd percentile.^{16, 17} The overall fetal outcome was depicted by the number of pregnancies resulting in a living baby 7 days post-partum.

Statistical analysis

Where applicable, in the case of sufficient events, statistical analysis was performed to test significance between previous pregnancies, index pregnancies and subsequent pregnancies. For categorical outcomes within patients, repeated measures ordinal logistic regression was performed to test significance. For repeated continuous outcomes, a linear mixed model was used to test significance. $P \leq 0.05$ was considered statistically significant. Analyses were performed using the SPSS statistics software (version 23.0, Armonk, NY: IBM Corp).

Results

Selection of cases

Forty-five placentas with an intervillous infiltrate were identified from the UMCU pathology archives between 2000 and 2015. Three cases were excluded due to intercurrent infection, including parvo virus and rubella. Four twin pregnancies were excluded. Thirty-eight cases fulfilled the diagnostic criteria for CIUE.⁵

Previous pregnancies

Maternal characteristics and pregnancy outcome are depicted in Table 1. Most women with CIUE in the index pregnancy were multigravida (28/38; 26.3%). The 28 women who had a previous pregnancy, had 78 pregnancies between them. Out of these 78 pregnancies there were 10 pre-term deliveries, 1 IUFD and 27 miscarriages. Twenty-seven spontaneous miscarriages were observed in 16 women (16/38; 42.1%) and 6 out of 27 miscarriages were late miscarriages (6/27; 22.2%). Furthermore, 7 out of 38 women have experienced recurrent miscarriages (7/38; 18.4%; 2 to 4 miscarriages per women). Twelve babies were FGR (12/43; 27.9%). Two early neonatal deaths in the same women were observed. Forty-one out of 78 pregnancies resulted in a living baby (41/78; 52.6%) and 14 women had at least one uneventful pregnancy before the index pregnancy (14/38; 36.8%). The placenta was available for re-assessment in 6 out of 78 (7.7%) previous pregnancies and CIUE was found in 2 placentas (2/6; 33.3%).

	Previous	Index	Subsequent	
Maternal characteristics				
Maternal age, mean (SD) [Range]		34 (4.5) [24-43]		
Gravidity, median [Range]		3 [1-8]		
Primigravida, n (%)		10 (26.3)		
Parity, median [Range]		1 [0-5]		
Nulliparous, n (%)		16 (42.1)		
Pregnancy characteristics				
Number of pregnancies	78	38	14	Significance
Term, n (%)	34 (43.6)	13 (34.2)	8 (57.1)	
Gestational age [weeks + days], mean (SD [days]) [range [weeks + days]]	40+2 (10) [37+5 - 42+0]	38+5 (9) [37+0 - 41+2]	39+6 (11) [37+2 - 42+1]	
IUFD, n (%)	0	2 (15.4)	0	
Pre-term, n (%)	10 (12.8)	21 (55.3)	2 (14.3)	
Gestational age [weeks + days], mean (SD [days]) [range [weeks + days]]	34+1 (23) [27+1 - 36+6]	31+5 (24) [25+3 - 36+6]	34+2 (6) [33+4 - 34+6]	
IUFD, n (%)	1 (10.0)	1 (4.8)	0	
Gestational age <24wkn, n (%)	34 (43.6)	4 (10.5)	4 (28.6)	
Gestational age [weeks + days], mean (SD [days]) [range [weeks + days]]	13+0 (39) [6+0 - 22+0]	22+5 (8) [21+2 - 23+6]	15+6 (37) [9+0 - 21+5]	
Miscarriage, n (%)	27 (79.4)	1 (25.0)	2 (50.0)	
Late miscarriage, n (%)	6 (22.2)	1 (100.0)	1 (50.0)	NA
Abortion, n (%)	3 (8.8)	0	0	NA
TOP, n (%)	3 (8.8)	3 (75.0)	2 (50.0)	0.011
TOP for congenital problem, n (%)	2 (66.7)	1 (33.3)	0	NA
TOP for FGR, n (%)	1 (33.3)	2 (66.7)	2 (100)	NA
Hypertensive complications of pregnancy, n (%)	5 (6.4)	13 (34.2)	1 (7.1)	<0.001
Pregnancy induced hypertension, n (%)	1 (20)	5 (38.5)	1 (100)	NA
Pre-eclampsia, n (%)	4 (80)	8 (61.5)	0	NA
Fetal characteristics				
Number of live born fetuses	43 (55.1)	31 (81.6)	10 (71.4)	0.05
Male, n (%)	23 (56.1)	15 (48.4)	3 (30.0)	0.786
FGR, n (%)	12 (27.9)	16 (51.6)	3 (30.0)	0.058
Early neonatal death, n (%)	2 (4.7)	2 (6.5)	1 (10.0)	0.198
Overall fetal outcome				
Living baby 7 days after birth, n (%)	41 (52.6)	29 (76.3)	9 (64.3)	0.084

Table 1

Table to show pregnancy outcomes for women with CIUE in the index pregnancy according to pregnancy order; previous pregnancy, index pregnancy or subsequent pregnancy.

TOP, hypertensive complications of pregnancy and number of live born fetuses was associated with pregnancy order. NA; not applicable.

Index pregnancy

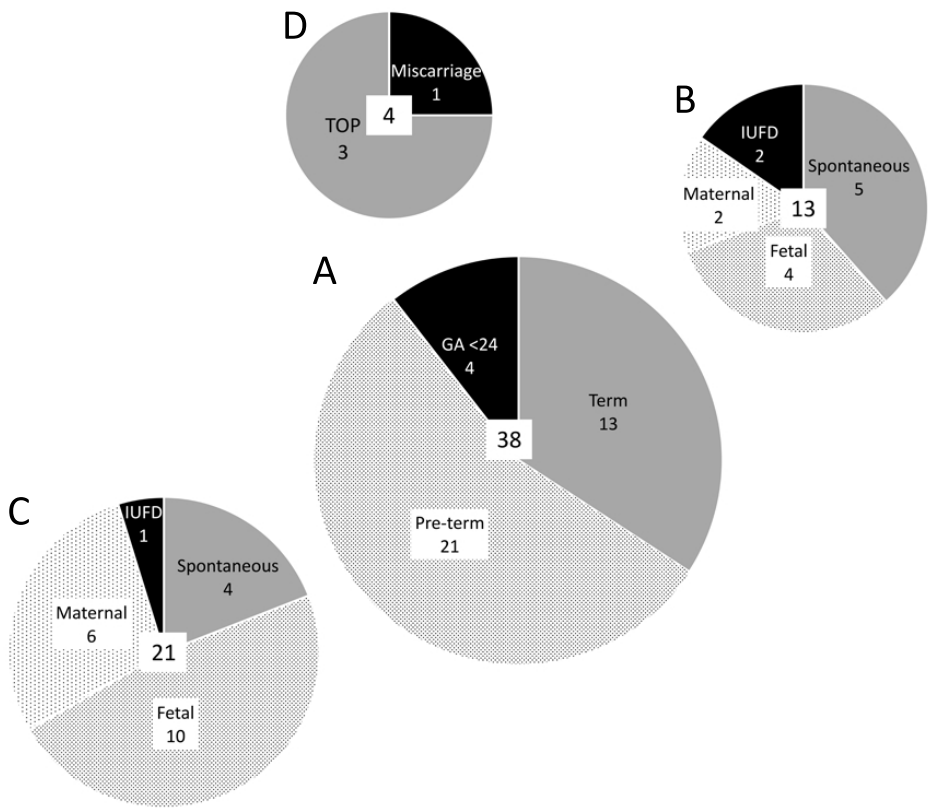
Out of 38 index pregnancies, 13 pregnancies resulted in term birth (13/38; 34.2%, Table 1, Figure 1 A and Figure 1 B), 21 fetuses were born pre-term (21/38; 55.3%, Figure 1 C) and 4 pregnancies resulted in a fetal loss before 24 weeks of gestation (4/38; 10.5%, Figure 1 D). Thirteen out of 38 pregnancies were complicated by hypertensive complications (34.2%, PIH or pre-eclampsia). Sixteen neonates were FGR (16/31; 51.6%). Five FGR neonates were born term and 11 FGR neonates were born pre-term. There were 2 early neonatal deaths: One baby died during induction of labour for severe pre-eclampsia at 25+6 weeks, weighing 580 grams. The other baby died 5 days post-partum, following an emergency caesarean section for fetal distress at 32+2 weeks, weighing 1240g. The 38 index pregnancies resulted in 29 living babies 7 days post-partum (76.3%).

Subsequent pregnancies

Follow-up information was available for 15 out of 38 women (39.5%). Seven of these 15 women did not have a further pregnancy after the index pregnancy. Eight women with previous CIUE had 14 subsequent pregnancies, which resulted in 2 miscarriages, 2 TOPs and 10 live births, but 9 living babies at 7 days post-partum (9/14; 64.3%). This early neonatal death was due to a congenital cardiomyopathy. This woman had experienced 2 earlier neonatal deaths due to a congenital cardiomyopathy as well. Seven out of 8 (87.5%) women who wished to get pregnant after the index pregnancy eventually had a pregnancy resulting in a living baby. None of the 9 successful pregnancies received prophylaxis against recurrent CIUE.

The placenta was analysed in 10 out of 14 subsequent pregnancies (71.4%) and recurrent CIUE was present in 5 placentas (5/10; 50%). Pregnancies accompanied by recurrent CIUE were observed in 3 women. The first woman had CIUE in 4 consecutive pregnancies with fetal losses. IUFD was diagnosed in combination with growth restriction in the index pregnancy at 22+1 weeks. Her second pregnancy was terminated for FGR at 21+5 weeks. During the third pregnancy, she was treated with aspirin and prednisone from 6 weeks of pregnancy. This pregnancy was terminated at 17+5 weeks for FGR. In her fourth pregnancy, the patient was treated with aspirin and low molecular weight heparin from 6 weeks of pregnancy. At 14+6 weeks, this pregnancy ended in a spontaneous miscarriage. The second woman had an index pregnancy which resulted in a FGR baby (2250g) born at 38 weeks. She has also had 3 miscarriages and one abortion. Her last pregnancy resulted in a healthy girl born at 37+4 weeks weighting 3255 grams. This last pregnancy was associated with CIUE and not treated. A third woman had 4 pregnancies in total of which 2 were accompanied by CIUE. Her first pregnancy was terminated for a psychosocial indication. Her second pregnancy was affected by CIUE and resulted in IUFD at 27+3 weeks with a baby weighting 450 grams. The third pregnancy was associated with CIUE and resulted in a FGR girl (2130g) at 37+2 weeks, without treatment. Last, this women had a pregnancy without histopathological confirmation of CIUE, which resulted in a preterm FGR baby (34+6 weeks, 1468g). This pregnancy was not treated as well. None of the

Figure 1 Pregnancy characteristics index pregnancies



A Twenty-one fetuses were born pre-term and 4 pregnancies ended before 24 weeks of gestation. **B** Four out of 6 iatrogenic births at term were for a fetal indication (66.7%; fetal distress, FGR or macrosomia associated with gestational diabetes). Maternal indications were pre-eclampsia and a caesarean section in the medical history. **C** In 10 out of 16 pre-term births induction of labour was for a fetal indication (10/16; 62.5%; fetal distress or FGR). Maternal indications were hypertensive complications of pregnancy, abruption of the placenta or a prolonged delivery. **D** Four pregnancies resulted in a fetal loss. One pregnancy ended in a late miscarriage, 2 pregnancies were terminated for severe FGR (associated with PIH) and 1 pregnancy was terminated for congenital defects.

pregnancies in these three women were accompanied by hypertensive pregnancy complications. TOP, hypertensive complications of pregnancy and number of live born fetuses was associated with whether a pregnancy was a previous pregnancy, index pregnancy or subsequent pregnancy (Table 1).

Discussion

The aim of this study was to investigate the clinical outcomes from pregnancies affected by CIUE. We identified 38 cases which fulfilled the diagnostic criteria for CIUE,⁵ also 78 previous pregnancies and 14 subsequent pregnancies were identified. Pregnancies affected by CIUE frequently resulted in FGR, pre-term birth and previous miscarriages are often observed in these women. Early neonatal survival was 76.3%. Recurrent CIUE was accompanied by FGR, miscarriage or the pregnancy was terminated due to severe FGR. However, a pregnancy with recurrent CIUE can also be without complications (1/5; 20%).

Other publications also mentioned FGR in pregnancies affected with CIUE,⁵ and in one study severity of the infiltrate was reported to associate with severity of FGR.⁶ FGR is tightly linked to chronic placental dysfunction and the intervillous infiltrate might contribute to a diminished exchange capacity in these placentas. Furthermore, CIUE is frequently associated with villitis of unknown etiology, massive perivillous fibrin depositions and trophoblast necrosis,⁵ which may also contribute to a diminished placenta function. Compared to other studies on CIUE, early neonatal survival seems higher in our study (76.3%).^{6, 8} Women with a pregnancy affected by CIUE had frequently had a previous miscarriage (16/38; 42.1%). Compared to women without CIUE in history, the frequency of miscarriage and recurrent miscarriages in our cohort of women with CIUE was 3 and 6 times higher, respectively.¹⁸⁻²⁰

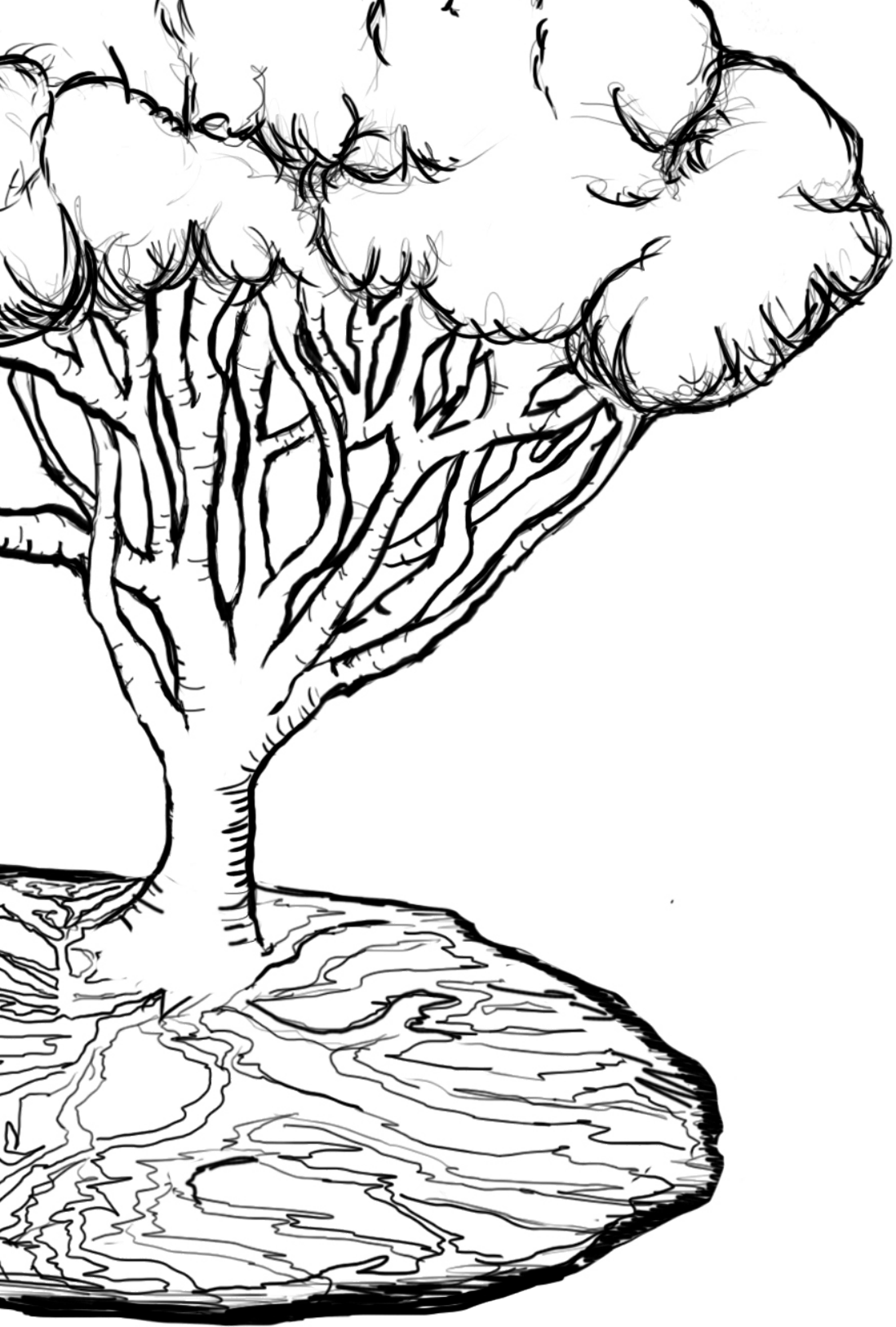
The pathophysiology of CIUE appears to be immunologically driven and several treatment strategies which suppress the immune system have been proposed for CIUE. Treatment with corticosteroids, hydroxychloroquine, intravenous immunoglobulin, aspirin and heparin have had variable efficacy to prevent recurrent CIUE.^{6, 7, 9, 21, 22} In our study, one patient was treated specifically for CIUE without an effect. Ten out of 14 (71.4%) subsequent pregnancies resulted in a living baby without treatment, although 3 out of 10 had FGR and 1 baby died within 7 days of birth. In 10 subsequent pregnancies the placenta was analysed, 5 had recurrent CIUE. Two pregnancies with recurrent CIUE were terminated for FGR, 1 pregnancy ended in a late miscarriage (14+3 weeks) and another resulted in term birth complicated by FGR. Recurrent CIUE can also be accompanied by an uncomplicated pregnancy without treatment (1/5; 20%). Henceforth, treatment for CIUE should be within the realms of well-conducted research and mindful of guidelines to prevent recurrent IUFD, FGR, pre-eclampsia or miscarriages.

Chronic intervillitis has been described before in cases with malaria, acute cytomegalovirus infection and dengue.²³⁻²⁵ We identified one case with a parvovirus infection and another with rubella virus infection early in pregnancy. These infections have not previously been described in association with a chronic intervillous infiltrate. These cases show again the importance of excluding an infectious cause when considering the diagnosis CIUE.⁵ Further research is needed to evaluate the diagnostic criteria for CIUE and should focus on inter-observer variability and associations between the intervillous infiltrate and clinical outcomes.

A strength of this study is the use of well-defined criteria to diagnose CIUE and associated clinical outcomes. Similar to most studies on CIUE, this study is limited by its retrospective design, which encourages selection bias. A retrospective design makes it more difficult to exclude for example, infectious causes for an intervillous infiltrate. Furthermore, indications for placenta examination and experience of the pathologist at a hospital also results in selection bias. The indications for placenta examination at our hospital can probably explain the significant association between TOP and hypertensive complications of pregnancy, and if a pregnancy was the previous, index or subsequent pregnancy. Interestingly, between 2006 and 2007, we studied 591 placentas from uncomplicated pregnancies¹⁰ and did not identify CIUE in any of these cases. It is unlikely therefore that we are overestimating the clinical impact of CIUE. Due to the presumed low incidence of CIUE, only a multi-center prospective study on placental histology in complicated and uncomplicated pregnancies could overcome selection bias and provide a clear answer to the prevalence of CIUE and the incidence of accompanied pregnancy complications. In conclusion, CIUE is often accompanied by unfavourable pregnancy outcomes, but can also be accompanied by an uncomplicated pregnancy. Our study provides additional insight into the clinical phenotype of CIUE. Therewith, this study emphasises the need for further research to understand the pathophysiology behind different pregnancy outcomes in CIUE.

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Summary and general discussion

Every pregnancy is unique due to distinct maternal, paternal and fetal factors. Presumably, the immunological response differs between pregnancies, and certain pregnancies could be immunologically more challenging for the mother than others. In the present thesis, pregnancies presumed to be more immunologically challenging were studied. For example, in oocyte donation pregnancies, the fetus is completely allogeneic for the mother. Therefore, these pregnancies provide an interesting setting in which to evaluate the effect of immunogenetic differences between the mother and the fetus on maternal adaptation to pregnancy. Based on findings from studies of pre-eclampsia and chronic intervillitis of unknown etiology (CIUE), it is postulated that the immune system plays a significant role in such pathophysiology. This thesis investigated oocyte donation pregnancies with and without pre-eclampsia and naturally conceived pregnancies complicated by pre-eclampsia or CIUE. The studies in this thesis aimed to examine maternal adaptation to pregnancy.

Placental thrombomodulin expression

Thrombomodulin is expressed on endothelial cells and the syncytiotrophoblast of the placenta. The diverse properties of thrombomodulin mediate coagulation, inflammation and cell survival. Based on the allogeneic nature of oocyte donation pregnancies, the role of the placenta in the pathogenesis of pre-eclampsia in oocyte donation pregnancies could be different from that in the pathogenesis of pre-eclampsia in naturally conceived pregnancies.¹⁻³ Hence, the expression, downstream effects, and regulation of thrombomodulin were studied in the placenta of women with pre-eclampsia after oocyte donation in **Chapter 2**. Thrombomodulin protein expression on syncytiotrophoblast was decreased in women with an oocyte donation pregnancy complicated by pre-eclampsia compared to that in women with uncomplicated *in vitro* fertilisation (IVF) pregnancies and uncomplicated naturally conceived pregnancies. Thrombomodulin mRNA expression was decreased in the placentas of oocyte donation pregnancies complicated by pre-eclampsia compared to those of uncomplicated oocyte donation pregnancies and uncomplicated naturally conceived pregnancies. Furthermore, thrombomodulin protein expression on the syncytiotrophoblast was also decreased in uncomplicated oocyte donation pregnancies compared with that in uncomplicated naturally conceived and uncomplicated IVF-induced pregnancies. Thus, placental thrombomodulin expression was decreased in both uncomplicated oocyte donation pregnancies and oocyte donation pregnancies complicated by pre-eclampsia. Loss of thrombomodulin was also associated with measurements of coagulation and inflammation. In oocyte donation pregnancies complicated by pre-eclampsia, thrombomodulin protein expression was associated with the number of fibrin deposits. In oocyte donation pregnancies complicated by pre-eclampsia, placental tissue factor and factor VIII mRNA expression were decreased compared to those in uncomplicated naturally conceived pregnancies. Therefore, in uncomplicated oocyte donation pregnancies,

placental factor VIII mRNA expression and tissue factor mRNA expression were associated with placental thrombomodulin mRNA expression, while in oocyte donation pregnancies complicated by pre-eclampsia, placental tissue factor mRNA expression was associated with placental thrombomodulin mRNA expression. This indicates that protective mechanisms were diminished in the placentas of uncomplicated oocyte donation pregnancies and oocyte donation pregnancies complicated by pre-eclampsia. Diminished endothelial protection in the placenta might be an explanation for the increased risk of pre-eclampsia in oocyte donation pregnancies (**Chapter 2**).

A previous study by Turner *et al.* suggested that pre-existing factors could contribute to the loss of thrombomodulin in the placenta. In this study, the extent of placental thrombomodulin loss was correlated with maternal body mass index (BMI), blood pressure and placental dysfunction.⁴ The presence of obesity and hypertension before pregnancy or early in pregnancy are clinical risk factors for pre-eclampsia^{5, 6} and both are known to influence the maintenance of the endothelium. When considering the diverse factors that contribute to the clinical syndrome of pre-eclampsia one can distinguish between placental, vascular and immunological risk factors. In **Chapter 2**, we studied a unique group of cases to evaluate the effect of the immunogenetic differences between the mother and fetus. It is indeed presumed that the maternal immune system needs to adapt differently or to a greater degree to an allogeneic fetus compared to a naturally conceived semi-allogeneic pregnancy.⁷⁻¹⁰ In the placentas of women who become pregnant after oocyte donation, signs of poor placentation and maternal to fetal reaction are more frequently observed, including, for instance, insufficient spiral artery remodelling, severe chronic deciduitis, villitis of unknown etiology and chronic intervillitis.^{3, 11-13} Functional and observational immunological studies have suggested that immunoreactivity is different between oocyte donation and naturally conceived pregnancies.^{14, 15} The decreased thrombomodulin expression in uncomplicated oocyte donation pregnancies found in this study could be caused by oocyte donation-specific immunoregulation. However, this could also be related to other pre-existing factors present in women who become pregnant through oocyte donation. Women who become pregnant after oocyte donation are often subject to an increased risk of several pregnancy complications due to advanced maternal age, primiparity, IVF and multiple gestations.^{5, 16-19} We found a negative association between thrombomodulin mRNA expression and maternal age in women with an oocyte donation pregnancy complicated by pre-eclampsia. No associations were found between placental thrombomodulin expression, parity, gravidity and maternal BMI. When placental thrombomodulin protein expression was studied in a group of advanced-aged mothers (>37 years) with an uncomplicated naturally conceived pregnancy, mainly a normal placental thrombomodulin protein expression pattern was found. The characteristics of women who became pregnant after IVF only differed from those of women with uncomplicated oocyte donation pregnancies in terms of maternal age, and in the placenta of women with uncomplicated IVF-induced pregnancies, the thrombomodulin protein expression pattern was normal as well (**Chapter 2**). Thus, placental thrombomodulin protein expression

was decreased in uncomplicated oocyte donation pregnancies, and this decrease was not caused by maternal factors related to oocyte donation and pre-eclampsia. In addition to predisposing vascular and immunological risk factors, pregnancy-specific factors can also contribute to the loss of placental thrombomodulin and endothelial maintenance in the placenta.

Experiments on mice have revealed that placental thrombomodulin expression is essential for a successful pregnancy. In heterozygous thrombomodulin-deficient mice (TM +/-), thrombomodulin mRNA and protein expression is 50% of that in wild-type mice. Heterozygous thrombomodulin-deficient mice are viable and free of thrombotic complications. However, homozygous thrombomodulin-deficient mice (TM -/-) die mid-gestation before the development of the cardiovascular system.²⁰ When thrombomodulin expression is restored in the extraembryonic tissues (i.e., trophoblasts of the placenta) of homozygous thrombomodulin-deficient mice, embryos develop normally through mid-gestation. However, later in gestation, homozygous thrombomodulin-deficient embryos develop consumptive coagulopathy, and none of them reach term.²¹ Based on these experiments, it is concluded that the expression of thrombomodulin in extraembryonic tissues is required for the development of the early placenta.²¹ Later in pregnancy, the endothelial expression of thrombomodulin is needed for the development of the cardiovascular system.²¹ Tissue-selective and temporary thrombomodulin gene inactivation results in a viable fetus. When the thrombomodulin gene is inactivated in female adult mice and this phenotype is rescued via the overexpression of a protein C transgene, coagulative complications do not occur.²² Interestingly, sudden mortality and severe morbidity due to coagulative complications occur during pregnancy in these mice.²² These mice were pregnant with fetuses with a thrombomodulin wild-type genotype. The phenotype manifested variably among them, and fetal development was arrested at various stages.²² The fetal development arrest suggests that maternal thrombomodulin deficiency interferes with fetal development before affecting maternal survival.²² Maternal mortality and morbidity in this mouse model is not yet fully understood, but it could be due to pregnancy-associated changes in haemostasis, haemostatic challenges of the placental vasculature or a maternal immune reaction towards the fetal thrombomodulin antigen.²² Thus, in addition to fetal thrombomodulin expression, sufficient maternal thrombomodulin expression is also needed for a successful pregnancy. These animal experiments mainly focused only on the anti-thrombotic effects of thrombomodulin, but none addressed its anti-inflammatory or complement regulatory effects.

The aforementioned experiments in mice showed that placental thrombomodulin expression is essential for a successful pregnancy. However, the precise role of thrombomodulin in the placenta is not yet understood. From *in vitro* experiments in endothelial cells, several factors have been identified that regulate thrombomodulin expression and degradation. For example, vascular endothelial growth factor (VEGF) results in the upregulation of thrombomodulin, whereas TNF α and shear stress result in its downregulation.²³⁻²⁵ However, the regulation of thrombomodulin expression in the placenta has not yet been extensively studied. In **Chapter 2**,

we focused on the effect of vitamin D on thrombomodulin expression in the placenta. A study describing cell culture experiments using aorta cells shows that the administration of vitamin D could increase thrombomodulin expression.²⁶ Therefore, decreased placental vitamin D receptor expression is associated with impaired vitamin D signalling and subsequent placental dysfunction and growth restriction.^{27, 28} Furthermore, vitamin D deficiency is associated with decreased pregnancy rates in recipients of oocyte donation and a decreased live birth rate in women pregnant after IVF/ICSI.^{29, 30} We showed that vitamin D receptor expression was decreased in the placenta of women with pre-eclampsia who became pregnant after oocyte donation. This implies the disruption of vitamin D signalling in these cases. The addition of vitamin D to cultured placental cells resulted in increased thrombomodulin mRNA expression (**Chapter 2**). Oral supplementation with vitamin D can increase vitamin D serum levels.³¹ Hence, we hypothesise that supplementation of vitamin D in women who become pregnant after oocyte donation could contribute to syncytiotrophoblast maintenance. Another approach could be treatment via the administration of recombinant thrombomodulin. The effect of recombinant thrombomodulin administration has been studied in a pre-eclamptic rat model. In this study, the administration of recombinant thrombomodulin resulted in the improved perfusion of fetal tissues and a decrease in the amount of circulating VEGF receptor-1 compared to that in untreated animals.³² In a mouse model of recurrent miscarriages, administration of recombinant thrombomodulin resulted in reduced fetal absorption.³³ Litter size and fetal weight were also increased in the treated animals.³³ More research is necessary to understand the role of thrombomodulin in the placenta as well as the non-coagulative properties of thrombomodulin. Moreover, the therapeutic effect of recombinant thrombomodulin in rat pre-eclampsia and mouse miscarriage models has set the stage for further exploration of the therapeutic effects of thrombomodulin on diseases characterised by placental dysfunction, such as pre-eclampsia.

Thrombomodulin in the kidney of women with pre-eclampsia

Thrombomodulin expression in the kidneys of women with pre-eclampsia has been discussed in **Chapter 3** of this thesis. The kidney is frequently affected in women with pre-eclampsia¹⁶, resulting in renal dysfunction caused by disruption of the glomerular filtration barrier.^{34, 35} For instance, increased levels of circulating anti-angiogenic factors lead to the decreased availability of VEGF in the glomerulus.^{34, 35} Many studies have indicated that impaired glomerular VEGF signalling leads to disruption of the glomerular filtration barrier, resulting in proteinuria and renal lesions comparable to aberrations seen in the kidneys of women with pre-eclampsia.³⁶⁻⁴¹ Pathological changes in the kidneys of women with pre-eclampsia are characterised by endotheliosis, podocyte foot process effacement, and podocyte loss.^{42, 43} However, what causes these changes is incompletely understood.

We found that glomerular thrombomodulin expression was significantly higher in the kidneys of women with pre-eclampsia than in the kidneys of nonpregnant hypertensive and pregnant normotensive controls. Additionally, glomerular

thrombomodulin expression was studied in rats exposed to sunitinib. This exposure results in disrupted VEGF signalling and a pre-eclampsia-like phenotype in a dose-dependent manner.⁴⁴ Glomerular thrombomodulin protein expression was increased in rats exposed to low and intermediate sunitinib doses, while renal thrombomodulin mRNA expression was increased in rats exposed to an intermediate sunitinib dose. In women with pre-eclampsia and rats exposed to sunitinib, both of which are characterised by impaired VEGF signalling, increased glomerular thrombomodulin protein expression was found (**Chapter 3**). In line with our findings, several studies have reported elevated serum levels of soluble thrombomodulin in women with pre-eclampsia,⁴⁵ which reflects the increased production and/or cleavage of thrombomodulin in the vascular endothelium.⁴⁶ As we previously showed that placental thrombomodulin is downregulated in women with pre-eclampsia,⁴ the renal endothelium may be the source of the increase in soluble thrombomodulin in the circulation. Increased renal thrombomodulin expression under antiangiogenic conditions such as pre-eclampsia or exposure to an angiogenesis inhibitor seems counterintuitive, as *in vitro* experiments have identified VEGF as a strong upregulator of thrombomodulin in endothelial cells.⁴⁷ Thrombomodulin expression can also be regulated via thrombin levels, shear stress and TNF α signalling.²³⁻²⁵ The precise regulation of thrombomodulin expression in glomerular endothelial cells remains to be elucidated to better understand the role of thrombomodulin and thrombomodulin dynamics in the glomerulus.

Histopathological analysis of kidneys of rats exposed to sunitinib revealed endothelial cell swelling in the kidneys of rats exposed to intermediate and high doses of sunitinib, whereas fibrin deposits were only seen in the glomerular capillaries of animals exposed to a high dose of sunitinib.⁴⁴ Renal lesions are preceded by an increase in glomerular thrombomodulin expression; renal histopathological changes are only seen in animals exposed to intermediate and high doses of sunitinib, while thrombomodulin is increased in animals exposed to low and intermediate doses of sunitinib. This could suggest that thrombomodulin expression is a mechanism to protect the glomerular filtration barrier against damage (**Chapter 3**). Glomerular thrombomodulin protein expression is also increased in glomerulonephritis and is reported to be associated with disease severity.⁴⁸ A study in diabetic mice revealed that the thrombomodulin-dependent activation of protein C plays an important role in the maintenance of the glomerular filtration barrier.⁴⁹

The effect of endothelin receptor antagonists was also studied in animals exposed to sunitinib. Systemic angiogenic imbalances result in the activation of endothelial cells and increased endothelin signalling.⁵⁰⁻⁵² Increased endothelin signalling is an important contributor to the development of hypertension in pre-eclampsia.^{53, 54} Furthermore, different experimental models of pre-eclampsia and angiogenesis inhibition have been shown to be responsive to endothelin receptor antagonists.^{53, 55} Treatment with sitaxentan (selective endothelin A receptor antagonist) as well as macitentan (dual endothelin A/B receptor antagonist) resulted in the normalisation of blood pressure, but only treatment with sitaxentan could decrease sunitinib-induced albuminuria.⁵⁶ Furthermore, only sitaxentan normalised the increase in

thrombomodulin mRNA expression in animals exposed to an intermediate sunitinib dose. Therefore, renal thrombomodulin mRNA expression and renal endothelin type A receptor mRNA expression are positively associated (**Chapter 3**). Based on these results, we hypothesise that thrombomodulin upregulation in the kidney and the renal protective effects of thrombomodulin may be mediated by the endothelin A receptor in a blood pressure-independent manner.

The administration of thrombomodulin could be an interesting approach to protect the renal endothelium in women with pre-eclampsia. Treatment with recombinant thrombomodulin improved fetal outcomes but not blood pressure in a pre-eclamptic rat model.³² Adeno-associated virus delivery of thrombomodulin improved renal outcomes in a type-2 diabetic nephropathy mouse model.⁵⁷ Albuminuria, renal interstitial inflammation and glomerular sclerosis were improved by the single administration of an adeno-associated viral thrombomodulin vector.⁵⁷ In humans, treatment with recombinant thrombomodulin can improve kidney function in severe lupus nephritis with thrombotic microangiopathy.⁵⁸ Thrombotic microangiopathy is a histopathological lesion that can be also be found in the kidneys of pre-eclampsia patients. Furthermore, recombinant soluble thrombomodulin has been used for the treatment of infection- and cancer-associated disseminated intravascular coagulation.⁵⁹ In a retrospective cohort study, the effect of recombinant thrombomodulin was evaluated in pregnant women with suspected disseminated intravascular coagulation. The majority of cases had postpartum haemorrhage or placental abruption; some cases of pre-eclampsia were also studied. In this small cohort, treatment with recombinant thrombomodulin significantly improved the platelet counts, D-dimer levels, fibrinogen levels and PT-INR.⁶⁰ However, the administration of recombinant thrombomodulin failed to improve the function of both the liver and kidney.⁶⁰ Treatment with recombinant thrombomodulin could be an interesting approach for use in women with pre-eclampsia to protect the endothelium and improve the fetal condition. A study in a more homogeneous cohort would reveal the safety and efficacy of recombinant thrombomodulin in pregnancies complicated by pre-eclampsia.

Pre-eclampsia-related kidney pathology and other manifestations of systemic endothelial dysfunction in women with pre-eclampsia are generally reversible. However, a proportion of women who have had pre-eclampsia are at risk of developing cardiovascular and kidney diseases later in life. For instance, women who have suffered from pre-eclampsia have a 5- to 12-fold increased risk of developing end-stage renal disease later in life.⁶¹ A nationwide study in Norway identified pre-eclampsia as an independent risk factor for the development of end-stage renal disease.⁶²⁻⁶⁴ A systematic review and meta-analysis showed that 31% of women who had pre-eclampsia developed microalbuminuria 7.1 years postpartum, which represents a four-fold increased risk compared to that of women with uncomplicated pregnancies.⁶⁵ It might be the case that the degree of endothelial damage during an episode of pre-eclampsia is related to the risk of developing renal or cardiovascular complications later in life. This possible relationship between the severity of endothelial damage and cardiovascular

complications later in life needs further investigation; for example, there is a need to investigate pregnancy and postpartum soluble thrombomodulin levels in relation to the development of cardiovascular diseases or kidney dysfunction later in life. Furthermore, the administration of recombinant thrombomodulin after a complicated pregnancy might be a promising treatment method to prevent cardiovascular disease later in life.

Reactive species interactome in oocyte donation pregnancies

Based on the allogeneic conditions observed in oocyte donation pregnancies, we evaluated the reactive species interactome status in naturally conceived and oocyte donation pregnancies in the absence and presence of pre-eclampsia in **Chapter 4** of this thesis. Hypoxia, inflammation and vascular stress are accompanied by the aberrant production of reactive oxygen species, and the pre-eclamptic placenta is characterised by an increased production of reactive species.⁶⁶⁻⁶⁸ Interestingly, in allograft organ transplantation, it has been shown that the systemic redox status predicts graft survival and mortality,⁶⁹⁻⁷¹ and the reduction of oxidative damage is associated with an improved kidney transplant outcome.⁷² Based on the allogeneic conditions in oocyte donation pregnancies, oocyte donation may translate into alterations in the concentrations of the constituents of the reactive species interactome compared with those in normal pregnancies. Hence, one would expect an increase in oxidative stress in oocyte donation pregnancies. However, the total free thiol and total 8-iso-prostaglandin F_{2α} concentrations were unchanged in uncomplicated oocyte donation pregnancies compared to those in uncomplicated naturally conceived pregnancies. Curiously, increased nitrite concentrations were observed in uncomplicated oocyte donation pregnancies compared to those in naturally conceived pregnancies. Diastolic blood pressure was slightly higher in uncomplicated oocyte donation pregnancies than in uncomplicated naturally conceived pregnancies. Furthermore, the diastolic blood pressure is inversely associated with protein-bound NO (RxNO) concentrations in uncomplicated naturally conceived pregnancies (**Chapter 4**). RxNO functions as an NO storage pool,⁷³ and decreased RxNO levels may reflect the increased utilisation of this alternative source of NO. The importance of NO in the regulation of blood pressure in pregnancy was also shown in an animal experiment in which NO production was blocked in virgin and pregnant rats. The blockage of NO synthases resulted in the development of hypertension in pregnant as well as virgin rats.⁷⁴ Increased nitrite concentrations in uncomplicated oocyte donation pregnancies, as shown in **Chapter 4**, could influence blood pressure regulation in these pregnancies and prevent the development of pre-eclampsia.

Both naturally conceived and oocyte donation pregnancies complicated by pre-eclampsia appear to be characterised by systemic redox stress; total free thiol levels and nitrite concentrations were significantly lower, while RxNO and sulfate concentrations were significantly higher in pregnancies complicated by pre-eclampsia compared to those in uncomplicated pregnancies. These changes have been described before⁷⁵⁻⁷⁷, but the literature is inconsistent.^{78, 79} Reactive species interactome measurements were not different between naturally conceived

and oocyte donation pregnancies complicated by pre-eclampsia (**Chapter 4**). In both naturally conceived and oocyte donation pregnancies complicated by pre-eclampsia, the reactive species interactome could be a link between placental dysfunction and systemic endothelial dysfunction during the development of pre-eclampsia.⁸⁰

Readouts of the reactive species interactome could be valuable predictors of pre-eclampsia. It is of high interest to develop reliable predictors for the development of pre-eclampsia. This would give every patient the needed care in time and prevent overtreatment, especially due to the unpredictable clinical course of pre-eclampsia. Currently, anti-angiogenic markers are used for the prediction of the short-term absence of pre-eclampsia, but the positive predictive value of sFlt-1/PIGF is limited.⁸¹ Other possible biomarkers are endoglin, TGF- β , cytokines and fetal mRNA/DNA levels in the maternal circulation. Their predictive value has not yet been investigated. Readouts of the reactive species interactome could also be useful as a biomarker of other syndromes causing placental dysfunction.⁶⁸ For example, free thiols are associated with graft survival in renal transplants.⁶⁹ A study of the predictive value of markers of the reactive species interactome has not yet been performed but should be considered. It would also be interesting to find a marker to predict the complications of pre-eclampsia later in life, such as cardiovascular problems or renal dysfunction. Perhaps markers of the reactive species interactome would be useful for this purpose as well.

Diagnostic criteria for chronic intervillitis of unknown etiology

Chapter 5 aimed to formulate diagnostic criteria for chronic intervillitis of unknown aetiology (CIUE) to improve research on CIUE and contribute to clinical care for affected patients. It also provided an overview of histopathological findings, maternal characteristics and clinical findings. While previous studies used diverse criteria to select cases, the presence of an intervillous infiltrate was the common criterion used by all groups. Studying the overall outcomes of cases in various studies showed that CIUE is associated with unfavourable pregnancy outcomes. Miscarriage was reported in 62 out of 256 (24.2%) described pregnancies. Impaired fetal growth was reported in nearly 65% of pregnancies. Only 59 out of 182 pregnancies (32.4%) resulted in term birth, while 55 out of 190 pregnancies ended in stillbirth (28.9%). Six neonatal deaths were documented. The rate of recurrence among 199 women was 25.1%, ranging from 4.2% to 100%. A high recurrence rate underlines the clinical importance of diagnosing CIUE. The described obstetric outcomes vary among studies, and the statuses of individual patients range from having mildly complicated pregnancies to pregnancies recurrently resulting in late miscarriage or intrauterine fetal demise.

Based on our thorough analysis of previous studies, we formulated the following diagnostic criteria for CIUE.

Inclusion criteria

Criterion I: An infiltrate is present in the intervillous space.

Criterion II: Approximately 80% of the mononuclear-cells in the intervillous space are CD68-positive cells.

Criterion III: The intervillous space should be occupied by an infiltrate for 5% or more.

Exclusion criterion

Criterion IV: Cases with clinical or histopathological signs of infection should be excluded.

A wide consensus does not yet exist for these criteria, and a consensus should be established in future projects. Defining the diagnosis criteria is the first step in improving research on CIUE and will eventually lead to the improvement of clinical care for patients with pregnancy accompanied by CIUE. In a clinical setting, a diagnosis should have a prognostic value for subsequent pregnancies. It should eventually provide direction for treatment. In the research setting, uniform diagnosis criteria are especially important to be able to compare different cohorts of patients.

The drawbacks of our criteria are the low cut-off point for the number of CD68-positive cells and the absence of criteria involving other histological changes in the placentas. Evidence for the relationship between the number of CD68-positive cells and clinical outcomes is not very convincing.⁸² Therefore, a low cut-off point is indicated for studying CIUE to enable the investigation of the full clinical spectrum of cases accompanied by intervillous infiltrate instead of only cases with more obvious intervillous infiltrate. When a strong correlation is found between the number of intervillous cells and clinical outcomes, the cut-off point can be changed. CIUE is frequently associated with villitis of unknown etiology, massive perivillous fibrin depositions and trophoblast necrosis.⁸³ The relationship of CIUE with other histopathological changes should be investigated and could provide useful insights into the pathophysiology of CIUE.

Clinical phenotype of chronic intervillitis of unknown etiology

As the clinical phenotype of CIUE is not fully understood, we conducted a case study of a cohort selected based on our previously defined diagnosis criteria (Chapter 5). In Chapter 6, we extensively described the obstetric characteristics of 38 women with at least one pregnancy accompanied by CIUE. The 38 women had 78 previous and 14 subsequent pregnancies. Pregnancies accompanied by CIUE frequently resulted in fetal growth restriction (16/31; 52%) and preterm birth (21/38; 55.3%). Twenty-nine out of 38 pregnancies with CIUE resulted in a living baby (76.3%). It was also noted that women with a pregnancy affected by CIUE frequently had multiple miscarriages (16/38; 42.1%). Recurrent CIUE can also be present in uncomplicated pregnancies. As in Chapter 5, we conclude from the findings in Chapter 6 that CIUE shows a broad spectrum of clinical effects. This

study emphasises the need for further research to understand the pathophysiology contributing to the different pregnancy outcomes in CIUE.

Based on our findings, we can debate whether CIUE is truly a self-contained entity or an epiphenomenon in some complicated pregnancies. The presence of CIUE in uncomplicated pregnancies and the discrepancy between clinical outcomes and the recurrence of placental lesions in subsequent pregnancies suggests that CIUE could be an epiphenomenon in some pregnancies. A discrepancy between the presence of CIUE and clinical outcomes has been suggested before⁸⁴, while its recurrence has been described in many cases.⁸⁴⁻⁸⁷ In case-control studies, clinical outcomes in patients with CIUE are generally worse than those in the chosen control groups.^{88, 89} Although there seems to be an association between the degree of infiltrate and fetal growth restriction,⁸² this has not yet been confirmed in other cohorts. A prospective cohort study of placentas from complicated and uncomplicated pregnancies should be performed to firmly determine whether CIUE is a self-contained entity. Between 2006 and 2007, 591 placentas from uncomplicated pregnancies were studied at the Utrecht University Medical Center⁹⁰; CIUE was not found in any of them.

The pathophysiology of CIUE appears to be immunologically driven. However, it is unclear whether the accumulation of maternal CD68-positive cells in the intervillous space is a cause or a consequence of another pathophysiological process.^{86, 91-95} The presence of complement deposition in the placenta supports the hypothesis that CIUE is an immune-based pathophysiology.^{82, 86, 96} Mixed lymphocyte cultures suggest a pathophysiological basis for human leukocyte antigen mismatches between the mother and fetus.⁹³ CIUE has also been found in cases of fetal and neonatal alloimmune thrombocytopenia.^{97, 98} This association supports the conclusion that CIUE is an allo-immune pathophysiology. Although the precise immunological pathophysiology underlying CIUE remains elusive, several treatment strategies that suppress the immune system have been proposed. Treatment regimens with corticosteroids, hydroxychloroquine, intravenous immunoglobulin, aspirin and heparin have had variable efficacy in preventing recurrent CIUE.^{82, 84, 85, 99-104} More research on the etiology of CIUE is warranted and will contribute to the development of more evidence-based treatment opportunities.

In **Chapter 6**, four twin pregnancies with CIUE were excluded from the study. Dichorionic twins with different genders were observed in 3 out of 4 of the pregnancies. Reassessment of the histology slides revealed that in 3 out of the 4 twin pregnancies, CIUE was only present in the placenta of one of the fetuses. These 3 cases could provide new insights into the pathophysiology of CIUE. For example, it is possible to correlate the presence of an intervillous infiltrate with fetal outcome. Therefore, it would be interesting to investigate human leukocyte antigen (HLA) mismatches between the fetus and mother in these cases.

Future perspectives

The immunological paradox of pregnancy has been recognised for decades. However, a conclusive hypothesis that can fully address its complexity has not yet been proposed. Approaching the problem from different angles and actively encouraging scientific cross-pollination will contribute to eventually unravelling the complex interaction between the mother and fetus. The work described in this thesis focused specifically on pregnancies that were assumed to be immunologically challenging.

Studying the fetal-maternal interface throughout pregnancy

Pregnancy is a dynamic process in which a pregnant woman's body experiences anatomical and physiological changes to adapt to pregnancy in such a way that homeostasis is maintained and fetal growth is facilitated. Adaptation to pregnancy starts just after conception and evolves throughout pregnancy. Investigating pregnancy at different organic levels (e.g., molecule, cell, organ, organism, and population) and at different time points during pregnancy is the next step to better understand the different physiological and pathophysiological changes that occur.

Several imaging techniques can be used to investigate organ function and fetal development throughout pregnancy. Ultrasound examinations can be used to follow fetal growth, and fetal growth restriction could indicate placental dysfunction.¹⁰⁵ Improved ultrasound techniques also allow the measurement of placental vascular flow in great detail. For example, the detection of diminished placental vascular flow in the second trimester has the potential to indicate women at risk for pre-eclampsia.¹⁰⁶ New imaging techniques also enable the study of cellular and molecular changes in the tissue of interest. Antibody-based tracers for PET (positron emission tomography) and SPECT (single-photon emission computed tomography) can be used to follow immune cells and trace endothelial cells, cytokines or enzymes *in vivo*.^{107, 108} These antibody-based tracers could be used to monitor the presence of immune cells at the fetal-maternal interface, which will provide insight into how immune cell levels change throughout pregnancy. PET- and SPECT-based techniques are not very suitable for use in pregnant women due to the radiation load, but they can be used in animal models. Antibody-based tracers have also been developed for MRI and ultrasound.¹⁰⁹ Once tracers are found to be safe to use in pregnant women, they will tremendously improve the opportunities to monitor the dynamics at the fetal-maternal interface at the cellular and molecular levels during pregnancy.

Collection of urine and peripheral blood samples is minimally invasive and can provide information on immune cell dynamics and organ function via different biochemical readouts. Both types of samples can be collected at different times during pregnancy. Urine samples can be used to measure angiogenic factors or components of the reactive species interactome.^{110, 111} Mass spectrometry allows the measurement of many different metabolites at the same time in urine and peripheral blood. As a hypothesis-free technique, it may also lead to the

discovery of new pathways of interest. Podocytes that detach from the glomerular filtration barrier can be isolated from urine¹¹² and used for cell culture experiments, enabling us to gain new insights into podocyte biology and renal dysfunction during pre-eclampsia.

In peripheral blood, the amounts and phenotypes of different maternal immune cells can be determined.¹¹³ Techniques such as mass cytometry allow for the study of up to 40 immune cell markers at the same time.¹¹⁴ Therefore, immune cells in peripheral blood are easily isolated, allowing for the study of their functionality and reactivity. For instance, the fetus-specific alloreactivity of maternal peripheral blood leukocytes can be measured in a mixed leukocyte reaction⁸, and the reactivity of peripheral blood immune cells to different stimuli can be determined. It is also possible to retrieve fetal cells from the maternal peripheral blood. Significant numbers of placental cells and cell fragments are released into the maternal circulation throughout pregnancy.¹¹⁵⁻¹¹⁸ In clinical practice, circulating cell-free fetal DNA analysis is already used to detect trisomies 21, 18 and 13.¹¹⁹ Furthermore, it is possible to detect cell-free fetal RNA transcripts in the maternal circulation. A combination of cell-free RNA analysis with data from single-cell transcriptomics can provide useful information on placental cell dynamics during pregnancy.¹²⁰ RNA transcriptomes from different subsets of placental cells from healthy and complicated pregnancies at different gestational ages can be used to investigate placental function on a cellular level throughout pregnancy.¹²⁰ Another approach to collect placental cells during pregnancy is TRIC (trophoblast retrieval and isolation from the cervix). Between 5 and 20 weeks of pregnancy, trophoblast cells can be collected via a cervix smear.¹²¹ From endocervical specimens, trophoblasts are isolated using anti-HLA G antibodies coupled to magnetic nanoparticles.¹²² Approximately 750 trophoblast cells can be isolated per sample.¹²² The isolated trophoblasts are of an extravillous phenotype.¹²² As a minimally invasive method, TRIC could be feasible for noninvasive prenatal screening.¹²² Therefore, isolated trophoblasts can be used in cell culture experiments. Experiments with trophoblasts retrieved by TRIC at different times during pregnancy could provide new insights into the cellular dynamics of trophoblasts throughout pregnancy. The aforementioned approaches for studying the fetal-maternal interface during pregnancy will contribute to increasing the knowledge of placental function, immunological adaptation and endothelial dysfunction. In particular, retrieving maternal and fetal cells throughout pregnancy is of high interest. These cells can be used for functional tests to provide insights into the dynamics at the fetal-maternal interface on a cellular level.

Paradoxical pregnancy complications

Some pregnancy complications provide an interesting setting to investigate the dynamics at the fetal-maternal interface more in-depth.

Pregnancy complications characterised by alloimmunisation set the stage for understanding maternal immunological tolerance against fetal epitopes. Allo-antibodies associated with pregnancy can be directed against HLA or antigens on erythrocytes or platelets.¹²³⁻¹²⁵ In particular, fetal and neonatal alloimmune

thrombocytopenia (FNAIT) caused by maternal allo-antibodies directed against fetal/paternal human platelet antigens is an interesting disease to study.¹²⁶ Allo-antibodies involved in FNAIT can cause thrombocytopenia and mild to severe bleeding complications in the fetus and neonates.¹²⁶⁻¹²⁸ HPA-1a, the most important antigen involved in FNAIT, is expressed not only on platelets but also on endothelial cells and syncytiotrophoblast of the placenta.¹²⁹⁻¹³¹ A high proportion of children with FNAIT have a reduced birth weight,^{132, 133} and associations of FNAIT with miscarriages and intrauterine fetal demise have been suggested in the literature. It might be the case that the interaction between maternal anti-HPA-1a alloantibodies and fetal HPA-1a on placental tissue leads to placental dysfunction, causing fetal growth restriction.¹³⁴ This was suggested by a FNAIT mouse model, in which immunised mice had pups with significantly decreased fetal weight and suffered from more miscarriages.¹³⁵ Studying the placentas from FNAIT cases can provide new insights into the effect of allo-antibodies directed against epitopes on placental cells.

Ectopic pregnancies are interesting for the study of placentation without the presence of the endometrium and placental function without the presence of decidual cells. Ectopic pregnancy refers to a pregnancy occurring outside of the uterus. Approximately 1% of pregnancies are ectopic and are an important cause of maternal mortality and morbidity.¹³⁶ Most commonly, ectopic pregnancies are seen in fallopian tubes. An unnoticed ectopic pregnancy might result in the rupture of the fallopian tube with consequent severe bleeding. The use of transvaginal ultrasound and the quantitative measurement of beta-hCG have led to the earlier diagnosis of ectopic pregnancies in fallopian tubes and hence earlier intervention.¹³⁶ Approximately 8% of ectopic pregnancies are non-tubal and are found in the cervix, a caesarean scar, the ovary or the abdominal cavity. Nontubal ectopic pregnancies are associated with high morbidity due to a late clinical presentation and diagnostic difficulties.¹³⁷ Interestingly, ectopic pregnancies in the abdominal cavity can reach full term and may result in a viable neonate.^{138, 139} When an ectopic pregnancy is removed by surgical intervention, the tissue can be studied using histopathological techniques to evaluate the phenotypes of trophoblasts without interaction with maternal endometrial cells/decidua.

Pregnancies complicated by the presence of placenta accreta, increta and percreta provide a setting to study the placenta when it has a more invasive phenotype. In these pregnancies, the placenta either grows into or through the myometrium. Major risk factors for placenta accreta include a history of caesarean section, previous uterine surgery and placenta previa.¹⁴⁰ Changes in trophoblasts observed in these cases are probably secondary to the unusual myometrial environment and not a primary defect of trophoblast biology leading to the excessive invasion of the myometrium.¹⁴¹ Studying placenta accreta cases could provide new insights into the interaction between trophoblasts, decidual cells, and maternal immune cells.

Conclusion

The work described in this thesis focused on the nature of immunologically challenging pregnancies and provides new insights into uncomplicated oocyte donation pregnancies and pregnancies complicated by pre-eclampsia or CIUE. Investigating immunologically challenging pregnancies improved our understanding of naturally conceived pregnancies and provided an interesting setting in which to test hypotheses relevant to transplantation immunology and tumour immunology.^{9, 142, 143} Henceforth, new discoveries in reproductive immunology could result in novel insights into the dynamics of the immune system. Future research should focus on more continuous study of the fetal-maternal interface during pregnancy and exploit the unique circumstances provided by some of the specific complications of pregnancy, such as FNAIT, ectopic pregnancies and placenta accreta.

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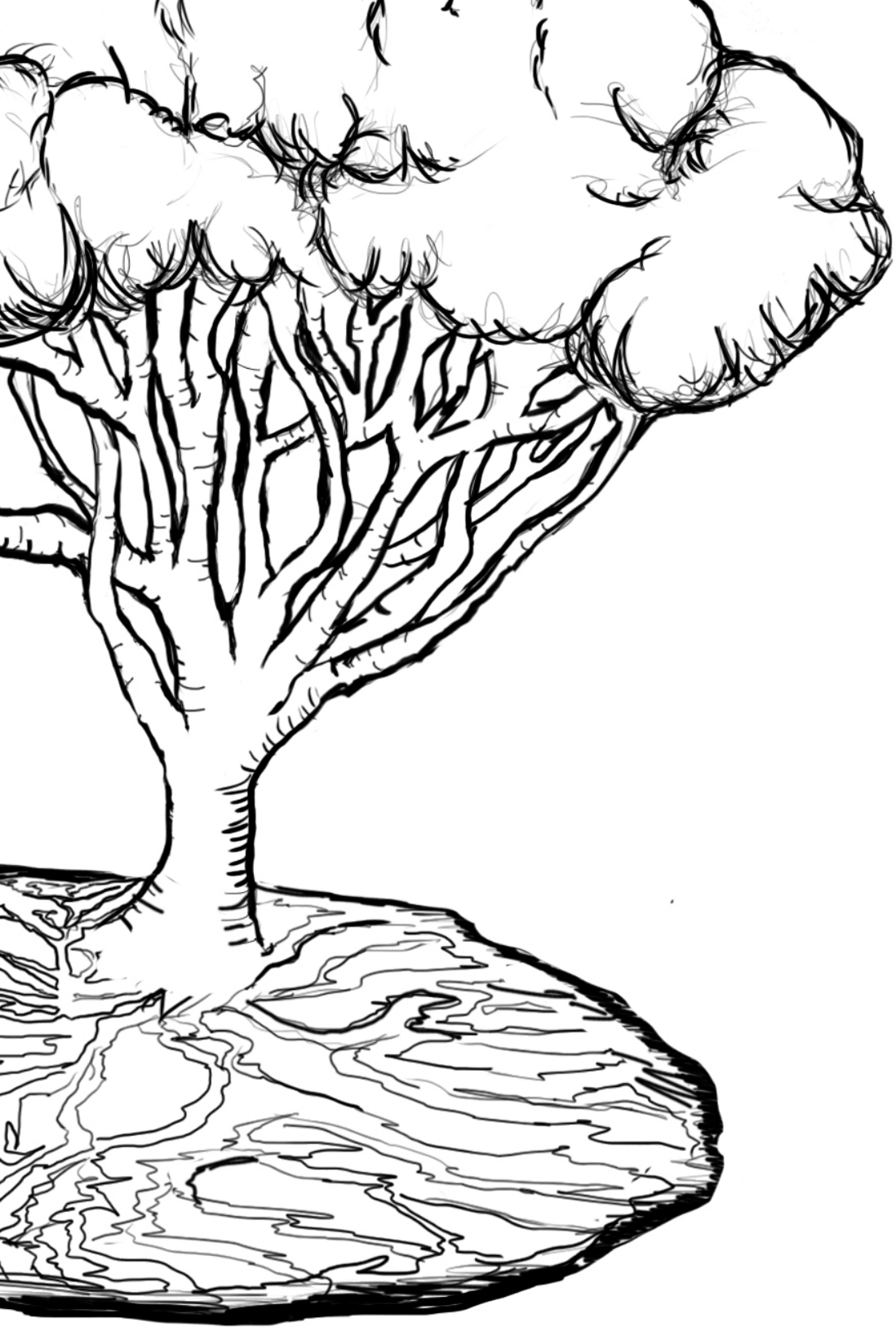
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Nederlandse samenvatting

De immunologische paradox van de zwangerschap

Vanuit een immunologisch perspectief is zwangerschap een paradoxale situatie. De foetus kan worden beschouwd als gedeeltelijk lichaamsvreemd (semi-allogeen), aangezien zowel genen van de moeder als van de vader door de foetus tot expressie worden gebracht. Desondanks wordt de foetus gedurende de zwangerschap niet afgestoten door het immuunsysteem van de moeder. De eerste wetenschapper die deze immunologische paradox in 1953 beschreef was Peter Brian Medawar. Samen met zijn collega Rupert Billingham gaf Medawar de volgende mogelijke verklaringen voor het feit dat een zwangerschap toch kon slagen: de moeder en foetus zijn anatomisch van elkaar gescheiden, de foetus brengt nog geen antigenen tot expressie, het immuunsysteem van de moeder is tolerant voor de foetus en de uterus heeft immunologisch gezien een unieke status. Latere wetenschappelijke studies tonen aan dat deze hypothesen de complexiteit van de reproductieve immunologie niet volledig konden omvatten. Allereerst is het niet zo dat de foetus en de moeder anatomisch van elkaar gescheiden zijn: in de placenta, het orgaan dat de foetus van voedingsstoffen voorziet, komen foetale cellen in direct contact met cellen van de moeder. Gedurende de zwangerschap komen ook grote hoeveelheden foetale cellen en celfragmenten in het bloed van de moeder terecht (**Hoofdstuk 1, Figuur 5**). Ten tweede brengen de foetus en de placenta diverse antigenen tot expressie die door het immuunsysteem van de moeder worden herkend. Ten derde blijkt de moeder niet geheel tolerant voor de foetus: er kunnen bijvoorbeeld antilichamen ontstaan welke gericht zijn tegen de foetus. Deze antilichamen, genaamd allo-antilichamen, zijn gericht tegen de, voor de moeder, lichaamsvreemde eiwitten van de foetus op onder meer immuuncellen, rode bloedcellen of bloedplaatjes. Bovendien is de uterus niet de enige plaats in het lichaam waar een embryo zich kan innestelen, zwangerschappen kunnen ook voorkomen in de eileiders en buikholte. Een zwangerschap in de buikholte kan zelfs leiden tot een voldragen, levend kind.

Dit proefschrift

Voor dit proefschrift is onderzoek gedaan naar immunologisch uitdagende zwangerschappen zoals eiceldonatiezwangerschappen en autologe zwangerschappen gecompliceerd door pre-eclampsie en chronische intervillitis van onbekende oorzaak (CIUE, *chronic intervillitis of unknown etiology*). In **Hoofdstuk 2** is de expressie van het eiwit trombomoduline in de placenta van vrouwen die zwanger zijn na een eiceldonatie onderzocht. Ook is bij vrouwen die zwanger zijn na een eiceldonatie gekeken naar het reactieve species-interactoom

(**Hoofdstuk 4**). **Hoofdstuk 3** behandelt ons onderzoek naar de trombomoduline-expressie in de nieren van vrouwen met pre-eclampsie. Tot slot beschrijven **Hoofdstuk 5** en **Hoofdstuk 6** de diagnosecriteria en het klinisch fenotype van CIUE.

Trombomoduline-expressie in de placenta

Trombomoduline is een eiwit dat tot expressie komt op endotheelcellen en trofoblastcellen van de placenta. Trombomoduline heeft vele functies, waaronder het reguleren van de stolling, ontsteking en de overleving van cellen. Omdat bij eiceldonatiezwangerschappen de foetus meer lichaamsvreemd (allogeen) kan zijn ten opzichte van de moeder dan in autologe zwangerschappen, is de rol van het immuunsysteem in eiceldonatiezwangerschappen mogelijk anders dan bij autologe zwangerschappen. Daarbij worden eiceldonatiezwangerschappen vaker gecompliceerd door pre-eclampsie. Pre-eclampsie is een aandoening die voornamelijk voorkomt in het derde trimester van de zwangerschap en uit zich in hypertensie en in veel gevallen proteïnurie. De oorzaak van pre-eclampsie is nog niet opgehelderd, maar het is zeker dat placentadisfunctie en schade aan de maternale bloedvaten er ten grondslag aan liggen. Daarom is het juist in eiceldonatiezwangerschappen, al dan niet gecompliceerd door pre-eclampsie, interessant om naar de expressie van trombomoduline in de placenta te kijken. Ons onderzoek heeft aangetoond dat de trombomoduline-eiwitexpressie op de syncytiotrofoblast verlaagd is in de placenta van vrouwen met een eiceldonatiezwangerschap gecompliceerd door pre-eclampsie. De trombomoduline-mRNA-expressie is ook verlaagd in eiceldonatiezwangerschappen gecompliceerd door pre-eclampsie. Daarnaast is de trombomoduline-eiwitexpressie verlaagd in ongecompliceerde eiceldonatiezwangerschappen ten opzichte van ongecompliceerde autologe zwangerschappen. De verlaagde trombomoduline-eiwitexpressie is geassocieerd met stolling en ontsteking. Deze associaties suggereren dat beschermende mechanismen in de placenta verstoord zijn in ongecompliceerde eiceldonatiezwangerschappen en eiceldonatiezwangerschappen gecompliceerd door pre-eclampsie. De verstoorde beschermingsmechanismen in deze placenta's zouden een verklaring kunnen zijn voor een verhoogd risico op pre-eclampsie in eiceldonatiezwangerschappen.

Proefdierexperimenten hebben laten zien dat trombomoduline-expressie in de placenta noodzakelijk is voor een succesvolle zwangerschap. De precieze rol en regulatie van trombomoduline in de placenta is nog niet goed begrepen. Wij hebben in **Hoofdstuk 2** ook laten zien dat de vitamine D-receptorexpressie verlaagd is in de placenta's van vrouwen met pre-eclampsie die zwanger zijn geworden door middel van een eiceldonatie. Daarbij resulteert het toevoegen van vitamine D aan gekweekte placentacellen in een toename van de trombomoduline-mRNA-expressie. Vitamine D lijkt dus de trombomoduline-expressie te verhogen. Vitamine D-suppletie of behandeling met recombinant-trombomoduline zou kunnen bijdragen aan de bescherming van de placentafunctie van vrouwen die zwanger zijn na een eiceldonatie. Maar er is meer onderzoek nodig om de precieze rol van trombomoduline in de placenta te begrijpen.

Trombomoduline in de nieren van vrouwen met pre-eclampsie

Vrouwen met pre-eclampsie hebben veelal proteïnurie, wat duidt op een beschadiging van de nieren. In **Hoofdstuk 3** hebben we een significant hogere trombomoduline-expressie gevonden in de nieren van vrouwen met pre-eclampsie in vergelijking met niet-zwangere hypertensieve controles en normotensieve zwangere controles. Daarnaast hebben we de trombomoduline-expressie onderzocht in een rattenproefdiermodel waar het pre-eclampsie fenotype veroorzaakt wordt door blootstelling aan sunitinib. De expressie van trombomoduline in de glomeruli was hoger in ratten die blootgesteld waren aan de lage en intermediaire sunitinibdosering. Histopathologische schade in de nieren is echter alleen aanwezig in dieren blootgesteld aan een intermediaire en hoge dosis sunitinib. In dieren blootgesteld aan een intermediaire en hoge sunitinibdosering wordt endotheliose vastgesteld, en fibrinedeposities worden geconstateerd in de nieren van dieren blootgesteld aan een hoge sunitinibdosering. Een studie in muizen met diabetes heeft laten zien dat trombomoduline een belangrijke rol speelt in de instandhouding van de glomerulaire filtratiebarrière. De verhoogde trombomoduline-expressie in dieren blootgesteld aan een lage en intermediaire dosis sunitinib in onze studie, suggereert dat trombomoduline mogelijk een beschermingsmechanisme is van de glomerulaire filtratiebarrière, terwijl het verlies van trombomoduline bij de hoge dosering kan duiden op een tekortschieten van dit beschermingsmechanisme.

Het toedienen van trombomoduline zou een therapie kunnen zijn om het nierendotheel te beschermen in vrouwen met pre-eclampsie. Behandeling met trombomoduline in een pre-eclampsieproefdiermodel zorgt voor betere foetale uitkomsten. De trombomodulinebehandeling heeft echter geen effect op de maternale bloeddruk in deze ratten. In een cohort van zwangere vrouwen met diffuse intraveneuze stolling zorgde behandeling met trombomoduline voor een verbetering van verschillende bloedwaarden. De behandeling had echter geen effect op zowel de lever- als nierfunctie.

Reactieve species-interactoom in eiceldonatiezwangerschappen

In reactie op zuurstofgebrek en ontsteking maakt de placenta allerlei stoffen aan, zoals anti-angiogene stoffen, pro-inflammatoire cytokines en zuurstofradicalen. In **Hoofdstuk 4** zijn verschillende componenten van het reactieve species-interactoom onderzocht in eiceldonatiezwangerschappen met en zonder pre-eclampsie. Het reactieve species-interactoom is de interactie tussen reactieve stoffen, zoals zuurstof, stikstof- en sulfaatradicalen en hun omgeving. Door de allogene situatie in eiceldonatiezwangerschappen hypothetiseren we dat in deze zwangerschappen veranderingen kunnen optreden in het reactieve species-interactoom. Veranderingen in het reactieve species-interactoom worden namelijk ook gezien bij patiënten die een niertransplantatie hebben ondergaan. In ongecompliceerde eiceldonatiezwangerschappen werd geen toename gevonden van reactieve species. In eiceldonatiezwangerschappen en controlezwangerschappen gecompliceerd door pre-eclampsie was dit wel het geval. In zowel eiceldonatiezwangerschappen als controlezwangerschappen gecompliceerd door pre-eclampsie zou het reactieve species-interactoom een link kunnen zijn tussen placentadisfunctie en

systemische endotheeldisfunctie. Opvallend was dat in ongecompliceerde eiceldonatiezwangerschappen de nitrietconcentraties hoger zijn ten opzichte van ongecompliceerde controlezwangerschappen. Deze verhoogde nitrietconcentraties in ongecompliceerde eiceldonatiezwangerschappen kunnen van invloed zijn op de bloeddruk van vrouwen en zo mogelijk de ontwikkeling van pre-eclampsie voorkomen. De resultaten uit **Hoofdstuk 4** ondersteunen dat er meer onderzoek nodig is naar de verschillende regulerende mechanismen in autologe zwangerschappen en eiceldonatiezwangerschappen.

Diagnosecriteria voor chronische intervillitis van onbekende oorzaak

CIUE is een zeldzame aandoening waarbij immuuncellen worden waargenomen in de intervillieuze ruimte van de placenta. De aanwezigheid van deze cellen in de intervillieuze ruimte is geassocieerd met zwangerschapscomplicaties zoals een achterblijvende foetale groei en intra-uterine foetale sterfte. In voorgaande studies zijn echter verschillende diagnosecriteria gebruikt om casus met CIUE te selecteren. Door de afwezigheid van eenduidige diagnosecriteria zijn studies onderling niet goed met elkaar te vergelijken en is het niet mogelijk de klinische consequenties van CIUE volledig te begrijpen. Derhalve was het doel van **Hoofdstuk 5** het formuleren van diagnosecriteria voor CIUE. Uit de literatuur blijkt de aanwezigheid van een intervillous celfiltraat het enige selectie criterium voor CIUE is dat in alle studies wordt gebruikt. In onze literatuurstudie is ook gekeken naar de zwangerschapsuitkomsten in casus met CIUE. Een miskraam werd gerapporteerd in 62 van de 256 (24.3%) zwangerschappen. In 65 procent van de zwangerschappen bleef de foetale groei achter. Slechts 59 van de 182 (32.4%) zwangerschappen resulteerden in een à terme geboren kind en maar liefst 55 van 190 (28.9%) zwangerschappen eindigden in foetale sterfte. De herhalingskans voor CIUE in een volgende zwangerschap was 25 procent (**Hoofdstuk 5, Tabel 4**). De hoge herhalingskans maakt de diagnose CIUE klinisch relevant. In de bestudeerde studies verschilden de obstetrische uitkomsten en ook in individuele patiënten kunnen obstetrische uitkomsten verschillen, van mild tot foetale sterfte.

Op basis van onze literatuurstudie formuleerden wij de onderstaande diagnosecriteria voor CIUE:

Inclusiecriteria

I: Aanwezigheid van een infiltraat in de intervillieuze ruimte.

II: Ongeveer 80% van de mononucleaire-cellen in de intervillieuze ruimte zijn CD68+-cellen.

III: 5% of meer van de intervillieuze ruimte is gevuld.

Exclusie criterium

IV: Casus met klinische of histopathologische tekenen van een infectie.

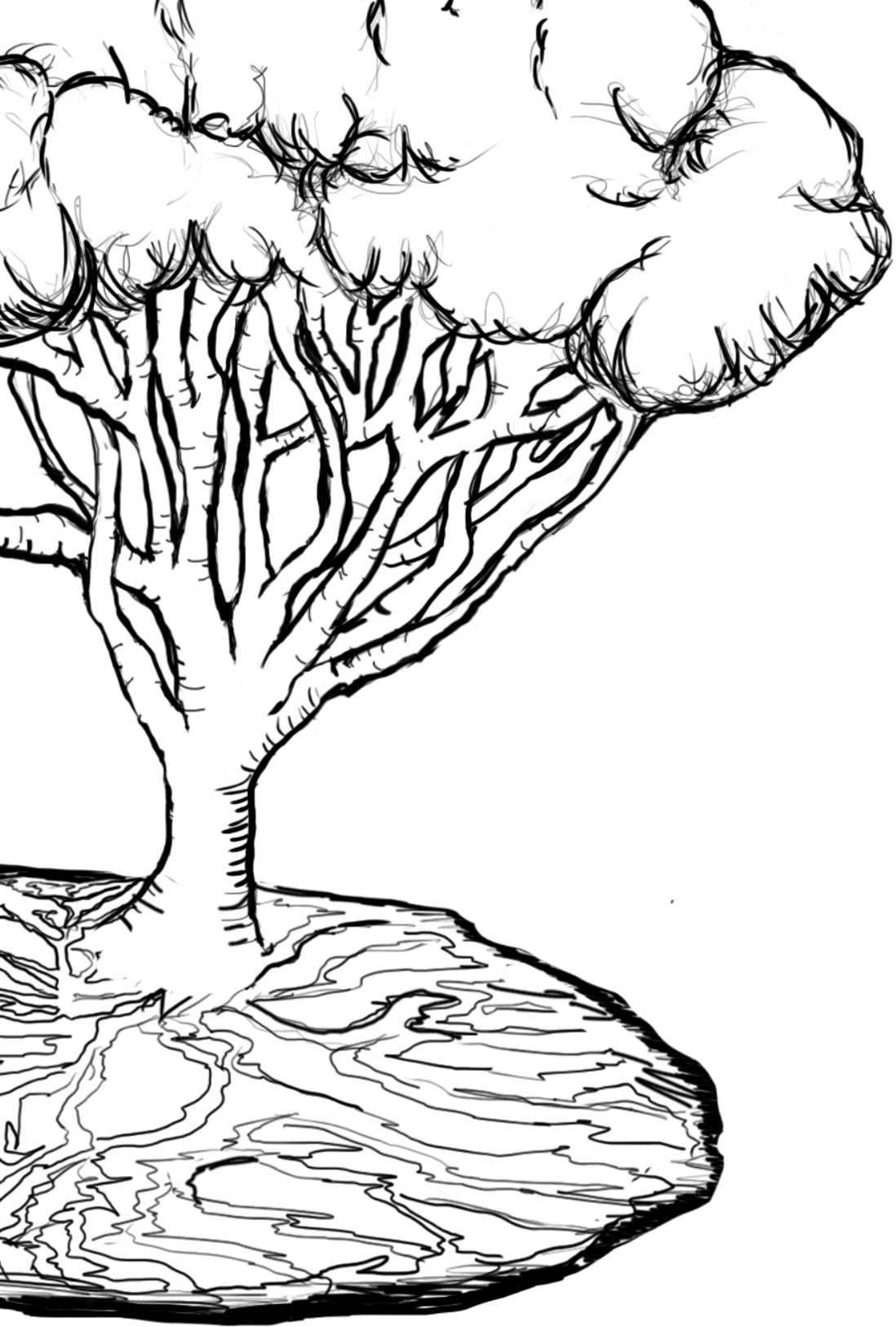
Door het introduceren van diagnosecriteria voor CIUE zijn toekomstige studies beter met elkaar te vergelijken en zal er meer inzicht komen in de klinische consequenties van CIUE.

Het klinisch fenotype van chronische intervillitis van onbekende origine

In **Hoofdstuk 6** beschrijven we uitgebreid de karakteristieken van 38 vrouwen die tenminste één zwangerschap hebben gehad met CIUE. Deze 38 vrouwen hadden gezamenlijk in totaal 78 zwangerschappen voorafgaand aan de diagnose CIUE en veertien zwangerschappen hierna. Zwangerschappen gecompliceerd door CIUE resulteerden vaak in foetale groeirestrictie (16/31; 52%) en vroeggeboorte (21/38; 55.3%). Negenentwintig van de 38 zwangerschappen met CIUE resulteerden in een levend kind (76.3%). Verder viel het op dat vrouwen met een zwangerschap gecompliceerd door CIUE voorafgaande aan die zwangerschap vaak één of meer miskramen hebben gehad (16/38; 42.1%). CIUE kan ook aanwezig zijn in ongecompliceerde zwangerschappen. Zoals in **Hoofdstuk 5** ook al opviel, concluderen we op basis van de gegevens in **Hoofdstuk 6** dat CIUE een gevarieerd klinisch spectrum heeft. CIUE gaat vaak gepaard met ernstige complicaties, maar herhaalde CIUE kan ook gepaard gaan met een ongecompliceerde zwangerschap.

Conclusie

Het onderzoek dat beschreven is in dit proefschrift is gericht op immunologisch uitdagende zwangerschappen en verschaft nieuwe inzichten in ongecompliceerde eiceldonatiezwangerschappen, zwangerschappen gecompliceerd door pre-eclampsie en zwangerschappen gecompliceerd door CIUE. Het onderzoeken van deze immunologisch uitdagende zwangerschappen vergroot onze kennis over ongecompliceerde autologe zwangerschappen. Daarbij biedt zwangerschap een interessante setting om hypothesen te testen uit de transplantatie-immunologie en tumorimmunologie en ontdekkingen in de reproductieve immunologie kunnen nieuwe inzichten geven in de dynamiek van het immuunsysteem in het algemeen. Toekomstig onderzoek kan bovendien gebruikmaken van de unieke omstandigheden van eiceldonatiezwangerschappen en specifieke zwangerschapscomplicaties zoals CIUE.



Appendices

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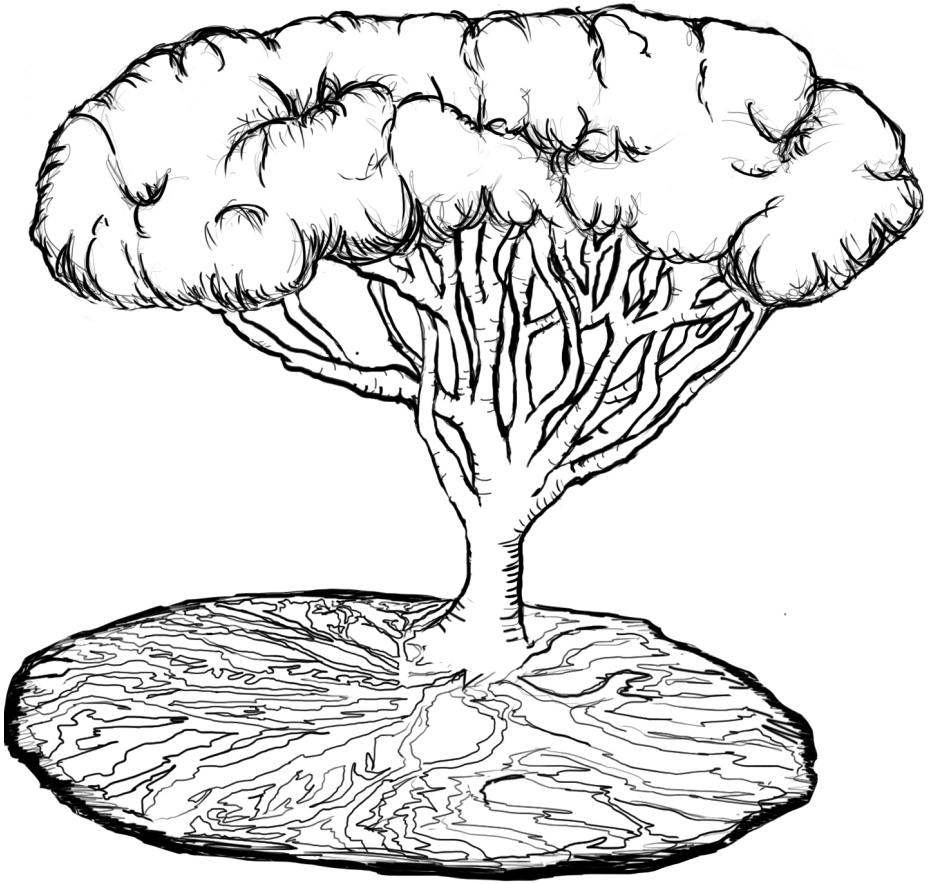
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Curriculum vitae

Manon Bos is geboren op 23 maart 1993 te Woerden en zij heeft haar jeugd doorgebracht in de Meije en Alphen aan den Rijn. In 2011 behaalde Manon haar VWO-diploma (Natuur & Gezondheid, Natuur & Techniek met Wiskunde D en Filosofie) aan het Groene Hart Lyceum te Alphen aan den Rijn. Datzelfde jaar startte zij met de studie Geneeskunde aan de Universiteit Leiden en werd tevens lid bij de K.S.R.V. "Njord". Bij Njord heeft zij fanatiek gewedstrijdroeid en diverse commissies gedaan. Al vroeg in de bachelor Geneeskunde raakte Manon geïnteresseerd in wetenschappelijk onderzoek, wat resulteerde in extracurriculair onderzoek bij diverse afdelingen en deelname aan de Honours College BW-track. Manon heeft ook al geruime tijd belangstelling in medisch leiderschap, onderwijs en Global Health. Derhalve volgde zij keuzevakken op het gebied van Global Health, heeft zij een minor Gezondheidsmanagement afgerond aan de Erasmus Universiteit Rotterdam en zette zij zich in voor de opleidingscommissie Geneeskunde. Daarnaast is Manon militair werkstudent bij het Ministerie van Defensie via het Defensy College Werkstudenten Programma.

Vanaf 2016 heeft Manon fulltime onderzoek gedaan bij de afdeling Pathologie en het Lab Reproductieve Immunologie (afdeling Gynaecologie en Verloskunde, afdeling Immunohematologie en Bloedtransfusie) onder begeleiding van Dr. J.J. Baelde, Dr. M.L.P. van der Hoorn en Prof. Dr. J.A. Bruijn. Haar onderzoek werd ondersteund door een Kolff studentonderzoekersbeurs van de Nierstichting en een tweejarige MD/PhD-beurs van het Leids Universitair Medisch Centrum. De resultaten van het gedane onderzoek presenteerde Manon op diverse nationale en internationale congressen waaronder de SRI (2016 Montreal), het Gynaecongres (2017 Amersfoort), de ASN Kidney Week (2016 Chicago, 2018 San Diego), IFPA (2017 Manchester), ISSHP (2018 Amsterdam) en ESRI (2018 Aalborg). Voor de presentaties op deze congressen ontving zij diverse beurzen en prijzen zoals de ASN Kidney Stars beurs, Y.W. (Charlie) Loke Award, Young Investigator ISSHP Travel Award en de MCR2017 Travel Award.

In maart 2019 is Manon begonnen met coschappen, in 2021 verwacht zij haar artsexamen te behalen en de masteropleiding Biomedische Wetenschappen af te ronden. Manon heeft de ambitie om na het afronden van haar studie een wetenschappelijke en klinische carrière te combineren.



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Collega's van de afdelingen Pathologie, Gynaecologie en Verloskunde en Immuno-hematologie en Bloedtransfusie, bedankt voor het delen van jullie ervaring, technische vaardigheden en enthousiasme.

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