

Cover Page



Universiteit Leiden



The handle <http://hdl.handle.net/1887/21710> holds various files of this Leiden University dissertation.

Author: Buurma, Aletta

Title: On the pathology of preeclampsia : genetic variants, complement dysregulation, and angiogenesis

Issue Date: 2013-09-11

ON THE PATHOLOGY OF
PREECLAMPSIA

*genetic variants, complement dysregulation,
and angiogenesis*



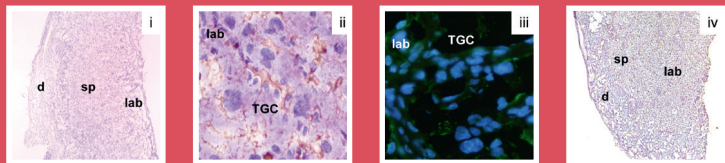
Aletta Buurma

ON THE PATHOLOGY OF PREECLAMPSIA

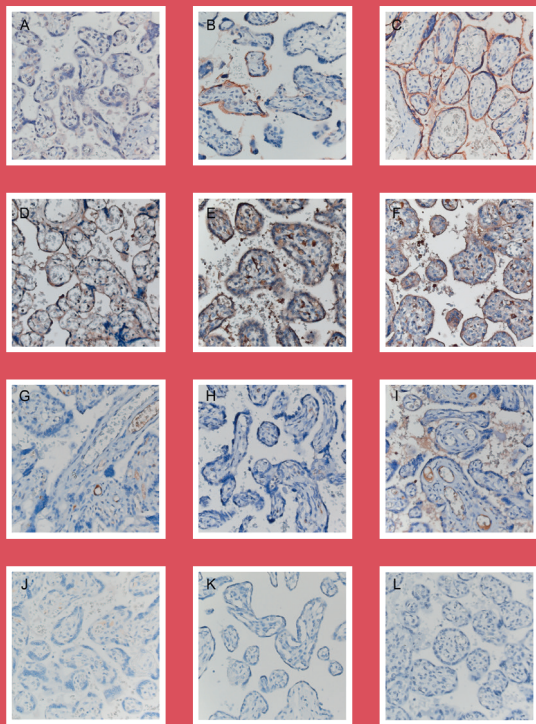
genetic variants, complement dysregulation, and angiogenesis

Aletta Buurma

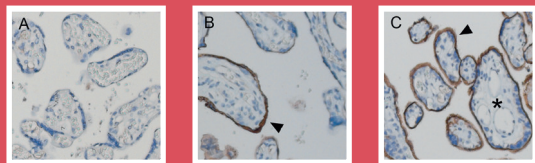
III
FIG 1A-B



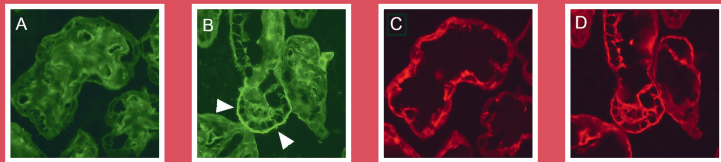
III
FIG 3



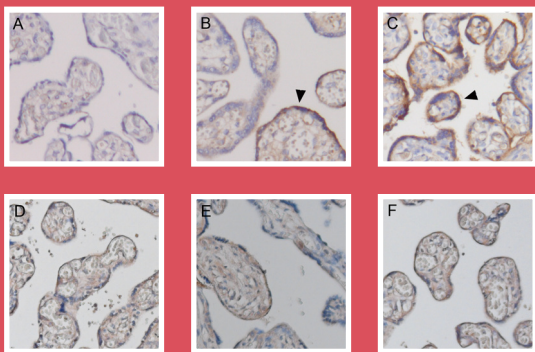
IV
FIG 1



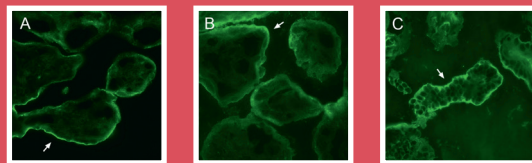
IV
FIG 3



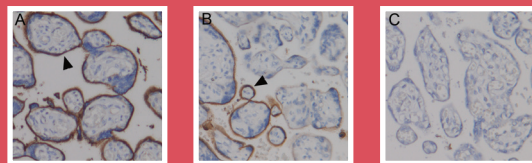
IV
FIG S1



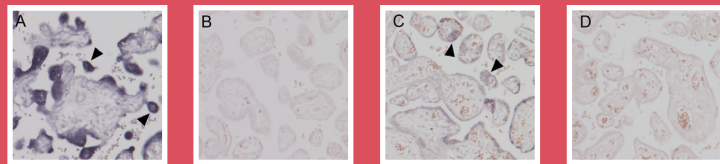
IV
FIG S2



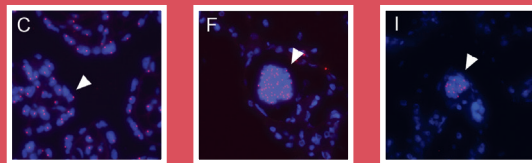
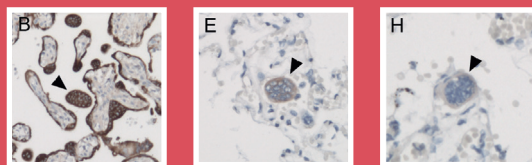
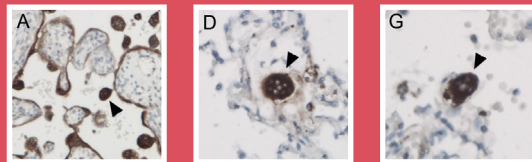
V
FIG 1



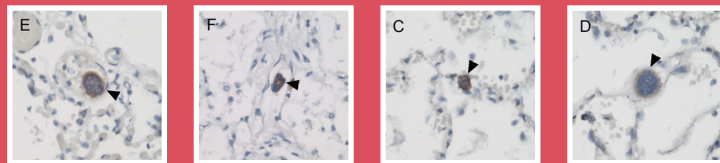
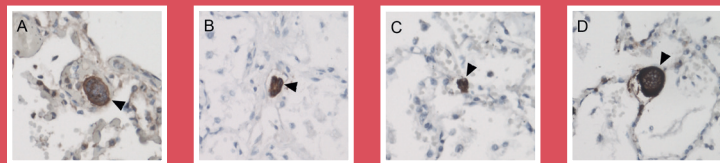
VI
FIG 1B



VI
FIG 2A



VI
FIG 2B



ON THE PATHOLOGY OF PREECLAMPSIA

genetic variants, complement dysregulation, and angiogenesis

*Aan mijn ouders en grootouders
die mij leerden om
zorgvuldig te planten
geduldig te zorgen
dankbaar te oogsten
en liefdevol te delen*

ON THE PATHOLOGY OF PREECLAMPSIA

*genetic variants, complement dysregulation,
and angiogenesis*

PROEFSCHRIFT

ter verkrijging van
de graad van Doctor aan de Universiteit Leiden
op gezag van Rector Magnificus prof. mr. C.J.J.M. Stolker,
volgens besluit van het College voor Promoties
te verdedigen op woensdag 11 september 2013
klokke 16:15 uur

DOOR

Aletta Atje Jacoba Buurma
Geboren te Lelystad
in 1988

PROMOTIECOMMISSIE

Promotor:	Prof. dr. J.A. Bruijn
Co-promotores:	Dr. J.J. Baelde Dr. K.W.M. Bloemenkamp
Overige Leden:	Prof. dr. F.H.J. Claas Prof. dr. C. van Kooten Prof. dr. C.J.M. de Groot (<i>VUMC, Amsterdam</i>)

ISBN 9789082101003

The studies described in this thesis were performed at the Department of Pathology of the Leiden University Medical Center, the Netherlands.

The publication of this thesis was financially supported by:
The departments of Pathology and Obstetrics of the Leiden University Medical Center, Stichting HELLP-syndroom Nederland, NVLE fonds, Rabobank Medicidesk, Medical Dynamics, ABN Amro, PerkinElmer, BMA BV (Mosos)

Cover: Patience Anders
Design: Jelmer Buurma (jelmer@jelmerbuurma.nl)

TABLE OF CONTENTS

I	General introduction	6
II	Genetic variants in preeclampsia: a meta-analysis	36
III	Classical complement activation as a footprint for murine and human antiphospholipid antibody- induced fetal loss	74
IV	Preeclampsia is characterized by placental complement dysregulation	100
V	Placental complement activation is related to placental dysfunction in the setting of preeclampsia	126
VI	Preeclampsia and placental fragments in the maternal lung	144
VII	Summary and general discussion	160
	Nederlandse samenvatting	178
	Curriculum Vitae	184
	Bibliography	188
	Acknowledgements	190

CHAPTER I

GENERAL INTRODUCTION

Introduction

PREECLAMPSIA – A HETEROGENEOUS DISEASE WITH SERIOUS CONSEQUENCES

Preeclampsia is a pregnancy-specific syndrome that is characterized by endothelial dysfunction, presenting clinically as hypertension and proteinuria after 20 weeks of gestation. The syndrome can be accompanied by a variety of complications including headache, visual disturbances, epigastric pain, generalized seizures (eclampsia) and HELLP (hemolysis, elevated liver enzymes, low platelets) syndrome.^{1,2} Because the etiology of preeclampsia is largely unknown and probably as heterogeneous as its presentation, accurate prediction, prevention, and rational therapy of pre-eclampsia are still not possible. The only cure for preeclampsia is delivery—often preterm—which rescues the mother but frequently compromises fetal outcome. Preeclampsia is not uncommon, complicating 2-8% of pregnancies, and therefore contributes significantly to maternal and fetal morbidity and mortality worldwide.³ In addition, the burden of preeclampsia is not limited to the period around pregnancy; the condition is associated with an increased prevalence of cardiovascular risk factors later in life, in both mothers and children.⁴⁻⁶

PREECLAMPSIA AND THE PLACENTA Although the exact causes of preeclampsia are still unidentified, the condition is believed to originate in the placenta, as evidenced by the fact that the placenta alone, not the fetus, is required for the development of preeclampsia.⁷⁻⁹ The placenta is a newly formed organ, composed of both fetal and maternal tissues. The placenta supports life throughout the gestational period of a developing fetus by supplying oxygen, protective antibodies, and nutrients while eliminating waste products. Preeclampsia is frequently characterized by disturbed placental development, which is already observable during the early stages of pregnancy. In a subset of preeclamptic women, however, aberrant placental development is a less prominent feature. Strikingly, the extent of placental dysfunction is related to

an earlier onset of preeclampsia and therefore, preeclampsia is often subdivided into early-onset and late-onset preeclampsia.¹⁰ Early-onset preeclampsia is considered a disorder in which abnormal placental morphology, reduced placental volume, intra-uterine growth restriction, and adverse maternal and neonatal outcomes are prominent features.¹⁰⁻¹³ Late-onset preeclampsia, on the contrary, is considered a maternal disorder. It is often associated with a normal placental morphology, larger placental volume, normal fetal growth, and more favorable maternal and neonatal outcomes.^{10;11;13}

IMMUNOLOGIC, GENETIC AND ANGIOGENIC ASPECTS OF PREECLAMPSIA A variety of risk factors have been identified that predispose women to preeclampsia. The most significant risk factors for developing preeclampsia are a history of preeclampsia and the presence of antiphospholipid antibodies.¹⁴ Other important risk factors for preeclampsia include nulliparity, a family history of preeclampsia, maternal age ≥ 40 , obesity and several underlying medical conditions, including pre-existent hypertension, pre-existent diabetes and autoimmune disease.^{1;14} In these epidemiological data lays the clue why this thesis is focused towards the immunologic and genetic aspects of preeclampsia; the strong relation between preeclampsia and autoimmune disorders supports a role for the immune system in preeclampsia. Likewise, the clear familial component that characterizes preeclampsia suggests that genetic factors contribute to the development of preeclampsia.

In the first part of this introductory chapter (*Part 1 - Placental development*), the development and function of the placenta during normal pregnancy will be explained. The second part (*Part 2 - Preeclampsia*) focuses on the pathophysiology of preeclampsia. This section will go into depth by focusing on two putative causes of preeclampsia: the presence of genetic variants and dysregulation of the complement system. In addition, the consequences of placental dysfunction in the setting of preeclampsia will be described,

including angiogenic dysregulation and the subsequent generalized endothelial dysfunction.

The third part of this chapter will focus on the research questions that form the basis for the experiments and the studies described in this thesis (*Part 3 - This thesis*). Finally, an outline of the chapters will be given.

Part 1 - Placental development

IMPLANTATION Shortly after conception, the placenta starts to develop in the uterus. However, before the fertilized ovum reaches the uterus, the lining of the uterus has already undergone a variety of important changes. The thickness and the vascularity of the uterine lining have increased, and the uterine glands have become elongated. Furthermore, the tissue between the uterine glands now contains a great number of large round, oval or polygonal cells, termed decidual cells. At the time all these changes have taken place, the lining of the uterus is termed the decidua.

On reaching the cavity of the uterus, the fertilized ovum—now termed the blastocyst—adheres to the decidua. This is the site where the placenta will subsequently develop. The attached blastocyst excavates a cavity in the decidua, in which it becomes embedded. The structure of the blastocyst that is responsible for this process of excavation is termed the trophoblast. The trophoblast proliferates rapidly and forms a network of branching projections—the chorionic villi—which infiltrate the uterine decidua and absorb from it the nutrients for the growth of the embryo (Figure 1).¹⁵

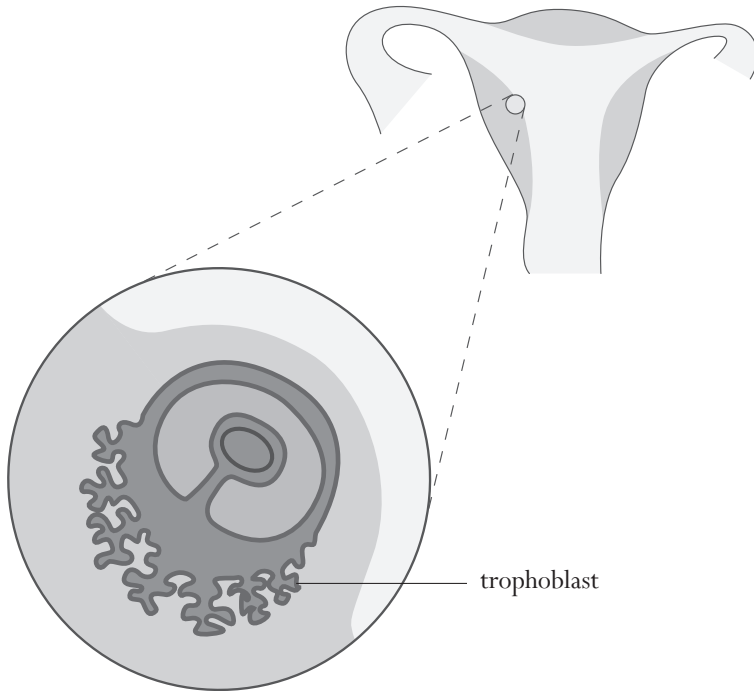


Figure 1: Implantation and trophoblast development

THE PLACENTA FORMS THE FETAL-MATERNAL INTERFACE

While infiltrating the maternal tissues, the trophoblast grows into the maternal blood vessels that are present within the uterine wall. The invasive phenotype of trophoblast cells enables them not only to invade but also to remodel the uterine spiral arteries. As a result, these uterine vessels change from narrow, muscular arterioles into flaccid, high-capacity vessels of low resistance, which fill the spaces in the trophoblastic network with maternal blood.^{16;17} All the spaces in the trophoblastic network—which communicate freely with one another—together form the intervillous space. The decidua and the intervillous space together form the maternal portion of the placenta (Figure 2).

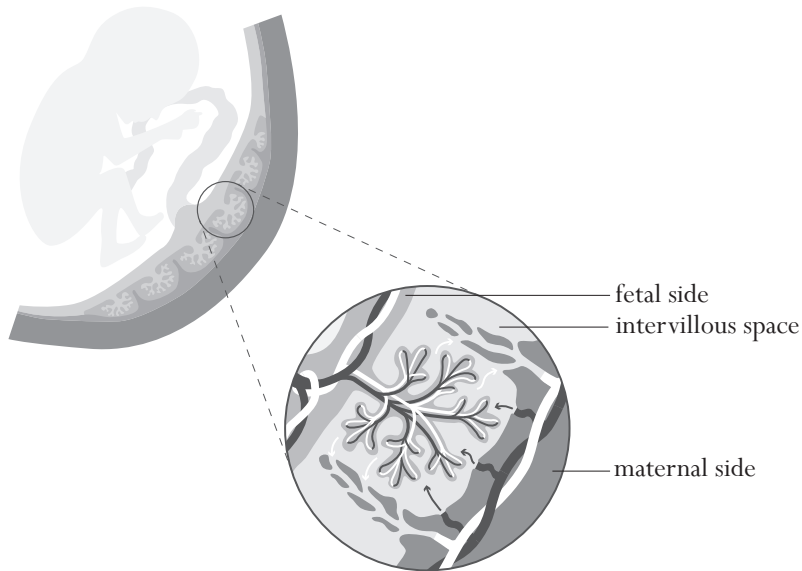


Figure 2: The fetal and maternal portions of the placenta. The fetus and the mother are in direct contact with each other but their blood currents are separated from one another by the delicate walls of the villi.

The chorionic villi are of fetal origin and therefore they make up the fetal portion of the placenta. These villi branch repeatedly and as a result, the fetal part of the placenta has an enormous surface area. During the course of pregnancy, the chorionic villi increase in size and become vascularized. The great majority of the villi float freely in the intervillous space, being surrounded by maternal blood. All villi are entirely covered by syncytiotrophoblast, which therefore forms the continuous physical barrier between mother and fetus, also called the fetal-maternal interface. So throughout pregnancy, the placenta is composed of a fetal portion and a maternal portion that are in direct contact with each other (Figure 2).

EXTENSIVE CONTACT FACILITATES THE MAIN FUNCTIONS OF THE PLACENTA Although both the fetal and the maternal blood currents traverse the placenta, their blood does not intermingle. However, oxygen, nutrients and protective antibodies from the maternal blood can be transported to the fetal circulation. At the same time, the placenta receives the waste products from the fetus and discharges them into the maternal circulation for disposal. The large surface area of all villi together facilitates this exchange of nutrients, waste products and protective antibodies between mother and fetus.

EXTENSIVE CONTACT ALSO IMPOSES AN IMMUNOLOGICAL CHALLENGE Although the large surface area of the placenta facilitates the main functions of the placenta, the extensive contact between mother and fetus also imposes an immunological challenge. As early as 1953, Peter Medawar put forward the idea of the fetus as a semi-allograft; the trophoblast is derived from the fetus and so it expresses paternal antigens which the mother's immune system may recognize as foreign.¹⁸ These paternal antigens can consequently elicit an immune response, possibly leading to fetal rejection if the maternal immune response is not adequately regulated. Therefore, in order to prevent maternal anti-fetal rejection, an immune privileged site must be created at the fetal-maternal interface. Trophoblasts have several characteristics that contribute to the prevention of both cellular and humoral maternal immune responses, together creating this immune privileged site.

INHIBITING IMMUNE MECHANISMS WITHIN THE PLACENTA

Cellular immune mechanisms are prevented in several ways. Importantly, trophoblast cells do not express the classical HLA-A, HLA-B, HLA-DR, HLA-DQ, and HLA-DP molecules that are the main targets for alloreactive T-cells in the setting of organ transplantation. However, they do express HLA-C, HLA-E, and HLA-G molecules by which they can avoid cell-mediated cytotoxicity.^{19;20} This is illustrated by the observation that HLA-

G-expressing cells induce regulatory T-cell activity and avoid NK-mediated cytotoxicity.^{21;22} T cell activity is furthermore suppressed by the expression of indoleamine-2,3-dioxygenase (IDO) on the trophoblast membrane.²³

Although cellular immune mechanisms have been subject of extensive investigation, less is known about the role of humoral immunity and the interplay with the complement system during pregnancy. Trophoblast expresses fetal antigens to which maternal lymphocytes are exposed and to which maternal B-cells can form targeted antibodies. The presence of anti-HLA or anti-paternal antibodies is indeed observed in approximately 30% of pregnant women, but it is normally not associated with pregnancy complications.²⁴ A possible explanation for this phenomenon is that trophoblast is well prepared for maternal anti-fetal antibody-mediated attack. A mechanism by which trophoblasts can repress humoral immune mechanisms is the expression of complement regulatory proteins on their cell membrane. Indeed, all three membrane-bound complement regulatory proteins (DAF, MCP and CD59) are highly expressed on trophoblast cells, strategically positioned at the fetal-maternal interface to protect the fetus from maternal immune responses.^{25;26} Because a large part of this thesis deals with complement activation at the fetal-maternal interface, an introduction of the complement system will be given below.

THE COMPLEMENT SYSTEM: BALANCE BETWEEN ACTIVATION AND REGULATION The complement system is an essential component of the human immune system.^{27;28} It is composed of over 30 soluble- and membrane-bound proteins that are constantly in a 'controlled active state', which means that the system is ready for action at all times. Specific triggers, such as invading pathogens, can activate the complement system by setting off a cascade of enzymatic reactions and positive feedback loops. The complement cascade is capable of achieving enormous amplification, as an initially small number of activated complement molecules produced early in the cascade

eventually leads to the generation of a large number of effector molecules. The result is destruction of potentially dangerous targets within seconds. The complement system also provides a link between innate and adaptive immune systems, thereby further facilitating the removal of potentially dangerous pathogens. Although the main role of the complement system is to protect the host against invading pathogens, the system is also involved in tissue regeneration and clearance of necrotic and apoptotic cells.²⁹

WHAT CAUSES ACTIVATION OF THE COMPLEMENT SYSTEM? The complement system (Figure 3) can become activated by three distinct pathways: the classical, the lectin and the alternative pathway.²⁷ Each pathway has specific triggers and recognition molecules by which it can become activated. Although the three pathways differ in how they are activated, they do share a final common pathway. All pathways converge at the level of C3. From this point, a final common pathway leads to the effector functions of complement, namely chemotaxis, opsonization and cell lysis. The classical pathway of complement is activated when its recognition molecule C1q binds to immune complex deposits, antibody-antigen binding, charged molecules, and apoptotic and necrotic cell debris. This pathway links innate immunity to adaptive B-cell mediated immunity after an antibody response has developed.²⁸ However, this pathway also forms an important threat in the setting of autoimmune diseases and transplantation, as deposition of auto- or allo-antibodies can activate the classical pathway leading to complement-mediated organ damage or graft rejection. The lectin pathway is activated when mannose-binding lectin (MBL) or ficolins recognize carbohydrates that are present on the surface of a wide range of pathogens. The recognition molecule of the lectin pathway is MBL. However, further downstream the pathway follows the same steps as classical complement activation, via C2, C4 and the final common pathway. Consequently, the lectin pathway is functionally similar to the classical pathway and it can be challenging to distinguish between

classical and lectin pathway activation. Finally, the alternative pathway is initiated spontaneously. The pathway is characterized by continuous hydrolysis of C3, and, at the same time, tight regulation to prevent uncontrolled activation. Activation of the alternative pathway can be considered as a positive feedback loop, as it amplifies complement activation initiated by the classical- or lectin pathway. Whenever C3 is formed by the classical- or lectin pathway activation, factor B and D immediately bind to it. This newly formed complex is subsequently stabilized by properdin, forming an enzyme that leads to more C3 activation. Thereby, a positive feedback loop is created, leading to instant amplification of the inflammatory reaction initiated by classical or lectin pathway activation.³⁰

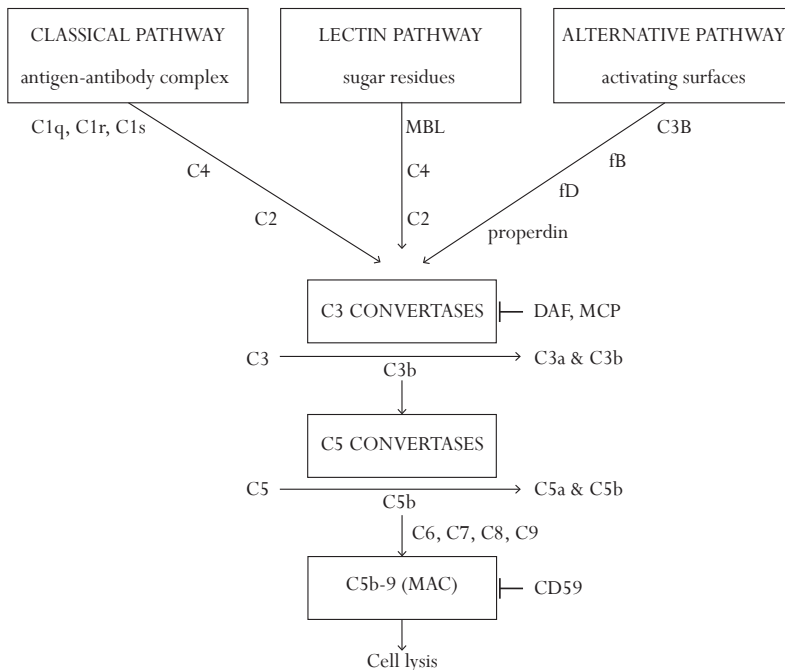


Figure 3: A schematic overview of the complement system

WHAT ARE THE EFFECTS OF COMPLEMENT ACTIVATION?

Activation of the complement cascade has various effects, which all have the purpose to start eliminating pathogens in the time before the adaptive immune system is ready for action. Firstly, complement activation results in the generation of the potent anaphylatoxins C3a and C5a, which attract neutrophils and monocytes to the site of inflammation. Aside from chemotaxis, these anaphylatoxins also cause vasodilatation, smooth muscle contraction, histamine release from mast cells, and oxidative burst from neutrophils.^{31;32} Secondly, the cleavage of certain complement proteins results in the generation of split products that coat the surface of target cells and pathogens, thereby facilitating the process of phagocytosis. Finally, complement activation can lead to the formation of the membrane attack complex (MAC). The MAC is a complex of proteins that work together to create a pore-like structure on cell membranes. Insertion of this MAC into cell membranes leads to cell lysis and, as a result, cell death. Importantly, even sublytic quantities or partial membrane attack complexes—which do not lead to cell death—are important for signaling effects.^{33;34}

HOW IS THE COMPLEMENT SYSTEM REGULATED?

Complement components are not capable of distinguishing foreign agents from self-tissues, and therefore the system operates rather non-specifically. This characteristic makes complement a potentially dangerous system for the host, as complement components have the capacity to bind and damage the host's own tissues. To prevent complement-mediated damage to host cells, tight regulation is necessary. Indeed, a complex array of protective mechanisms exists, illustrated by the fact that nearly half of all complement components have a regulatory function. Soluble and membrane-bound complement regulators act in concert to protect cells and tissues from unintended complement-mediated injury.³⁵ Their goal is to prevent damage to host tissues by inhibiting the cascade on three vital points: activation, amplification and the formation of

the membrane attack complex. The membrane bound regulatory proteins include CD59, Decay Accelerating Factor (DAF) and Membrane Cofactor Protein (MCP).³⁶ These factors are widely expressed by host tissues to prevent complement from inducing auto-injury. CD59 binds to components of the MAC, preventing its assembly and, therefore, its insertion into the cell membrane. DAF prevents the formation and accelerates the destruction of C3-convertases, which are enzymes derived from C4 that normally lead to C3 activation and the formation of the anaphylatoxins C5a and C3a. MCP binds the complement components C3b and C4b and facilitates their degradation by the soluble complement regulator factor I, thereby preventing further activation and amplification of the complement cascade. Tissues that are in direct contact with blood—such as endothelium and placental tissues—are constantly exposed to complement components. As a consequence, these tissues need to express many different complement regulatory proteins to prevent the potentially deleterious effects of complement activation. Soluble regulatory proteins are factor I, factor H, and the C4-binding protein. These soluble regulators mainly affect enzymes involved in the alternative pathway, thereby preventing amplification of the cascade and blocking positive feedback loops.

When the complement system manages to damage host tissues, the extent of activation has either exceeded the host's regulatory capacity, or the host's regulatory mechanisms worked inadequately. Excessive activation could result from an overload of immune complexes, for instance in the setting of autoimmune diseases, in which auto-antibodies result in the generation of immune complexes by binding antigens on host tissues. Constant exposure of host tissues to auto-antibodies may overwhelm complement regulatory mechanisms, thereby allowing direct complement-mediated injury. The consequences of inadequate regulation can be observed in patients with mutations in their genes coding for complement regulatory proteins, for instance patients with hemolytic uremic syndrome³⁷ but also in women with the HELLP syndrome as well as preeclampsia.^{38;39}

WHAT IS THE ROLE OF COMPLEMENT IN PHYSIOLOGICAL PREGNANCY? Although the presence of complement deposits is generally a pathologic finding, complement split products are frequently observed in placentas from healthy women with uncomplicated pregnancies. Limited amounts of both early (C1q and C4) and late (C5, C6, C9, MAC) complement components have been demonstrated in normal placentas.⁴⁰⁻⁴⁴ In addition, serum levels of C3, C4 and total hemolytic complement (CH50) gradually rise with 10-50% during physiological pregnancy, likely due to increased synthesis.⁴⁵ Furthermore, plasma concentrations of anaphylatoxins and other complement activation products are significantly higher in normal pregnant women as compared to non-pregnant women.^{46;47} In conclusion, low grade complement activation is compatible with successful pregnancy.

Importantly, placental complement deposits are not accompanied by widespread tissue damage. This absence of tissue injury can be explained by the extensive expression of complement regulatory proteins at the fetal-maternal interface.^{25;26} Murine studies have underscored the importance of complement regulation during pregnancy. Embryos deficient in Crry—a murine complement regulator—are characterized by compromised survival, and their placentas show marked C3 depositions and inflammation. Mice deficient in both Crry (Crry^{-/-}) and C3 (C3^{-/-}) are rescued from lethality⁴⁸, which elegantly demonstrates that complement activation at the fetal-maternal interface causes fetal loss. These observations also underline the critical role of complement regulation in successful pregnancy.

Importantly, it has been suggested that the classical pathway component C1q contributes to trophoblast invasion of the decidua. Invading trophoblasts actively synthesize C1q, which promotes trophoblast adhesion and migration.^{49;50} Defective local production of C1q may be involved in pregnancy disorders that are characterized by impaired trophoblast invasion, such as preeclampsia.⁵¹

Part 2 - Preeclampsia

WHAT IS THE ROLE OF THE PLACENTA IN PREECLAMPSIA?

The pathophysiology of preeclampsia is generally subdivided into two stages.⁵² The early, preclinical stage is associated with abnormal placental development, whereas the late, clinical stage is characterized by widespread endothelial dysfunction and dysregulation of angiogenic factors. During the early stage, the mother has not yet developed the typical manifestations of preeclampsia. However, profound changes can already be observed in the placenta. During normal pregnancy, the trophoblast acquires an invasive phenotype and remodels the uterine spiral arteries to assure sufficient placental perfusion.^{16;17} In preeclampsia, on the contrary, remodeling of the uterine spiral arteries into flaccid, high capacity vessels fails (Figure 4).⁵³⁻⁵⁵ The result is increased uterine vascular resistance and diminished placental perfusion. In a subset of women destined to develop preeclampsia, these changes can be detected long before the onset of the clinical syndrome.⁵⁶⁻⁵⁸ However, poor placental development should not be regarded as the cause of preeclampsia but rather as a powerful predisposing factor. Importantly, not all pregnancies characterized by poor placentation have adverse outcomes.⁵⁹ Moreover, not all women with preeclampsia have profound placental dysfunction.

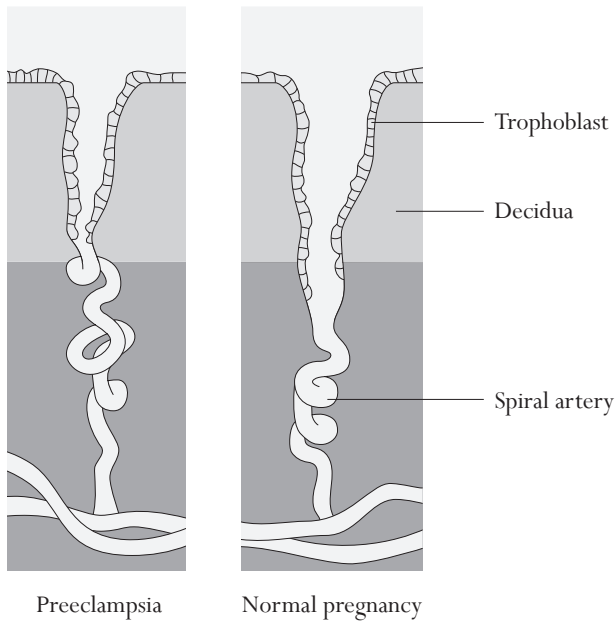


Figure 4: During normal pregnancy, the trophoblast acquires an invasive phenotype and remodels the uterine spiral arteries to assure sufficient placental perfusion. In preeclampsia, on the contrary, remodeling of the uterine spiral arteries into flaccid, high capacity vessels fails.

LATE-ONSET PREECLAMPSIA; A RESULT OF MATERNAL SUSCEPTIBILITY? In case of late-onset of preeclampsia—in which placental dysfunction is less prominent—maternal constitutive factors likely contribute to the pathogenesis of preeclampsia. Important risk factors for developing preeclampsia include a patient history of preeclampsia as well as a family history of preeclampsia, suggesting that preeclampsia may have a genetic background. Similarly, conditions characterized by endothelial dysfunction increase the risk for developing preeclampsia, suggesting that maternal cardiovascular and metabolic syndrome-like disorders contribute to the pathogenesis of preeclampsia.¹

Evidence for a genetic component of preeclampsia comes from family studies, which have shown that primigravid women with a family history of preeclampsia have a two- to five-fold increased risk of developing preeclampsia as compared to primigravid women with no such history.⁶⁰⁻⁶⁴ Additionally, the risk of preeclampsia is increased more than seven-fold in women who have suffered from preeclampsia in any previous pregnancy.¹⁴ Interestingly, paternal genetic components also predispose to preeclampsia, which is illustrated by the fact that fathers who were born from a preeclamptic pregnancy, have a greater chance of fathering a child who is also born from a preeclamptic pregnancy.^{65;66} Because the paternal genes are passed on to the fetus, this finding indicates that the genotype of the fetus contributes to the overall risk of preeclampsia. Additionally, there are marked differences in the incidence of preeclampsia among various ethnic groups, which further supports a role for a genetic component in the pathogenesis of preeclampsia.¹ In conclusion, epidemiologic data suggest that both maternal and paternal genes contribute to the development of preeclampsia. However, the underlying genetics are complex, and it is currently unclear which genes are involved and how individual genetic variants contribute to preeclampsia. Therefore, numerous genetic association studies have been performed to elucidate the genetic background of preeclampsia. These genetic association studies have mainly focused on so-called “candidate genes” which are genes that are likely to be involved in the pathogenesis of preeclampsia. Despite a sharp increase in the number of published studies regarding genetic associations in preeclampsia, attempts to replicate findings from these studies have frequently yielded inconsistent results. This lack of reproducibility can be due to population diversity, but it is often the result of small sample sizes or false-positive results.⁶⁷ Because the prior probabilities of genetic associations are low, the number of false-positive associations that are generated by chance alone is high. Therefore, replicating an association in independent studies is essential for identifying true genetic associations among the large number of false-positives.

As previously mentioned, women suffering from pre-existent hypertension, diabetes, renal disease and obesity have a significantly higher risk for developing preeclampsia. Importantly, the genes that predispose to the aforementioned conditions could indirectly also predispose to preeclampsia. For instance, certain genetic variants may increase the risk for developing hypertension, which in turn causes an increased susceptibility for developing preeclampsia. Such predisposing disorders could set off a cascade of placental and systemic inflammation as well as oxidative stress, resulting in late onset pre-eclampsia.^{1;52}

PLACENTAL DYSFUNCTION IN PREECLAMPSIA: AN

IMMUNOLOGICAL PROBLEM? As opposed to late-onset preeclampsia, early-onset preeclampsia is frequently characterized by reduced placental perfusion and marked histopathological changes in the placenta, including compromised villous volume and surface area.^{11;13;58} It has been proposed that an excessive or atypical maternal immune response to trophoblast underlies this aberrant placental development in the setting of preeclampsia.^{59;68} Evidence for immune dysregulation in the pathophysiology of preeclampsia comes from epidemiological data, which have pointed out that preeclampsia is relatively common among women with the autoimmune diseases antiphospholipid syndrome (APS) and systemic lupus erythematoses (SLE). The presence of anti-phospholipid antibodies increases the risk for developing preeclampsia nine-fold.¹⁴ Conversely, among women who have had preeclampsia, anti-phospholipid antibodies are more frequently found.⁶⁹ In women with APS/SLE, pregnancies are frequently complicated by recurrent miscarriage, intra-uterine fetal loss and preeclampsia, and the complement system often plays an essential role in mediating these pregnancy complications.⁷⁰ The relation between preeclampsia and the aforementioned autoimmune diseases, combined with the important role of the complement system in mediating pregnancy complications in women with APS/SLE, suggests that the complement system may also be involved

in the pathogenesis of preeclampsia in women without underlying autoimmune disease.

WHAT IS THE EVIDENCE FOR COMPLEMENT DYSREGULATION IN HUMAN PREECLAMPSIA?

Indeed, several lines of evidence support the idea that complement is involved in the pathogenesis of preeclampsia. In preeclampsia, serum levels of C3a, C4d, and soluble MAC are markedly increased as compared to healthy pregnant women.⁴⁷ In addition, women with increased serum levels of the alternative pathway fragment Bb during early pregnancy are more likely to develop preeclampsia later in pregnancy⁷¹, suggesting that early activation of the alternative pathway may play a role in the pathogenesis of preeclampsia. Although the distribution of placental complement components is similar to physiological pregnancy, the quantity of these depositions is generally increased in cases of preeclampsia.^{40;44}

A putative mechanism by which the complement system can be activated in the setting of preeclampsia is the presence of circulating (auto)-antibodies. A subgroup of women with preeclampsia have circulating auto-antibodies directed against the angiotensin-I type II receptor (AT1-AA).⁷² These antibodies could result in hypertension through stimulating the angiotensin-I receptor. However, these antibodies may also cause complement activation, as demonstrated in a murine model. Importantly, injecting mice with these auto-antibodies results in complement activation as well as angiogenic dysregulation, an important hallmark in the pathophysiology of preeclampsia.⁷³ It is important to note that not only excessive activation but also inadequate regulation of the complement system could be responsible for complement deposits in women with preeclampsia. Importantly, mutations in complement regulatory proteins predispose to preeclampsia.³⁹ These mutations have also been found in women with the HELLP syndrome, which is closely linked to preeclampsia.³⁸

In summary, increasing evidence suggests that the complement

system is involved in the pathogenesis of preeclampsia. However, relatively little is known about which pathway is activated and how (changes in) complement regulation contribute to the pathogenesis of preeclampsia.

LATE MANIFESTATIONS OF PLACENTAL DYSFUNCTION:

ANGIOGENIC DYSREGULATION During the second stage of preeclampsia, the clinical manifestations of the syndrome develop. During this stage, reduced uteroplacental flow and episodes of irregular placental perfusion likely lead to the generation of reactive oxygen species⁷⁴, resulting in placental oxidative stress and placental dysfunction.⁷⁵ Subsequently, the preeclamptic placenta releases excessive amounts of the anti-angiogenic factors soluble fms-like tyrosine kinase (sFlt-1) and soluble endoglin (sEng).⁷⁶⁻⁷⁸ The anti-angiogenic factor sFlt-1 is a splice variant of the vascular endothelial growth factor (VEGF) receptor. By sequestering VEGF and placental growth factor (PlGF), sFlt-1 prevents their interaction with their membrane-bound receptors. Endoglin is a co-receptor for transforming growth factor B (TGF-B). Its soluble form, sEng, binds and thereby neutralizes TGF-B. As VEGF, PlGF and TGF-B are all growth factors essential for endothelial integrity, relative shortage of these angiogenic factors results in endothelial dysfunction. Therefore, these anti-angiogenic factors likely cause the generalized endothelial dysfunction that characterizes preeclampsia (Figure 5).^{79;80} Indeed, animal models have demonstrated that administration of sFlt-1 and sEng recapitulates the features of preeclampsia.^{77;78} Importantly, it is the syncytiotrophoblast—the outer layer of the placental villi—that produces these anti-angiogenic factors.⁸¹

TROPHOBLAST SHEDDING LIKELY CONTRIBUTES TO THIS

ANGIOGENIC IMBALANCE In addition to anti-angiogenic factors, the preeclamptic placenta also releases excessive amounts of syncytiotrophoblast fragments. During normal pregnancy, syncytiotrophoblast fragments are constantly shed into the maternal

circulation but this shedding is markedly increased in preeclampsia.⁸² This increase in shedding may be due to oxidative stress and subsequent apoptosis and necrosis of the syncytiotrophoblast.⁸³ These syncytiotrophoblast fragments release sFlt-1 and remain transcriptionally active up to 48 hours after their release from the placenta.⁸⁴ This implies that syncytial fragments may form an important source of sFlt-1 in the maternal circulation and that they could play an important role in causing systemic endothelial dysfunction (Figure 5). The fragments may contribute to endothelial dysfunction, as they inhibit endothelial proliferation *in vitro*.⁸⁵

THE END-RESULT: GENERALIZED ENDOTHELIAL

DYSFUNCTION Regardless whether placental dysfunction or maternal susceptibility underlies the development of preeclampsia, the end-result is endothelial dysfunction. Various studies have revealed that preeclampsia is associated with generalized endothelial dysfunction. Firstly, women with preeclampsia are characterized by impaired flow-mediated vasodilation^{86,87}, as well as by impaired acetylcholine-mediated vasorelaxation.⁸⁸ In addition, preeclampsia is associated with an enhanced vascular reactivity to angiotensin II⁸⁹ and serum from preeclamptic women induces endothelial activation *in vitro*.⁹⁰ Importantly, endothelial dysfunction can explain the clinical manifestations of preeclampsia.⁹¹ Hypertension in the setting of preeclampsia may result from impaired endothelial control of vascular tone, whereas proteinuria could be a manifestation of increased vascular permeability. Headache, seizures, visual disturbances, epigastric pain and fetal growth restriction could result from endothelial dysfunction in the vasculature of target organs, including the brain, liver, and placenta. When expectant management of preeclampsia is no longer possible due to the severity of the aforementioned symptoms, delivery is the only option left. As a result, preeclampsia continues to contribute to maternal and fetal morbidity and mortality worldwide.

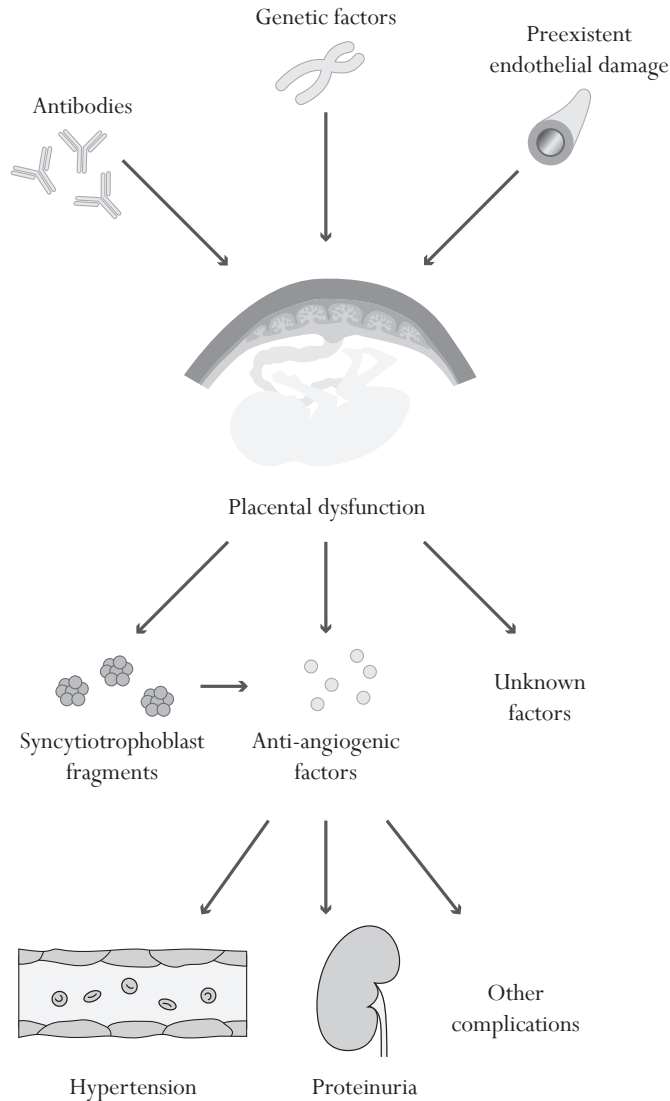


Figure 5: The pathophysiology of preeclampsia. The dysfunctional placenta releases excessive amounts of anti-angiogenic factors and syncytiotrophoblast fragments. These factors lead to endothelial dysfunction, which, in turn, can explain the clinical manifestations of preeclampsia.

Part 3 - This thesis

Despite decades of research, it remains largely unknown what drives placental dysfunction in preeclamptic women. Likewise, it is uncertain what exactly underlies maternal susceptibility in the setting of preeclampsia. Further research into the pathophysiological mechanisms underlying preeclampsia may provide targets for prevention, diagnosis and therapy. This thesis touches upon genetic, immunologic and angiogenic aspects of preeclampsia, and particularly on:

- The contribution of genetic variants in the development of preeclampsia
- Placental complement dysregulation in the setting of preeclampsia and the autoimmune disorders APS and SLE
- The source of sFlt-1 and the spread of transcriptionally active syncytiotrophoblast fragments throughout the maternal body

AIMS OF THIS THESIS

- To determine which genetic variants are reproducibly and significantly associated with preeclampsia
- To determine the role of placental C4d in SLE and antiphospholipid syndrome related adverse pregnancy outcome
- To investigate the relation between placental complement dysregulation and preeclampsia
- To explore the relationship between placental C4d and the extent of placental dysfunction in the setting preeclampsia
- To investigate whether placenta-derived fragments are retained in the maternal lung, and whether they remain transcriptionally active *in situ*

THESIS OUTLINE The role of genetic variants in preeclampsia is explored in **Chapter 2**, in which the results are described of a meta-analysis of genetic association studies that have been performed in the setting of preeclampsia.

Complement activation plays an important role in the pathogenesis of pregnancy complications in the setting of autoimmune disease. In **Chapter 3** the potential of the classical pathway component C4d as a biomarker for pregnancy complications in women with the autoimmune disorders antiphospholipid syndrome and systemic lupus erythematosus is explored.

Preeclampsia is strongly related to the autoimmune disorders APS and SLE. As complement activation plays an important role in mediating pregnancy complications in women with APS/SLE, this strong correlation suggests that the complement system may also be involved in the pathogenesis of preeclampsia. Therefore, **Chapters 4 and 5** focus on the role of placental complement activation in women with preeclampsia. **Chapter 4** describes the study in which we have investigated which complement pathway is activated within the placenta during preeclampsia. **Chapter 5** focuses on the relation between placental complement activation and the extent of placental dysfunction in the setting of preeclampsia.

Chapter 6 is devoted to angiogenic dysbalance in women with preeclampsia. In this chapter, the placental expression of sFlt-1 and the systemic spread of syncytiotrophoblast fragments is evaluated.

Overall, the work described in this thesis focuses on pathogenic mechanisms in the setting of preeclampsia. How do the genetic, immunologic and angiogenic aspects of preeclampsia together contribute to the endothelial dysfunction that is characteristic of preeclampsia? What is cause and what is consequence? These questions are addressed in **Chapter 7**, the general discussion, where the findings of this thesis will be summarized and placed in a more general perspective. The general discussion will be followed by a summary in Dutch.

REFERENCES

1. Steegers EA, von Dadelszen P, Duvekot JJ, Pijnenborg R. Pre-eclampsia. *Lancet* 2010; 376:631-644.
2. ACOG practice bulletin. Diagnosis and management of preeclampsia and eclampsia. Number 33, January 2002. *Obstet Gynecol* 2002; 99:159-167.
3. Khan KS, Wojdyla D, Say L, Gulmezoglu AM, Van Look PF. WHO analysis of causes of maternal death: a systematic review. *Lancet* 2006; 367:1066-1074.
4. Bellamy L, Casas JP, Hingorani AD, Williams DJ. Pre-eclampsia and risk of cardiovascular disease and cancer in later life: systematic review and meta-analysis. *BMJ* 2007; 335:974.
5. Davis EF, Lazdam M, Lewandowski AJ, Worton SA, Kelly B, Kenworthy Y *et al.* Cardiovascular risk factors in children and young adults born to preeclamptic pregnancies: a systematic review. *Pediatrics* 2012; 129:e1552-e1561.
6. Fraser A, Nelson SM, Macdonald-Wallis C, Cherry L, Butler E, Sattar N *et al.* Associations of pregnancy complications with calculated cardiovascular disease risk and cardiovascular risk factors in middle age: the Avon Longitudinal Study of Parents and Children. *Circulation* 2012; 125:1367-1380.
7. Berkowitz RS, Goldstein DP. Chorionic tumors. *N Engl J Med* 1996; 335:1740-1748.
8. Matsuo K, Kooshesh S, Dinc M, Sun CC, Kimura T, Baschat AA. Late postpartum eclampsia: report of two cases managed by uterine curettage and review of the literature. *Am J Perinatol* 2007; 24:257-266.
9. Nugent CE, Punch MR, Barr M, Jr., LeBlanc L, Johnson MP, Evans MI. Persistence of partial molar placenta and severe preeclampsia after selective termination in a twin pregnancy. *Obstet Gynecol* 1996; 87:829-831.
10. Raymond D, Peterson E. A critical review of early-onset and late-onset preeclampsia. *Obstet Gynecol Surv* 2011; 66:497-506.
11. Eghor M, Ansari T, Morris N, Green CJ, Sibbons PD. Morphometric placental villous and vascular abnormalities in early- and late-onset pre-eclampsia with and without fetal growth restriction. *BJOG* 2006; 113:580-589.
12. Onah HE, Iloabachie GC. Conservative management of early-onset pre-eclampsia and fetomaternal outcome in Nigerians. *J Obstet Gynaecol* 2002; 22:357-362.
13. James JL, Whitley GS, Cartwright JE. Pre-eclampsia: fitting together the placental, immune and cardiovascular pieces. *J Pathol* 2010; 221:363-378.
14. Duckitt K, Harrington D. Risk factors for pre-eclampsia at antenatal booking: systematic review of controlled studies. *BMJ* 2005; 330:565.
15. James JL, Carter AM, Chamley LW. Human placentation from nidation to 5 weeks of gestation. Part I: What do we know about formative placental development following implantation? *Placenta* 2012; 33:327-334.
16. Zhou Y, Damsky CH, Chiu K, Roberts JM, Fisher SJ. Preeclampsia is associated with abnormal expression of adhesion molecules by invasive cytotrophoblasts. *J Clin Invest* 1993; 91:950-960.

17. Zhou Y, Damsky CH, Fisher SJ. Preeclampsia is associated with failure of human cytotrophoblasts to mimic a vascular adhesion phenotype. One cause of defective endovascular invasion in this syndrome? *J Clin Invest* 1997; 99:2152-2164.
18. Billington WD. The immunological problem of pregnancy: 50 years with the hope of progress. A tribute to Peter Medawar. *J Reprod Immunol* 2003; 60:1-11.
19. Hunt JS, Petroff MG, McIntire RH, Ober C. HLA-G and immune tolerance in pregnancy. *FASEB J* 2005; 19:681-693.
20. Hunt JS. Stranger in a strange land. *Immunol Rev* 2006; 213:36-47.
21. Guerin LR, Prins JR, Robertson SA. Regulatory T-cells and immune tolerance in pregnancy: a new target for infertility treatment? *Hum Reprod Update* 2009; 15:517-535.
22. Le Bouteiller P, Mallet V. HLA-G and pregnancy. *Rev Reprod* 1997; 2:7-13.
23. Munn DH, Zhou M, Attwood JT, Bondarev I, Conway SJ, Marshall B *et al*. Prevention of allogeneic fetal rejection by tryptophan catabolism. *Science* 1998; 281:1191-1193.
24. Balasch J, Ercilla G, Vanrell JA, Vives J, Gonzalez-Merlo J. Effects of HLA antibodies on pregnancy. *Obstet Gynecol* 1981; 57:444-446.
25. Holmes CH, Simpson KL, Wainwright SD, Tate CG, Houlihan JM, Sawyer IH *et al*. Preferential expression of the complement regulatory protein decay accelerating factor at the fetomaternal interface during human pregnancy. *J Immunol* 1990; 144:3099-3105.
26. Holmes CH, Simpson KL, Okada H, Okada N, Wainwright SD, Purcell DF *et al*. Complement regulatory proteins at the feto-maternal interface during human placental development: distribution of CD59 by comparison with membrane cofactor protein (CD46) and decay accelerating factor (CD55). *Eur J Immunol* 1992; 22:1579-1585.
27. Walport MJ. Complement. First of two parts. *N Engl J Med* 2001; 344:1058-1066.
28. Walport MJ. Complement. Second of two parts. *N Engl J Med* 2001; 344:1140-1144.
29. Flierman R, Daha MR. The clearance of apoptotic cells by complement. *Immunobiology* 2007; 212:363-370.
30. Harboe M, Mollnes TE. The alternative complement pathway revisited. *J Cell Mol Med* 2008; 12:1074-1084.
31. Haas PJ, van Strijp J. Anaphylatoxins: their role in bacterial infection and inflammation. *Immunol Res* 2007; 37:161-175.
32. Klos A, Tenner AJ, Johswich KO, Ager RR, Reis ES, Kohl J. The role of the anaphylatoxins in health and disease. *Mol Immunol* 2009; 46:2753-2766.
33. Cole DS, Morgan BP. Beyond lysis: how complement influences cell fate. *Clin Sci (Lond)* 2003; 104:455-466.
34. Muller-Eberhard HJ. The killer molecule of complement. *J Invest Dermatol* 1985; 85:47s-52s.
35. Zipfel PF, Skerka C. Complement regulators and inhibitory proteins. *Nat Rev Immunol* 2009; 9:729-740.
36. Morgan BP, Meri S. Membrane proteins that protect against complement lysis. *Springer Semin Immunopathol* 1994; 15:369-396.
37. Noris M, Mescia F, Remuzzi G. STEC-HUS, atypical HUS and TTP are all diseases of complement activation. *Nat Rev Nephrol* 2012; 8:622-33.

38. Fakhouri F, Jablonski M, Lepercq J, Blouin J, Benachi A, Hourmant M *et al.* Factor H, membrane cofactor protein, and factor I mutations in patients with hemolysis, elevated liver enzymes, and low platelet count syndrome. *Blood* 2008; 112:4542-4545.
39. Salmon JE, Heuser C, Triebwasser M, Liszewski MK, Kavanagh D, Roumenina L *et al.* Mutations in complement regulatory proteins predispose to preeclampsia: a genetic analysis of the PROMISSE cohort. *PLoS Med* 2011; 8:e1001013.
40. Sinha D, Wells M, Faulk WP. Immunological studies of human placenta: complement components in pre-eclamptic chorionic villi. *Clin Exp Immunol* 1984; 56:175-184.
41. Weir PE. Immunofluorescent studies of the uteroplacental arteries in normal pregnancy. *Br J Obstet Gynaecol* 1981; 88:301-307.
42. Wells M, Bennett J, Bulmer JN, Jackson P, Holgate CS. Complement component deposition in uteroplacental (spiral) arteries in normal human pregnancy. *J Reprod Immunol* 1987; 12:125-135.
43. Rampersad R, Barton A, Sadovsky Y, Nelson DM. The C5b-9 membrane attack complex of complement activation localizes to villous trophoblast injury in vivo and modulates human trophoblast function in vitro. *Placenta* 2008; 29:855-861.
44. Tedesco F, Radillo O, Candussi G, Nazzaro A, Mollnes TE, Pecorari D. Immunohistochemical detection of terminal complement complex and S protein in normal and pre-eclamptic placentae. *Clin Exp Immunol* 1990; 80:236-240.
45. Abramson SB, Buyon JP. Activation of the complement pathway: comparison of normal pregnancy, preeclampsia, and systemic lupus erythematosus during pregnancy. *Am J Reprod Immunol* 1992; 28:183-187.
46. Richani K, Soto E, Romero R, Espinoza J, Chaiworapongsa T, Nien JK *et al.* Normal pregnancy is characterized by systemic activation of the complement system. *J Matern Fetal Neonatal Med* 2005; 17:239-245.
47. Derzsy Z, Prohaszka Z, Rigo J, Jr., Fust G, Molvarec A. Activation of the complement system in normal pregnancy and preeclampsia. *Mol Immunol* 2010; 47:1500-1506.
48. Xu C, Mao D, Holers VM, Palanca B, Cheng AM, Molina H. A critical role for murine complement regulator *cr1* in fetomaternal tolerance. *Science* 2000; 287:498-501.
49. Bulla R, Agostinis C, Bossi F, Rizzi L, Debeus A, Tripodo C *et al.* Decidual endothelial cells express surface-bound C1q as a molecular bridge between endovascular trophoblast and decidual endothelium. *Mol Immunol* 2008; 45:2629-2640.
50. Agostinis C, Bulla R, Tripodo C, Gismondi A, Stabile H, Bossi F *et al.* An alternative role of C1q in cell migration and tissue remodeling: contribution to trophoblast invasion and placental development. *J Immunol* 2010; 185:4420-4429.
51. Singh J, Ahmed A, Girardi G. Role of complement component C1q in the onset of preeclampsia in mice. *Hypertension* 2011; 58:716-724.
52. Roberts JM, Hubel CA. The two stage model of preeclampsia: variations on the theme. *Placenta* 2009; 30 Suppl A:S32-S37.

53. Meekins JW, Pijnenborg R, Hanssens M, McFadyen IR, van AA. A study of placental bed spiral arteries and trophoblast invasion in normal and severe pre-eclamptic pregnancies. *Br J Obstet Gynaecol* 1994; 101:669-674.
54. Brosens I, Pijnenborg R, Vercruysse L, Romero R. The "Great Obstetrical Syndromes" are associated with disorders of deep placentation. *Am J Obstet Gynecol* 2011; 204:193-201.
55. Lim KH, Zhou Y, Janatpour M, McMaster M, Bass K, Chun SH *et al.* Human cytotrophoblast differentiation/ invasion is abnormal in pre-eclampsia. *Am J Pathol* 1997; 151:1809-1818.
56. Plasencia W, Maiz N, Bonino S, Kaihura C, Nicolaides KH. Uterine artery Doppler at 11 + 0 to 13 + 6 weeks in the prediction of pre-eclampsia. *Ultrasound Obstet Gynecol* 2007; 30:742-749.
57. Gomez O, Figueras F, Martinez JM, del RM, Palacio M, Eixarch E *et al.* Sequential changes in uterine artery blood flow pattern between the first and second trimesters of gestation in relation to pregnancy outcome. *Ultrasound Obstet Gynecol* 2006; 28:802-808.
58. Li H, Gudnason H, Olofsson P, Dubiel M, Gudmundsson S. Increased uterine artery vascular impedance is related to adverse outcome of pregnancy but is present in only one-third of late third-trimester pre-eclamptic women. *Ultrasound Obstet Gynecol* 2005; 25:459-463.
59. Redman CW, Sacks GP, Sargent IL. Preeclampsia: an excessive maternal inflammatory response to pregnancy. *Am J Obstet Gynecol* 1999; 180:499-506.
60. Chesley LC, Cooper DW. Genetics of hypertension in pregnancy: possible single gene control of pre-eclampsia and eclampsia in the descendants of eclamptic women. *Br J Obstet Gynaecol* 1986; 93:898-908.
61. Cincotta RB, Brennecke SP. Family history of pre-eclampsia as a predictor for pre-eclampsia in primigravidas. *Int J Gynaecol Obstet* 1998; 60:23-27.
62. Nilsson E, Salonen RH, Cnattingius S, Lichtenstein P. The importance of genetic and environmental effects for pre-eclampsia and gestational hypertension: a family study. *BJOG* 2004; 111:200-206.
63. Sutherland A, Cooper DW, Howie PW, Liston WA, MacGillivray I. The incidence of severe pre-eclampsia amongst mothers and mothers-in-law of pre-eclamptics and controls. *Br J Obstet Gynaecol* 1981; 88:785-791.
64. Mogren I, Hogberg U, Winkvist A, Stenlund H. Familial occurrence of preeclampsia. *Epidemiology* 1999; 10:518-522.
65. Esplin MS, Fausett MB, Fraser A, Kerber R, Mineau G, Carrillo J *et al.* Paternal and maternal components of the predisposition to preeclampsia. *N Engl J Med* 2001; 344:867-872.
66. Skjaerven R, Vatten LJ, Wilcox AJ, Ronning T, Irgens LM, Lie RT. Recurrence of pre-eclampsia across generations: exploring fetal and maternal genetic components in a population based cohort. *BMJ* 2005; 331:877.
67. Ioannidis JP, Ntzani EE, Trikalinos TA, Contopoulos-Ioannidis DG. Replication validity of genetic association studies. *Nat Genet* 2001; 29:306-309.
68. Jauniaux E, Poston L, Burton GJ. Placental-related diseases of pregnancy: Involvement of oxidative stress and implications in human evolution. *Hum Reprod Update* 2006; 12:747-755.

69. Heilmann L, Schorsch M, Hahn T, Fareed J. Antiphospholipid syndrome and pre-eclampsia. *Semin Thromb Hemost* 2011; 37:141-145.
70. Cohen D, Berger SP, Steup-Beekman GM, Bloemenkamp KW, Bajema IM. Diagnosis and management of the antiphospholipid syndrome. *BMJ* 2010; 340:c2541.
71. Lynch AM, Murphy JR, Byers T, Gibbs RS, Neville MC, Giclas PC *et al.* Alternative complement pathway activation fragment Bb in early pregnancy as a predictor of preeclampsia. *Am J Obstet Gynecol* 2008; 198:385-389.
72. Dechend R, Muller DN, Wallukat G, Homuth V, Krause M, Dudenhausen J *et al.* Activating auto-antibodies against the AT1 receptor in preeclampsia. *Autoimmun Rev* 2005; 4:61-65.
73. Wang W, Irani RA, Zhang Y, Ramin SM, Blackwell SC, Tao L *et al.* Autoantibody-mediated complement C3a receptor activation contributes to the pathogenesis of preeclampsia. *Hypertension* 2012; 60:712-721.
74. Burton GJ. Oxygen, the Janus gas; its effects on human placental development and function. *J Anat* 2009; 215:27-35.
75. Hung TH, Skepper JN, Burton GJ. In vitro ischemia-reperfusion injury in term human placenta as a model for oxidative stress in pathological pregnancies. *Am J Pathol* 2001; 159:1031-1043.
76. Levine RJ, Lam C, Qian C, Yu KF, Maynard SE, Sachs BP *et al.* Soluble endoglin and other circulating antiangiogenic factors in preeclampsia. *N Engl J Med* 2006; 355:992-1005.
77. Maynard SE, Min JY, Merchan J, Lim KH, Li J, Mondal S *et al.* Excess placental soluble fms-like tyrosine kinase 1 (sFlt1) may contribute to endothelial dysfunction, hypertension, and proteinuria in preeclampsia. *J Clin Invest* 2003; 111:649-658.
78. Venkatesha S, Toporsian M, Lam C, Hanai J, Mammoto T, Kim YM *et al.* Soluble endoglin contributes to the pathogenesis of preeclampsia. *Nat Med* 2006; 12:642-649.
79. Lam C, Lim KH, Karumanchi SA. Circulating angiogenic factors in the pathogenesis and prediction of preeclampsia. *Hypertension* 2005; 46:1077-1085.
80. Redman CW, Sargent IL. Latest advances in understanding preeclampsia. *Science* 2005; 308:1592-1594.
81. Clark DE, Smith SK, He Y, Day KA, Licence DR, Corps AN *et al.* A vascular endothelial growth factor antagonist is produced by the human placenta and released into the maternal circulation. *Biol Reprod* 1998; 59:1540-1548.
82. Redman CW, Sargent IL. Circulating microparticles in normal pregnancy and pre-eclampsia. *Placenta* 2008; 29 Suppl A:S73-S77.
83. Redman CW, Sargent IL. Placental debris, oxidative stress and pre-eclampsia. *Placenta* 2000; 21:597-602.
84. Rajakumar A, Cerdeira AS, Rana S, Zsengeller Z, Edmunds L, Jeyabalan A *et al.* Transcriptionally active syncytial aggregates in the maternal circulation may contribute to circulating soluble fms-like tyrosine kinase 1 in preeclampsia. *Hypertension* 2012; 59:256-264.

85. Smarason AK, Sargent IL, Starkey PM, Redman CW. The effect of placental syncytiotrophoblast microvillous membranes from normal and pre-eclamptic women on the growth of endothelial cells in vitro. *Br J Obstet Gynaecol* 1993; 100:943-949.
86. McCarthy AL, Woolfson RG, Raju SK, Poston L. Abnormal endothelial cell function of resistance arteries from women with preeclampsia. *Am J Obstet Gynecol* 1993; 168:1323-1330.
87. Cockell AP, Poston L. Flow-mediated vasodilatation is enhanced in normal pregnancy but reduced in preeclampsia. *Hypertension* 1997; 30:247-251.
88. Pascoal IF, Lindheimer MD, Nalbantian-Brandt C, Umans JG. Preeclampsia selectively impairs endothelium-dependent relaxation and leads to oscillatory activity in small omental arteries. *J Clin Invest* 1998; 101:464-470.
89. Gant NF, Daley GL, Chand S, Whalley PJ, MacDonald PC. A study of angiotensin II pressor response throughout primigravid pregnancy. *J Clin Invest* 1973; 52:2682-2689.
90. Roberts JM, Edep ME, Goldfien A, Taylor RN. Sera from preeclamptic women specifically activate human umbilical vein endothelial cells in vitro: morphological and biochemical evidence. *Am J Reprod Immunol* 1992; 27:101-108.
91. Roberts JM, Taylor RN, Goldfien A. Clinical and biochemical evidence of endothelial cell dysfunction in the pregnancy syndrome preeclampsia. *Am J Hypertens* 1991; 4:700-708.

CHAPTER II

GENETIC VARIANTS IN

PREECLAMPSIA:

A META-ANALYSIS

ALETTA BUURMA • ROSANNE TURNER • ANNEMARIEK DRIESSEN
ANTIEN MOOYAART • JAN SCHOONES • JAN ANTHONIE BRUIJN
KITTY BLOEMENKAMP • OLAF DEKKERS • HANS BAELEDE

HUMAN REPRODUCTION UPDATE

Abstract

BACKGROUND Preeclampsia has a clear familial component, suggesting that the condition may be partly attributable to genetic susceptibility. The search for susceptibility genes has led to a drastic increase in the number of published studies associating genetic factors with preeclampsia. However, attempts to replicate these findings have yielded inconsistent results. This meta-analysis assessed the pooled effect of each genetic variant that is reproducibly associated with preeclampsia.

METHODS Studies that assessed the association between genes and preeclampsia were searched in PubMed, Embase and Web of Science. We selected all genetic variants that were significantly associated with preeclampsia in an initial study and were subsequently independently reproduced in at least one additional study. All studies that assessed these reproduced variants were then included. The association between genetic variants and preeclampsia was calculated at the allele level, and the main measure of effect was a pooled odds ratio in a random-effects model.

RESULTS The literature search yielded 2965 articles, of which 542 investigated genetic associations in preeclampsia. We identified 22 replicated genetic variants, of which seven remained significantly associated with preeclampsia following meta-analysis. These variants were in or near the following genes: ACE, CTLA4, F2, FV, LPL and SERPINE1.

CONCLUSIONS This meta-analysis identified seven genetic variants associated with preeclampsia. Importantly, many of these variants are also risk factors for developing cardiovascular disease, revealing that preeclampsia and cardiovascular disease have shared genetic risk factors. The contribution of the identified genetic variants in the pathogenesis of preeclampsia should be the focus of future studies.

Introduction

Preeclampsia is a severe pregnancy complication characterized by hypertension and proteinuria after 20 weeks of gestation. Globally, preeclampsia affects 5-8% of pregnancies and contributes significantly to maternal and fetal morbidity and mortality.¹ Furthermore, women with preeclampsia have an increased risk of developing cardiovascular disease later in life.² Because the precise etiology of preeclampsia remains unknown, accurate prediction and prevention of the condition are at present difficult.

Preeclampsia is believed to result from a complex interplay between genetic components and environmental factors. Evidence for a genetic component comes from family studies, which have shown that preeclampsia is relatively common among daughters and sisters of preeclamptic women.³⁻⁸ Furthermore, the prevalence of preeclampsia differs between various ethnic groups.¹ However, the underlying genetics are complex, and it is currently unclear what genes are involved and how individual genetic variants contribute to preeclampsia.

Numerous genetic association studies have been performed to elucidate the genetic background of preeclampsia. An overview of candidate genes investigated in the setting of preeclampsia indicates a sharp increase in the number of published studies regarding genetic associations in preeclampsia, with three published studies in 1996 in contrast to 30 published studies in 2004.⁹ However, attempts to replicate these studies have yielded inconsistent results. Although this lack of reproducibility can be due simply to population diversity, it is often the result of small sample sizes or false-positive results.¹⁰ Because the prior probabilities of genetic associations are low, the number of false-positive associations that are generated by chance alone is high. The likelihood of finding false-positive associations increases when low prior probabilities are not accounted for in the statistical analysis.

Therefore, independently replicating an association is essential for identifying true genetic associations among the large number of false-positives.

The aim of this study was to compile an overview of the genetic variants that are truly associated with preeclampsia. Therefore, we performed a meta-analysis to assess the pooled effect of genetic variants that have been reproducibly associated with preeclampsia.

Methods

LITERATURE SEARCH The databases PubMed, Embase and Web of Science were searched through February 2012 for studies that evaluated genetic variants in preeclampsia. A comprehensive search strategy was developed in collaboration with a trained librarian. The search terms that were used in the search strategy included: “Preeclampsia” and “Polymorphisms” or “Genes”. For these terms, all relevant keyword variants were included. The names of specific genes and polymorphisms that were yielded in the first search were added to the search strategy in subsequent searches. The search strategy was optimized for each database (see Supplementary data). Aside from limiting the searches to studies published in English, no other limits or filters were applied in the searches. In addition, references of other narrative and systematic reviews were checked for relevant articles.

ELIGIBILITY CRITERIA We searched for case-control studies that compared genetic variants between patients with preeclampsia and healthy women with uncomplicated pregnancies. We only included studies that defined preeclampsia as elevated blood pressure (with clear cut-off values for systolic and diastolic blood pressure) accompanied with proteinuria measured in at least a semi-quantitative way, in line with the ISSHP (International Society for the Study of Hypertension in Pregnancy) criteria.¹¹ For inclusion,

the study had to involve unrelated women. Studies in which the case group contained women with gestational hypertension were excluded, as were studies in which the control group contained subjects who had never been pregnant. All titles and abstracts were reviewed by two observers (A.B. and R.T.), who independently assessed whether the study investigated the relationship between preeclampsia and at least one genetic variant. Genetic association studies were screened for whether they contained a positive or negative association between an individual genetic variant and preeclampsia (based on the reported p-values, with association defined as significant when $p < 0.05$). When a genetic variant was found to be significantly associated with preeclampsia (either at the allelic or genotypic level, including the recessive and dominant model) in at least two independent studies, that variant was considered to be reproduced. For these reproduced genetic variants, all other genetic studies—irrespective of their p-values—were identified to estimate the pooled effect of the variant on preeclampsia in a meta-analysis.

DATA EXTRACTION Allele frequencies were extracted and entered into separate databases by two authors independently. These two databases were then compared, and disparities were resolved by consensus. Multiple studies published by the same author(s) were checked for overlapping (i.e. redundant) participant groups, and in cases in which the studies overlapped the study with the smaller dataset was excluded. When insufficient data was provided to calculate an odds ratio, at least two attempts were made to contact the corresponding author. When neither the published report nor the corresponding author provided sufficient data to calculate an odds ratio at the allele level, the study was excluded.

STATISTICAL ANALYSIS The main outcome of the meta-analysis was the pooled odds ratio (calculated at the allele level) for the association between reproduced genetic variants and preeclampsia.

The frequency of the minor allele was compared between women with preeclampsia and healthy control subjects who had an uncomplicated pregnancy. The data were pooled using a random-effects model to account for between-study heterogeneity. To estimate heterogeneity, we used I^2 , which reflects the percentage of total variation across studies that is due to heterogeneity rather than due to chance.¹² Bias due to small study size was tested using a stratified analysis for study size as described previously.¹³ This analysis was performed only for genetic variants that were significantly associated with preeclampsia after the meta-analysis and for which the number of studies that investigated the genetic variant was higher than ten. Publication bias was assessed using the Begg and Egger tests. In addition, we generated funnel plots of all reproduced genetic variants. All analyses were performed using STATA (StataCorp. 2011. Stata Statistical Software, Release 10. College Station, TX; StataC).

Results

The initial literature search yielded 2965 articles, 542 of which were genetic association studies regarding preeclampsia (Figure 1). We identified 22 polymorphisms in 15 genes that were reproducibly associated with preeclampsia. Associations between these 22 variants and preeclampsia were described in 163 articles, representing 283 studies. These articles were published from 1993 through 2012. The number of studies per genetic variant ranged from 2 to 45, and the number of cases included in these studies ranged from 7 to 808.

In a random-effects meta-analysis, seven genetic variants in or near six genes were significantly associated with preeclampsia (Figure 2a). The remaining 15 reproduced variants were not associated with preeclampsia following meta-analysis (Figure 2b). The odds ratios of the significant associations with preeclampsia ranged from 1.20 to 2.42. The genes with the largest effect had wider confidence intervals, indicating less certainty in the effect estimates. No

significant protective effect was found for any gene. Table I provides an overview of the analyses of all reproduced genetic variants as well as the location and the functional consequences of these genetic variants, and Table II provides the references of the included studies per genetic variant. The characteristics of all included studies, as well as forest plots of the individual reproduced genetic variants, funnel plots for assessment of publication bias and stratified analysis for study size are provided in Supplementary data. The cut-off values for hypertension and proteinuria in Table II of the Online Supplement show that some studies only included women with severe preeclampsia.

GENETIC VARIANTS INVOLVED IN COAGULATION AND

FIBRINOLYSIS Five genetic variants in four genes that are related to coagulation and fibrinolysis were associated reproducibly with preeclampsia. Of these five variants, four were still associated with preeclampsia after the meta-analysis. Two variants in coagulation factor V (FV), rs6025 and rs6020, remained associated with preeclampsia in the meta-analysis. The rs6025 variant, which is also known as Factor V Leiden, was a frequently studied polymorphism in preeclampsia, with 40 studies resulting in a pooled odds ratio of 1.94 (95% CI 1.56-2.45). In a sensitivity analysis, the pooled odds ratio decreased slightly with increasing study size, decreasing from 1.99 in studies with <100 cases to 1.71 in studies with ≥200 cases. The rs6020 variant was reported in only two studies, resulting in a pooled odds ratio of 1.94 (95% CI 1.05-3.60). A variant in methylenetetrahydrofolate reductase (MTHFR), rs1801133, was reported in 45 studies, resulting in a pooled odds ratio of 1.06 (95% CI 0.97-1.16). The rs1799963 variant of the coagulation factor II (F2) gene (also known as prothrombin) was investigated in 30 studies and was associated with preeclampsia with an odds ratio of 1.95 (95% CI 1.43-2.66). In a sensitivity analysis, the studies with the largest number of cases yielded the largest effect estimate, with an odds ratio of 3.84 (95% CI 2.18-6.78). The rs1799889 variant in

serpin peptidase inhibitor (SERPINE1, also known as plasminogen activator inhibitor type 1) was associated with preeclampsia in the meta-analysis with an odds ratio of 1.17 (95% CI 1.03-1.33). When subdividing the studies based on study size, the effect estimate diminished slightly from 1.21 in studies with < 100 cases to 1.17 in studies with 100-200 cases and 1.14 in studies with $\geq$ 200 cases.

GENETIC VARIANTS INVOLVED IN THE RENIN-ANGIOTENSIN SYSTEM The angiotensin I converting enzyme (ACE) rs4646994 variant has been studied frequently in preeclampsia, with 20 studies yielding a pooled odds ratio of 1.20 (95% CI 1.08-1.34). A stratified analysis revealed a diminishing effect as study size increased, with a pooled odds ratio of 1.45 (95% CI 1.21-1.73) for studies with <100 cases, which is in contrast with a pooled OR of 1.05 (95% CI 0.90-1.23) for pooled studies with $\geq$ 200 cases. The rs699 and rs4762 variants in angiotensinogen (AGT) were studied in 21 and five studies, respectively. The rs699 variant was not associated with preeclampsia after meta-analysis, with a pooled odds ratio of 1.23 (95% CI 0.98-1.54). The rs4762 variant was also not associated with preeclampsia, with an odds ratio of 1.25 (95% CI 0.67-2.30). Another variant in the renin-angiotensin system, rs5186 of angiotensin II receptor type 1 (AT1R), was investigated in nine studies and was not associated with preeclampsia after meta-analysis.

GENETIC VARIANTS INVOLVED IN OXIDATIVE STRESS

Three variants in the nitric oxide synthase 3 (NOS3) gene were reproducibly associated with preeclampsia, but none was still associated with preeclampsia following the meta-analysis. The 27 bp-VNTR in intron 4 yielded a pooled odds ratio of 1.14 (95% CI 0.90-1.43), and the rs2070744 and rs1799983 variants yielded pooled odds ratios of 1.08 (95% CI 0.95-1.23) and 1.19 (95% CI 1.00-1.42), respectively.

GENETIC VARIANTS INVOLVED IN INFLAMMATION

The cytotoxic T-lymphocyte-associated protein 4 (CTLA4) rs231775 variant was reported in four studies. The meta-analysis revealed an association with preeclampsia with a pooled odds ratio of 1.24 (95% CI 1.01-1.52). The rs1800896 variant of interleukin 10 (IL-10) was not associated with preeclampsia in the meta-analysis (OR 0.91, 95% CI 0.74-1.12). Two variants in the tumor necrosis factor alpha (TNF-alpha) gene (rs1800629 and rs1799724) were reproduced in preeclampsia but were not associated with preeclampsia after the meta-analysis, with odds ratios of 1.17 (95% CI 0.91-1.49) and 0.66 (95% CI 0.33-1.31), respectively.

GENETIC VARIANTS INVOLVED IN LIPID METABOLISM

The rs1800590 and rs268 variants in the lipoprotein lipase (LPL) gene were reproduced in preeclampsia, but only rs268 remained associated with preeclampsia following the meta-analysis (OR 2.42, 95% CI 1.25-4.68). The combined rs429358 and rs7412 polymorphisms (E2 allele) in the apolipoprotein E (APOE) gene was reported in eight studies, yielding a pooled odds ratio of 0.86 (95% CI 0.66-1.13).

GENETIC VARIANTS INVOLVED IN OTHER PATHWAYS

Two variants in the toll-like receptor 4 (TLR4) gene, rs4986790 and rs4986791, were reported in four and three studies, respectively. Neither variant remained associated with preeclampsia following the meta-analysis. The rs3025039 variant in the vascular endothelial growth factor (VEGF) gene was reproduced in preeclampsia, although the meta-analysis did not reveal a statistically significant association (OR 1.36, 95% CI 0.64-2.91).

Discussion

In this meta-analysis, seven genetic variants were found to be associated with preeclampsia. Meta-analysis for several individual genetic variants in the setting of preeclampsia has been performed previously. However, the present study provides the first complete and comprehensive overview of all genetic variants that are reproducibly associated with preeclampsia. These data may shed light on the pathogenesis of preeclampsia and thereby reveal molecular pathways that can be targeted in the management of this condition. Genetic variants in or near the ACE, CTLA4, F2, FV (two variants), LPL and SERPINE1 genes were associated with preeclampsia. The results of this meta-analysis suggest that the following systems may play a role in the pathogenesis of preeclampsia: the renin-angiotensin system, coagulation and fibrinolysis, lipid metabolism, and inflammation. Functional studies are needed to elucidate the contribution of these variants and pathways to the pathogenesis of preeclampsia.

One genetic variant involved in the renin-angiotensin system remained associated with preeclampsia following the meta-analysis; the D (deletion) allele of ACE rs4646994. This finding is in line with a previous meta-analysis, which also revealed evidence of small study bias.¹³ The ACE rs4646994 variant is known to be associated with increased activity of the angiotensin-converting enzyme¹⁴, which could increase the conversion of angiotensin I into angiotensin II, thus affecting the regulation of blood pressure and blood volume.

Preeclampsia is associated with an exaggerated maternal inflammatory response. Therefore, various candidate genes involved in inflammation have been studied in the setting of preeclampsia; only one genetic variant in CTLA-4 remained associated with preeclampsia after our meta-analysis. No previous meta-analysis of this variant in the setting of preeclampsia has been published to date. CTLA-4 plays an important role in the negative regulation of T-cell proliferation and activation. The G allele of CTLA4

rs231775 is associated with reduced surface expression of CTLA-4, possibly leading to increased T-cell proliferation and activation.¹⁵⁻¹⁶ Carrying the G allele of CTLA-4 could contribute to the maternal inflammatory response, thereby increasing the risk of developing preeclampsia.

With respect to genes involved in lipid metabolism, one variant in LPL remained associated with preeclampsia following the meta-analysis. No previous meta-analysis of this variant in the setting of preeclampsia has been published to date. The G allele of LPL rs268 is associated with reduced LPL activity and dyslipidemia.¹⁷ Because dyslipidemia can contribute to endothelial cell dysfunction, carriers of the G allele may have an increased risk for developing preeclampsia.¹⁸

After meta-analysis, several factors involved in coagulation and fibrinolysis remained associated with preeclampsia, which is largely in line with previous meta-analyses.¹⁹⁻²⁰ Normal pregnancy is associated with the development of a hypercoagulable, hypofibrinolytic state, which is exaggerated in preeclampsia. Thrombophilias can increase the risk of developing preeclampsia via placental thrombosis and effects on both trophoblast growth and differentiation.²¹ The A allele of F2 rs1799963 is associated with both higher prothrombin levels and an increased risk of thrombosis.²²⁻²³ Two variants in FV are associated with preeclampsia. FV rs6025 causes activated protein C resistance and subsequent thrombophilic events. The A allele of FV rs6020 is also associated with a weak response to activated protein C²⁴ and can therefore cause a predisposition to thrombotic events. The SERPINE1 gene encodes the plasminogen activator inhibitor 1 (PAI-1) protein, which is an important inhibitor of fibrinolysis. The 4G allele of SERPINE1 rs1799889 is associated with elevated plasma levels of PAI-1.²⁵ By increasing the inhibition of fibrinolysis, this genetic variant may contribute to the exaggerated hypercoagulable state that characterizes women with preeclampsia.

In accordance to previous meta-analyses, many genetic variants did not remain associated with preeclampsia following meta-

analysis.²⁶⁻²⁹ Perhaps this is due to the clinical variety of the cases that were included in the studies. Some studies, for instance, only included women with severe preeclampsia. It is, however, also likely that there is a true lack of association between preeclampsia and these genetic variants. Illustratively, publication bias can lead to the early publication of extreme, promising results, while subsequent (larger) studies often contradict these initial findings.³⁰⁻³¹

It is important to note that epidemiological studies have revealed a relationship between preeclampsia and cardiovascular morbidity and mortality later in life.³²⁻³⁶ Women who have had preeclampsia are more likely to develop cardiovascular disease, and preeclampsia and cardiovascular disease share various risk factors, including obesity, hypertension and diabetes.¹ Several of the variants that were associated with preeclampsia in this meta-analysis are also identified risk factors for developing cardiovascular disease. For example, the SERPINE1 rs1799889 variant, the FV rs6025 and the F2 rs1799963 variant are all associated with coronary disease.³⁷ In addition, carriers of select LPL alleles have an increased risk for developing coronary disease, and the rs268 variant of LPL is associated with adverse lipid profiles.³⁸ Thus, preeclampsia and cardiovascular disease have shared genetic risk factors as well as overlapping environmental risk factors. The presence of genetic variants may contribute to the increased risk of cardiovascular disease among women who have a history of preeclampsia. It would be interesting to investigate whether a combination of environmental and genetic risk factors can predict what women with preeclampsia will be more likely to develop cardiovascular disease later in life. In this way, preventive strategies that are tailored to the individual patient could be developed.

Our meta-analysis included only genetic variants that were associated with preeclampsia and for which independent replication was available. This approach has been described previously³⁹ and aims to reduce the likelihood of reporting false-positive associations. However, by selecting only the genetic variants that are reproducibly associated with preeclampsia, genetic variants with smaller effect

sizes might have been overlooked. For example, when variants were described in small studies that individually lacked sufficient power to detect modest effects, pooling these studies may have resulted in a significant association. Publication bias is an issue for concern in all meta-analyses. Studies yielding negative results are less likely to be published; as a result, authors might only report those associations that reach statistical significance, thereby omitting non-significant genetic associations. Together, these publication biases could result in an overrepresentation of significant effects. Therefore, the effect estimates that are reported in this study should be interpreted with caution, particularly when associations were based on a small number of studies and/or relatively small groups of participants. In addition, small study bias may have affected the outcomes of this meta-analysis. Small-study bias is a form of bias in which small studies regarding gene-disease associations report genetic effects that are not found—or are found at a much smaller magnitude—in larger studies. In addition to preeclampsia¹³, evidence for small-study bias has previously been reported with respect to both neurological and cardiovascular diseases.^{30;40} When many small studies that report false-positive associations are pooled in a meta-analysis, conclusions drawn from that meta-analysis are likely to be unreliable. Therefore, results that are drawn from meta-analyses in which there is evidence of small-study bias should be interpreted with caution. To investigate whether small-study bias played a role in our analyses, we subdivided the studies based on the number of cases and performed a stratified analysis. We found that the ACE rs4646994 variant appeared to be subject to small-study bias. The rs6020 variant in FV was reported in only two studies; therefore, no study size-based analysis was performed for this variant. For the remaining variants, no change—or only a slight change—in effect estimates was observed with increasing study size. Moreover, it is important to note that in this study, the genes with the largest effects were generally associated with wider confidence intervals, suggesting greater uncertainty in their effect estimates.

Because the precise etiology of preeclampsia remains unknown, effective strategies for preventing and treating preeclampsia are currently lacking. The identification of genetic variants associated with preeclampsia susceptibility can lead to novel biological insights⁴¹ and result in new targets for the prevention and treatment of preeclampsia. However, in order to prevent (small-study) bias, genetic association studies should preferably be performed using large (multi-center) cohorts. Furthermore, most genes that were studied in the setting of preeclampsia were investigated because they were previously shown to be involved in hypertension or cardiovascular disease. An alternate method for identifying new susceptibility genes is to use a hypothesis-free approach such as genome-wide association studies. In addition, next-generation sequencing—which allows the sequencing of DNA at unprecedented speeds—may identify rare causal variants that are associated with preeclampsia. Aside from searching for novel susceptibility genes, future studies should also focus on assessing the relevance of previously detected and reproduced genetic variants.

In summary, this meta-analysis identified seven genetic variants in or near six different genes that are associated with preeclampsia. These genetic variants are likely to represent true associations. Moreover, this is the first study to report that preeclampsia and cardiovascular disease have genetic risk factors in common. Further studies investigating the relative contribution of these variants and the mechanisms by which they affect the risk of developing preeclampsia are warranted.

FIG 1 **STUDY SELECTION PROCESS**

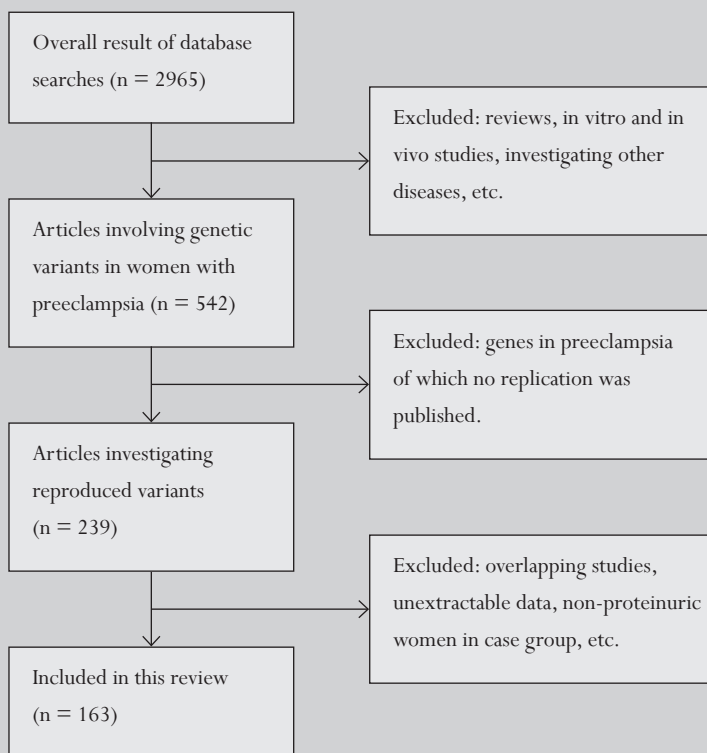


Figure 1: Flow chart illustrating how the studies were selected for the meta-analysis.

ODDS RATIOS FOR THE GENETIC VARIANTS THAT WERE REPRODUCIBLY ASSOCIATED WITH PREECLAMPSIA

FIG 2A

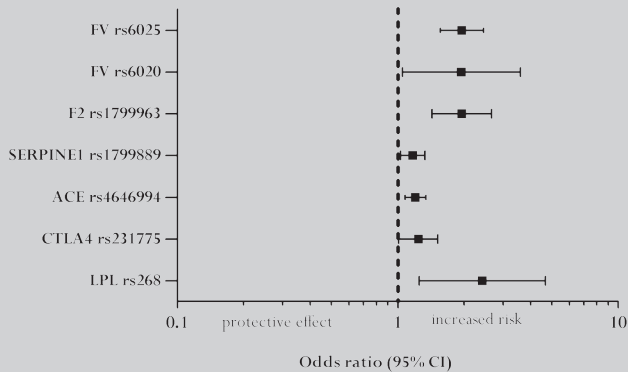


FIG 2B

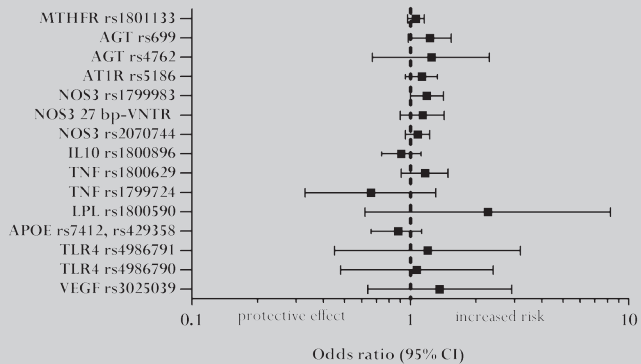


Figure 2 odds ratios (with 95% confidence intervals) for the genetic variants that were reproducibly associated with preeclampsia.

Figure 2A shows all the genetic variants that were reproduced in an independent study and significantly associated with preeclampsia following the meta-analysis.

Figure 2B shows all the genetic variants that were reproduced in an independent study, but were not significantly associated with preeclampsia following the meta-analysis.

TABLE 1 RANDOM-EFFECTS META-ANALYSIS OF REPRODUCED VARIANTS

VARIANT BY GENE	MINOR ALLELE	STUDIES (N)	CASES (N)	CONTROLS (N)	OR (95% CI)
ACE					
rs4646994	Deletion	20	2855	4582	1.20 (1.08-1.34)
AGT					
rs699	C	21	2104	4530	1.23 (0.98-1.54)
rs4762	T	5	497	1395	1.25 (0.67-2.30)
APOE					
rs429358, rs7412	E2	7	554	712	0.86 (0.66-1.13)
AT1R					
rs5186	C	9	886	1230	1.13 (0.95-1.33)
CTLA4					
rs231775	G	4	353	536	1.24 (1.01-1.52)
F2					
rs1799963	A	30	3329	4878	1.95 (1.43-2.66)
FV					
rs6020	A	2	266	336	1.94 (1.05-3.60)
rs6025	A	40	4373	6446	1.95 (1.56-2.45)
IL-10					
rs1800896	G	8	1075	1360	0.91 (0.74-1.12)
LPL					
rs1800590	G	3	395	579	2.27 (0.62-8.24)
rs268	G	4	530	933	2.42 (1.25-4.68)
MTHFR					
rs1801133	T	45	5418	7271	1.06 (0.97-1.16)
NOS3					
27 bp-VNTR in intron 4	4a	14	1593	2239	1.14 (0.90-1.43)
rs2070744	C	11	1571	2202	1.08 (0.95-1.23)
rs1799983	T	24	2825	4048	1.19 (1.00-1.42)
SERPINE1					
rs1799889	4G	11	1283	1661	1.17 (1.03-1.33)

FOR PREECLAMPSIA

I^2 (%)	PVALUE FOR FUNNEL PLOT ASYMMETRY*	LOCATION	FUNCTION/ CONSEQUENCE
47	0.044	in an intron	higher serum ACE levels ¹⁴
81	0.116	in the gene	higher plasma angiotensinogen levels ⁴²
80	0.327	in the gene	conflicting data ^{42 43}
2	0.881	in the gene	hyperlipoproteinemia ⁴⁴
0	0.022	in the 3' untranslated region	increased response to angiotensin II ⁴⁵
3	1	in the gene	higher T-cell activation and proliferation rates ¹⁶
8	0.133	in the 3'-untranslated region	elevated prothrombin levels ⁴⁶
60	0.317	in the gene	poor response to activated protein C ²⁴
34	0.456	in the gene	poor response to activated protein C ⁴⁷
64	0.621	in the promoter region	lower serum IL-10 levels ⁴⁸
72	0.602	in the 5' untranslated region	no changes in lipid profiles ³⁸
21	1	in the gene	adverse lipid profiles ³⁸
45	0.531	in the gene	elevated plasma homocysteine levels ⁴⁹
63	0.071	in an intron	altered nitrite and nitrate levels ⁵⁰
28	0.484	in the promoter region	reduced eNOS gene promoter activity ⁵¹
68	0.960	in the gene	reduced nitrate, nitrite and nitric oxide production ^{52 53}
10	0.102	in the promoter region	higher PAI-1 levels ^{25 54 55}

TABLE 1 *CONTINUED*

VARIANT BY GENE	MINOR ALLELE	STUDIES (N)	CASES (N)	CONTROLS (N)	OR (95% CI)
TLR4					
rs4986790	G	4	723	614	1.07 (0.48-2.40)
rs4986791	T	3	614	461	1.20 (0.45-3.20)
TNF-alpha					
rs1800629	A	12	1592	1837	1.17 (0.91-1.49)
rs1799724	T	3	390	385	0.66 (0.33-1.31)
VEGF					
rs3025039	T	3	377	514	1.36 (0.64-2.91)

* Begg test for funnel plot asymmetry, which is suggestive of publication bias

ACE: angiotensin converting enzyme

AGT: angiotensinogen

APOE: apolipoprotein E

AT1R: angiotensin II receptor type 1

CTLA: cytotoxic T-lymphocyte-associated protein 4

F2: factor 2

FV: factor V

IL10: interleukin 10

LPL: lipoprotein lipase

MTHFR: methylenetetrahydrofolate reductase

NOS3: nitric oxide synthase 3

SERPINE: serine peptidase inhibitor

TLR: toll like receptor

TNF: tumor necrosis factor

VEGF: vascular endothelial growth factor

I^2 (%)	PVALUE FOR FUNNEL PLOT ASYMMETRY*	LOCATION	FUNCTION/ CONSEQUENCE
78	0.497	in the gene	dampened inflammatory response ⁵⁶
79	0.602	in the gene	dampened inflammatory response ⁵⁶
55	0.237	in the gene	higher TNF-alpha gene expression ⁵⁷
85	0.602	near the gene	higher TNF-alpha serum levels ⁵⁸
88	0.602	in the gene	lower VEGF levels ^{59 60}

TABLE 2 REFERENCES OF INCLUDED ARTICLES, PER GENETIC VARIANT

VARIANT BY GENE		REFERENCES
ACE		61 62 63 64 65 66 67 68 69 70 71 72 73 74 75 76 77 13 13 78
	rs4646994	79
AGT		
	rs699	61 62 80 63 64 65 81 82** 83 84 85 86 75 76 87 79 88 89 90
	rs4762	65 81 84 75 79
APOE		
	rs429358, rs7412	91 92 93 94 95 96 97
AT1R		
	rs5186	98 63 64 71 99 100 75 77 101
CTLA4		
	rs231775	102 103 104 105
F2		106 107 108 109 110 111 112 113 114 115 116 117 118 119
	rs1799963	120 121 122 123 124 † 125 126 127 128 129 130 131 132 133 87 134
FV		
	rs6020	135 136 62 106 107 108 109 137 110 138 111 112 139 113 115 135
	rs6025	116 117 119 120 121 122 140 141 126 142 127 128 143 129 130 131 144 95 145 146 147 148 133 87 149 134
IL-10		
	rs1800896	150 151 152 153 154 155 156 157
LPL		
	rs1800590	158 159 160
	rs268	92 158 159 161
MTHFR		62 162 163 164 110 138 165 112 113 114 115 116 117 166
	rs1801133	119 167 120 168 121 141 169 170 171 126 127 130 131 144 172 146 173 174 175 176*** 177 178 148 179 97 136 180 181 89
NOS3		
	27 bp- VNTR in intron 4	61 80 63 182 183 184 185 186 187 188 189 190 191 192
	rs2070744	61 183 184 193 187 188 189 194 190 87 192
	rs1799983	61 195 182 183 184 196 197 193 198 199 200 186 160 188 189 190 201 202 87 203 204 205 206 192
SERPINE1		
	rs1799889	110 165 115 116 196 121 126 131 207 87 208

CONTINUED

VARIANT BY GENE	REFERENCES
TLR4	
rs4986790	209 210 211 212
rs4986791	210 211 212
TNF-alpha	
rs1800629	213 214 150 152 215 216 153 217 218 155 219 220 221 222 157
rs1799724	213 223 220
VEGF	
rs3025039	193 224 225

** Two datasets

*** Three datasets

† Similar control group to another article (Kupferminc et al., 2000); this and the subsequent citation (Kupferminc et al., 2000) are considered to be one dataset.

REFERENCES

1. Steegers EA, von Dadelszen P, Duvekot JJ, Pijnenborg R. Pre-eclampsia. *Lancet* 2010;376:631-44.
2. Bellamy L, Casas JP, Hingorani AD, Williams DJ. Pre-eclampsia and risk of cardiovascular disease and cancer in later life: systematic review and meta-analysis. *BMJ* 2007;335:974.
3. Arngrimsson R, Bjornsson S, Geirsson RT, Bjornsson H, Walker JJ, Snaedal G. Genetic and familial predisposition to eclampsia and pre-eclampsia in a defined population. *Br J Obstet Gynaecol* 1990;97:762-69.
4. Chesley LC, Cooper DW. Genetics of hypertension in pregnancy: possible single gene control of pre-eclampsia and eclampsia in the descendants of eclamptic women. *Br J Obstet Gynaecol* 1986;93:898-908.
5. Cincotta RB, Brennecke SP. Family history of pre-eclampsia as a predictor for pre-eclampsia in primigravidas. *Int J Gynaecol Obstet* 1998;60:23-27.
6. Esplin MS, Fausett MB, Fraser A, Kerber R, Mineau G, Carrillo J, *et al.* Paternal and maternal components of the predisposition to preeclampsia. *N Engl J Med* 2001;344:867-72.
7. Nilsson E, Salonen RH, Cnattingius S, Lichtenstein P. The importance of genetic and environmental effects for pre-eclampsia and gestational hypertension: a family study. *BJOG*. 2004;111:200-06.
8. Sutherland A, Cooper DW, Howie PW, Liston WA, MacGillivray I. The incidence of severe pre-eclampsia amongst mothers and mothers-in-law of pre-eclamptics and controls. *Br J Obstet Gynaecol* 1981;88:785-91.
9. Chappell S, Morgan L. Searching for genetic clues to the causes of pre-eclampsia. *Clin Sci (Lond)* 2006;110:443-58.
10. Ioannidis JP, Ntzani EE, Trikalinos TA, Contopoulos-Ioannidis DG. Replication validity of genetic association studies. *Nat Genet* 2001;29:306-09.
11. Brown MA, Lindheimer MD, de Swiet M, Van Assche A, Moutquin JM. The classification and diagnosis of the hypertensive disorders of pregnancy: statement from the International Society for the Study of Hypertension in Pregnancy (ISSHP). *Hypertens Pregnancy* 2001;20:IX-XIV.
12. Higgins JP, Thompson SG, Deeks JJ, Altman DG. Measuring inconsistency in meta-analyses. *BMJ* 2003;327:557-60.
13. Serrano NC, Diaz LA, Paez MC, Mesa CM, Cifuentes R, Monterrosa A, *et al.* Angiotensin-converting enzyme I/D polymorphism and preeclampsia risk: evidence of small-study bias. *PLoS Med* 2006;3:e520.
14. Sayed-Tabatabaei FA, Oostra BA, Isaacs A, van Duijn CM, Witteman JC. ACE polymorphisms. *Circ Res* 2006;98:1123-33.
15. Teft WA, Kirchhof MG, Madrenas J. A molecular perspective of CTLA-4 function. *Annu Rev Immunol* 2006;24:65-97.
16. Sun T, Zhou Y, Yang M, Hu Z, Tan W, Han X, *et al.* Functional genetic variations in cytotoxic T-lymphocyte antigen 4 and susceptibility to multiple types of cancer. *Cancer Res* 2008;68:7025-34.
17. Fisher RM, Humphries SE, Talmud PJ. Common variation in the lipoprotein lipase gene: effects on plasma lipids and risk of atherosclerosis. *Atherosclerosis* 1997;135:145-59.

18. Mayret-Mesquiti M, Perez-Mendez O, Rodriguez ME, Fortoul TI, Gorocica P, Bernal-Alcantara D, *et al.* Hypertriglyceridemia is linked to reduced nitric oxide synthesis in women with hypertensive disorders of pregnancy. *Hypertens Pregnancy* 2007;26:423-31.
19. Dudding T, Heron J, Thakkestian A, Nurk E, Golding J, Pembrey M, *et al.* Factor V Leiden is associated with pre-eclampsia but not with fetal growth restriction: a genetic association study and meta-analysis. *J Thromb Haemost* 2008;6:1869-75.
20. Lin J, August P. Genetic thrombophilias and preeclampsia: a meta-analysis. *Obstet Gynecol* 2005;105:182-92.
21. Isermann B, Sood R, Pawlinski R, Zogg M, Kalloway S, Degen JL, *et al.* The thrombomodulin-protein C system is essential for the maintenance of pregnancy. *Nat Med* 2003;9:331-37.
22. Ceelie H, Spaargaren-van Riel CC, Bertina RM, Vos HL. G20210A is a functional mutation in the prothrombin gene; effect on protein levels and 3'-end formation. *J Thromb Haemost* 2004;2:119-27.
23. Kyrle PA, Mannhalter C, Beguin S, Stumpflen A, Hirschl M, Weltermann A, *et al.* Clinical studies and thrombin generation in patients homozygous or heterozygous for the G20210A mutation in the prothrombin gene. *Arterioscler Thromb Vasc Biol* 1998;18:1287-91.
24. Le W, Yu JD, Lu L, Tao R, You B, Cai X, *et al.* Association of the R485K polymorphism of the factor V gene with poor response to activated protein C and increased risk of coronary artery disease in the Chinese population. *Clin Genet* 2000;57:296-303.
25. Ye S, Green FR, Scarabin PY, Nicaud V, Bara L, Dawson SJ, *et al.* 4G/5G genetic polymorphism in the promoter of the plasminogen activator inhibitor-1 (PAI-1) gene is associated with differences in plasma PAI-1 activity but not with risk of myocardial infarction in the ECTIM study. *Etude CasTemoins de l'Infarctus du Myocarde. Thromb Haemost* 1995;74:837-41.
26. Bombell S, McGuire W. Tumour necrosis factor (-308A) polymorphism in pre-eclampsia: meta-analysis of 16 case-control studies. *Aust N Z J Obstet Gynaecol* 2008;48:547-51.
27. Medica I, Kastrin A, Peterlin B. Genetic polymorphisms in vasoactive genes and preeclampsia: a meta-analysis. *Eur J Obstet Gynecol Reprod Biol* 2007;131:115-26.
28. Molvarec A, Jermendy A, Nagy B, Kovacs M, Varkonyi T, Hupucz P, *et al.* Association between tumor necrosis factor (TNF)-alpha G-308A gene polymorphism and preeclampsia complicated by severe fetal growth restriction. *Clin Chim Acta* 2008;392:52-57.
29. Xie C, Yao MZ, Liu JB, Xiong LK. A meta-analysis of tumor necrosis factor-alpha, interleukin-6, and interleukin-10 in preeclampsia. *Cytokine* 2011;56:550-59.
30. Healy DG, Abou-Sleiman PM, Casas JP, Ahmadi KR, Lynch T, Gandhi S, *et al.* UCHL-1 is not a Parkinson's disease susceptibility gene. *Ann Neurol* 2006;59:627-33.
31. Ioannidis JP, Trikalinos TA. Early extreme contradictory estimates may appear in published research: the Proteus phenomenon in molecular genetics research and randomized trials. *J Clin Epidemiol* 2005;58:543-49.
32. Hannaford P, Ferry S, Hirsch S. Cardiovascular sequelae of toxemia of pregnancy. *Heart* 1997;77:154-58.

33. Irgens HU, Reisaeter L, Irgens LM, Lie RT. Long term mortality of mothers and fathers after pre-eclampsia: population based cohort study. *BMJ* 2001;323:1213-17.
34. Jonsdottir LS, Arngrimsson R, Geirsson RT, Sigvaldason H, Sigfusson N. Death rates from ischemic heart disease in women with a history of hypertension in pregnancy. *Acta Obstet Gynecol Scand* 1995;74:772-76.
35. Rodie VA, Freeman DJ, Sattar N, Greer IA. Pre-eclampsia and cardiovascular disease: metabolic syndrome of pregnancy? *Atherosclerosis* 2004;175:189-202.
36. Smith GC, Pell JP, Walsh D. Pregnancy complications and maternal risk of ischaemic heart disease: a retrospective cohort study of 129,290 births. *Lancet* 2001;357:2002-06.
37. Ye Z, Liu EH, Higgins JP, Keavney BD, Lowe GD, Collins R, *et al.* Seven haemostatic gene polymorphisms in coronary disease: meta-analysis of 66,155 cases and 91,307 controls. *Lancet* 2006;367:651-58.
38. Sagoo GS, Tatt I, Salanti G, Butterworth AS, Sarwar N, van Maarle M, *et al.* Seven lipoprotein lipase gene polymorphisms, lipid fractions, and coronary disease: a HuGE association review and meta-analysis. *Am J Epidemiol* 2008;168:1233-46.
39. Mooyaart AL, Valk EJ, van Es LA, Bruijn JA, de Heer E, Freedman BI, *et al.* Genetic associations in diabetic nephropathy: a meta-analysis. *Diabetologia* 2011;54:544-53.
40. Keavney B, McKenzie C, Parish S, Palmer A, Clark S, Youngman L, *et al.* Large-scale test of hypothesised associations between the angiotensin-converting-enzyme insertion/deletion polymorphism and myocardial infarction in about 5000 cases and 6000 controls. International Studies of Infarct Survival (ISIS) Collaborators. *Lancet* 2000;355:434-42.
41. McCarthy MI, Abecasis GR, Cardon LR, Goldstein DB, Little J, Ioannidis JP, *et al.* Genome-wide association studies for complex traits: consensus, uncertainty and challenges. *Nat Rev Genet* 2008;9:356-69.
42. Jeunemaitre X, Soubrier F, Kotelevtsev YV, Lifton RP, Williams CS, Charrau A, *et al.* Molecular basis of human hypertension: role of angiotensinogen. *Cell* 1992;71:169-80.
43. Balam-Ortiz E, Esquivel-Villarreal A, Alfaro-Ruiz L, Carrillo K, Elizalde A, Gil T, *et al.* Variants and haplotypes in angiotensinogen gene are associated with plasmatic angiotensinogen level in Mexican population. *Am J Med Sci* 2011;342:205-11.
44. Utermann G. Apolipoprotein E polymorphism in health and disease. *Am Heart J* 1987;113:433-40.
45. van Geel PP, Pinto YM, Voors AA, Buikema H, Oostergera M, Crijns HJ, *et al.* Angiotensin II type 1 receptor A1166C gene polymorphism is associated with an increased response to angiotensin II in human arteries. *Hypertension* 2000;35:717-21.
46. Poort SR, Rosendaal FR, Reitsma PH, Bertina RM. A common genetic variation in the 3'-untranslated region of the prothrombin gene is associated with elevated plasma prothrombin levels and an increase in venous thrombosis. *Blood* 1996;88:3698-703.
47. Bertina RM, Koeleman BP, Koster T, Rosendaal FR, Dirven RJ, de Ronde H, *et al.* Mutation in blood coagulation factor V associated with resist-

- ance to activated protein C. *Nature* 1994;369:64-67.
48. Wang AH, Lam WJ, Han DY, Ding Y, Hu R, Fraser AG, *et al.* The effect of IL-10 genetic variation and interleukin 10 serum levels on Crohn's disease susceptibility in a New Zealand population. *Hum Immunol* 2011;72:431-35.
49. Frosst P, Blom HJ, Milos R, Goyette P, Sheppard CA, Matthews RG, *et al.* A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. *Nat Genet* 1995;10:111-13.
50. Wang XL, Wang J. Endothelial nitric oxide synthase gene sequence variations and vascular disease. *Mol Genet Metab* 2000;70:241-51.
51. Nakayama M, Yasue H, Yoshimura M, Shimasaki Y, Kugiyama K, Ogawa H, *et al.* T-786->C mutation in the 5'-flanking region of the endothelial nitric oxide synthase gene is associated with coronary spasm. *Circulation* 1999;99:2864-70.
52. Sofowora G, Dishy V, Xie HG, Imamura H, Nishimi Y, Morales CR, *et al.* In-vivo effects of Glu298Asp endothelial nitric oxide synthase polymorphism. *Pharmacogenetics* 2001;11:809-14.
53. Veldman BA, Spiering W, Doevendans PA, Vervoort G, Kroon AA, de Leeuw PW, *et al.* The Glu298Asp polymorphism of the NOS 3 gene as a determinant of the baseline production of nitric oxide. *J Hypertens* 2002;20:2023-27.
54. Lin S, Huiya Z, Bo L, Wei W, Yongmei G. The plasminogen activator inhibitor-1 (PAI-1) gene -844 A/G and -675 4G/5G promoter polymorphism significantly influences plasma PAI-1 levels in women with polycystic ovary syndrome. *Endocrine*. 2009;36:503-09.
55. Rallidis LS, Gialeraki A, Merkouri E, Liakos G, Dagres N, Sionis D, *et al.* Reduced carriership of 4G allele of plasminogen activator inhibitor-1 4G/5G polymorphism in very young survivors of myocardial infarction. *J Thromb Thrombolysis* 2010;29:497-502.
56. Kiechl S, Lorenz E, Reindl M, Wiedermann CJ, Oberhollenzer F, Bonora E, *et al.* Toll-like receptor 4 polymorphisms and atherogenesis. *N Engl J Med* 2002;347:185-92.
57. Kroeger KM, Carville KS, Abraham LJ. The -308 tumor necrosis factor-alpha promoter polymorphism effects transcription. *Mol Immunol* 1997;34:391-99.
58. Puthothu B, Bierbaum S, Kopp MV, Forster J, Heinze J, Weckmann M, *et al.* Association of TNF-alpha with severe respiratory syncytial virus infection and bronchial asthma. *Pediatr Allergy Immunol* 2009;20:157-63.
59. Ruggiero D, Dalmasso C, Nutile T, Sorice R, Dionisi L, Aversano M, *et al.* Genetics of VEGF serum variation in human isolated populations of cileto: importance of VEGF polymorphisms. *PLoS One* 2011;6:e16982.
60. Al-Habboubi HH, Sater MS, Almawi AW, Al-Khateeb GM, Almawi WY. Contribution of VEGF polymorphisms to variation in VEGF serum levels in a healthy population. *Eur Cytokine Netw* 2011;22:154-58.
61. Aggarwal PK, Jain V, Jha V. Endothelial nitric oxide synthase, angiotensin-converting enzyme and angiotensinogen gene polymorphisms in hypertensive disorders of pregnancy. *Hypertens Res* 2010;33:473-77.
62. Aggarwal S, Dimri N, Tandon I, Agarwal S. Preeclampsia in North Indian

- women: the contribution of genetic polymorphisms. *J Obstet Gynaecol Res* 2011;37:1335-41.
63. Benedetto C, Marozio L, Ciccone G, Chieppa G, Quaglia M, Matullo G, *et al.* Synergistic effect of renin-angiotensin system and nitric oxide synthase genes polymorphisms in pre-eclampsia. *Acta Obstet Gynecol Scand* 2007;86:678-82.
 64. Bouba I, Makrydimas G, Kalaitzidis R, Lolis DE, Siamopoulos KC, Georgiou I. Interaction between the polymorphisms of the renin-angiotensin system in preeclampsia. *Eur J Obstet Gynecol Reprod Biol* 2003;110:8-11.
 65. Choi H, Kang JY, Yoon HS, Han SS, Whang CS, Moon IG, *et al.* Association of Angiotensin-converting enzyme and angiotensinogen gene polymorphisms with preeclampsia. *J Korean Med Sci* 2004;19:253-57.
 66. Galao AO, de Souza LH, da Costa BE, Scheibe RM, Poli de Figueiredo CE. Angiotensin-converting enzyme gene polymorphism in preeclampsia and normal pregnancy. *Am J Obstet Gynecol* 2004;191:821-24.
 67. Gurdol F, Isbilen E, Yilmaz H, Isbir T, Dirican A. The association between preeclampsia and angiotensin-converting enzyme insertion/deletion polymorphism. *Clin Chim Acta* 2004;341:127-31.
 68. Heiskanen JT, Pirskanen MM, Hiltunen MJ, Mannermaa AJ, Punnonen KR, Heinonen ST. Insertion-deletion polymorphism in the gene for angiotensin-converting enzyme is associated with obstetric cholestasis but not with preeclampsia. *Am J Obstet Gynecol* 2001;185:600-03.
 69. Kaur R, Jain V, Khuller M, Gupta I, Sherawat BS. Association of angiotensin-converting enzyme gene polymorphism with pregnancy-induced hypertension. *Acta Obstet Gynecol Scand* 2005;84:929-33.
 70. Kobashi G, Hata A, Shido K, Ohta K, Yamada H, Kato EH, *et al.* Insertion/deletion polymorphism of the angiotensin-converting enzyme gene and preeclampsia in Japanese patients. *Semin Thromb Hemost* 2005;31:346-50.
 71. Li H, Ma Y, Fu Q, Wang L. Angiotensin-converting enzyme insertion/deletion (ACE I/D) and angiotensin II type 1 receptor (AT1R) gene polymorphism and its association with preeclampsia in Chinese women. *Hypertens Pregnancy* 2007;26:293-301.
 72. Mando C, Antonazzo P, Tabano S, Zanutto S, Pileri P, Somigliana E, *et al.* Angiotensin-converting enzyme and adducin-1 polymorphisms in women with preeclampsia and gestational hypertension. *Reprod Sci* 2009;16:819-26.
 73. Miskovic B, Sertic J, Stavljenic-Rukavina A, Stipoljev F. Association of angiotensin-converting enzyme insertion-deletion polymorphism with preeclampsia. *Coll Antropol* 2008;32:339-43.
 74. Morgan L, Foster F, Hayman R, Crawshaw S, Baker PN, Broughton PF, *et al.* Angiotensin-converting enzyme insertion-deletion polymorphism in normotensive and pre-eclamptic pregnancies. *J Hypertens* 1999;17:765-68.
 75. Procopciuc L, Caracostea G, Iordache G, Olteanu I, Stamatian F. Influence of RAS Polymorphisms on the Development and Perinatal Outcome of Preeclampsia. Genetic RAS Evaluation. *Gineco Ro* 2009;5:88-93.
 76. Roberts CB, Rom L, Moodley J, Pegoraro RJ. Hypertension-related

- gene polymorphisms in pre-eclampsia, eclampsia and gestational hypertension in Black South African women. *J Hypertens* 2004;22:945-48.
77. Salimi S, Mokhtari M, Yaghmaei M, Jamshidi M, Naghavi A. Association of angiotensin-converting enzyme intron 16 insertion/deletion and angiotensin II type 1 receptor A1166C gene polymorphisms with preeclampsia in South East of Iran. *J Biomed Biotechnol* 2011;2011:941515.
 78. Uma R, Forsyth SJ, Struthers AD, Fraser CG, Godfrey V, Murphy DJ. Polymorphisms of the angiotensin converting enzyme gene in early-onset and late-onset pre-eclampsia. *J Matern Fetal Neonatal Med* 2010;23:874-79.
 79. Wang L, Feng Y, Zhang Y, Zhou H, Jiang S, Niu T, *et al.* Prolylcarboxypeptidase gene, chronic hypertension, and risk of preeclampsia. *Am J Obstet Gynecol* 2006;195:162-71.
 80. Bashford MT, Hefler LA, Vertrees TW, Roa BB, Gregg AR. Angiotensinogen and endothelial nitric oxide synthase gene polymorphisms among Hispanic patients with preeclampsia. *Am J Obstet Gynecol* 2001;184:1345-50.
 81. Dissanayake VH, Giles V, Jayasekara RW, Seneviratne HR, Kalsheker N, Broughton PF, *et al.* A study of three candidate genes for pre-eclampsia in a Sinhalese population from Sri Lanka. *J Obstet Gynaecol Res* 2009;35:234-42.
 82. Guo G, Wilton AN, Fu Y, Qiu H, Brennecke SP, Cooper DW. Angiotensinogen gene variation in a population case-control study of preeclampsia/eclampsia in Australians and Chinese. *Electrophoresis* 1997;18:1646-49.
 83. Jenkins LD, Powers RW, Cooper M, Gallaher MJ, Markovic N, Ferrell R, *et al.* Preeclampsia risk and angiotensinogen polymorphisms M235T and AGT -217 in African American and Caucasian women. *Reprod Sci* 2008;15:696-701.
 84. Knyrim E, Muetze S, Eggermann T, Rudnik-Schoeneborn S, Lindt R, Ortlepp JR, *et al.* Genetic analysis of the angiotensinogen gene in pre-eclampsia: study of German women and review of the literature. *Gynecol Obstet Invest* 2008;66:203-08.
 85. Kobashi G, Hata A, Shido K, Kato EH, Yamada H, Fujimoto S, *et al.* Association of a variant of the angiotensinogen gene with pure type of hypertension in pregnancy in the Japanese: implication of a racial difference and significance of an age factor. *Am J Med Genet* 1999;86:232-36.
 86. Morgan L, Crawshaw S, Baker PN, Broughton PF, Kalsheker N. Maternal and fetal angiotensinogen gene allele sharing in pre-eclampsia. *Br J Obstet Gynaecol* 1999;106:244-51.
 87. Tempfer CB, Jirecek S, Riemer EK, Zeisler H, Denschlag D, Hefler L, *et al.* Polymorphisms of thrombophilic and vasoactive genes and severe preeclampsia: a pilot study. *J Soc Gynecol Investig* 2004;11:227-31.
 88. Ward K, Hata A, Jeunemaitre X, Helin C, Nelson L, Namikawa C, *et al.* A molecular variant of angiotensinogen associated with preeclampsia. *Nat Genet* 1993;4:59-61.
 89. Yoshida A, Miura K, Nakayama D, Masuzaki H. Correlation between preeclampsia and prevalence of polymorphism of angiotensinogen, methylenetetrahydrofolate reductase and factor V, prothrombin genes among Japanese women. *Acta Medica Nagasakiensis* 2008;53:37-41.
 90. Zhang XQ, Varner M, Dizon-Town-

- son D, Song F, Ward K. A molecular variant of angiotensinogen is associated with idiopathic intrauterine growth restriction. *Obstet Gynecol* 2003;101:237-42.
91. Belo L, Gaffney D, Caslake M, Santos-Silva A, Pereira-Leite L, Quintanilha A, *et al.* Apolipoprotein E and cholesteryl ester transfer protein polymorphisms in normal and preeclamptic pregnancies. *Eur J Obstet Gynecol Reprod Biol* 2004;112:9-15.
92. Bernard N, Girouard J, Forest JC, Giguere Y. The combination of ApoCIII, hepatic lipase and hormone sensitive lipase gene polymorphisms suggests an association with susceptibility to gestational hypertension. *J Hum Genet* 2007;52:244-54.
93. Chikosi AB, Moodley J, Pegoraro RJ, Lanning PA, Rom L. Apolipoprotein E polymorphism in South African Zulu women with preeclampsia. *Hypertens Pregnancy* 2000;19:309-14.
94. Francoual J, Audibert F, Trioche P, Chalas J, Capel L, Lindenbaum A, *et al.* Is a polymorphism of the apolipoprotein E gene associated with preeclampsia? *Hypertens Pregnancy* 2002;21:127-33.
95. Nagy B, Rigo J, Jr., Fintor L, Karadi I, Toth T. Apolipoprotein E alleles in women with severe pre-eclampsia. *J Clin Pathol* 1998;51:324-25.
96. Procopciuc LM, Hazi GM, Caracostea G, Nemeti G, Olteanu I, Stamatian F. Apolipoprotein E polymorphism - a risk factor in Romanian pregnant women with preeclampsia. *Gineco Ro* 2011;7:134-40.
97. Stiefel P, Miranda ML, Bellido LM, Luna J, Jimenez L, Pamies E, *et al.* Genotype of the CYBA promoter -930A/G, polymorphism C677T of the MTHFR and APOE genotype in patients with hypertensive disorders of pregnancy: an observational study. *Med Clin (Barc)* 2009;133:657-61.
98. Akbar SA, Khawaja NP, Brown PR, Tayyeb R, Bamfo J, Nicolaides KH. Angiotensin II type 1 and 2 receptors gene polymorphisms in pre-eclampsia and normal pregnancy in three different populations. *Acta Obstet Gynecol Scand* 2009;88:606-11.
99. Morgan L, Crawshaw S, Baker PN, Brookfield JF, Broughton PF, Kalsheker N. Distortion of maternal-fetal angiotensin II type 1 receptor allele transmission in pre-eclampsia. *J Med Genet* 1998;35:632-36.
100. Plummer S, Tower C, Alonso P, Morgan L, Baker P, Broughton-Pipkin F, *et al.* Haplotypes of the angiotensin II receptor genes AGTR1 and AGTR2 in women with normotensive pregnancy and women with preeclampsia. *Hum Mutat* 2004;24:14-20.
101. Seremak-Mrozikiewicz A, Dubiel M, Drews K, Breborowicz GH, Mrozikiewicz PM. 1166C mutation of angiotensin II type 1 receptor gene is correlated with umbilical blood flow velocimetry in women with preeclampsia. *J Matern Fetal Neonatal Med* 2005;17:117-21.
102. Best LG, Nadeau M, Davis K, Lamb F, Bercier S, Anderson CM. Genetic Variants, Immune Function, and Risk of Pre-Eclampsia among American Indians. *Am J Reprod Immunol* 2012;67:152-59.
103. Jaaskelainen E, Toivonen S, Keski-Niula L, Paattiniemi EL, Helisalmi S, Punnonen K, *et al.* CTLA-4 polymorphism 49A-G is associated with placental abruption and preeclampsia in Finnish women. *Clin Chem Lab Med*

- 2008;46:169-73.
104. Pendelowski KP, Sass N, Torloni MR, Mattar R, Moron AF, Franchim CS, *et al.* Immunoregulatory gene polymorphisms in women with preeclampsia. *Hypertens Res* 2011;34:384-88.
105. Samsami DA, Doroudchi M, Kalantari T, Pezeshki AM, Ghaderi A. Heterozygosity in CTLA-4 gene and severe preeclampsia. *Int J Gynaecol Obstet* 2005;88:19-24.
106. Agorastos T, Karavida A, Lambropoulos A, Constantinidis T, Tzitzimikas S, Chrisafi S, *et al.* Factor V Leiden and prothrombin G20210A mutations in pregnancies with adverse outcome. *J Matern Fetal Neonatal Med* 2002;12:267-73.
107. Alfirevic Z, Mousa HA, Martlew V, Briscoe L, Perez-Casal M, Toh CH. Postnatal screening for thrombophilia in women with severe pregnancy complications. *Obstet Gynecol* 2001;97:753-59.
108. Benedetto C, Marozio L, Salton L, Maula V, Chiappa G, Massobrio M. Factor V Leiden and factor II G20210A in preeclampsia and HELLP syndrome. *Acta Obstet Gynecol Scand* 2002;81:1095-100.
109. Best LG, Dorsam ST, Nadeau M, Burd L, Anderson CM. Genetic thrombophilia variants and risk for preeclampsia among American Indians. *Hypertens Pregnancy* 2009;28:85-94.
110. Dalmaz CA, Santos KG, Botton MR, Tedoldi CL, Roisenberg I. Relationship between polymorphisms in thrombophilic genes and preeclampsia in a Brazilian population. *Blood Cells Mol Dis* 2006;37:107-10.
111. de Groot CJ, Bloemenkamp KW, Duvekot EJ, Helmerhorst FM, Bertina RM, van der Meer F, *et al.* Preeclampsia and genetic risk factors for thrombosis: a case-control study. *Am J Obstet Gynecol* 1999;181:975-80.
112. Demir SC, Evruke C, Ozgunen T, Kadayifci O, Altintas UI, Kokangul S. The relationship between pregnancy induced hypertension and congenital thrombophilia. *Saudi Med J* 2006;27:1161-66.
113. Dogan OO, Simsek Y, Celen S, Danisman N. Frequency of hereditary thrombophilia, anticoagulant activity, and homocysteine levels in patients with hemolysis, elevated liver functions and low thrombocyte count (HELLP) syndrome. *Journal of Obstetrics and Gynaecology Research* 2011;37:527-33.
114. Dusse LM, Carvalho MG, Braganca WF, Paiva SG, Godoi LC, Guimaraes DA, *et al.* Inherited thrombophilias and pre-eclampsia in Brazilian women. *Eur J Obstet Gynecol Reprod Biol* 2007;134:20-23.
115. Fabbro D, D'Elia AV, Spizzo R, Driul L, Barillari G, Di Loreto C, *et al.* Association between plasminogen activator inhibitor 1 gene polymorphisms and preeclampsia. *Gynecol Obstet Invest* 2003;56:17-22.
116. Gerhardt A, Goecke TW, Beckmann MW, Wagner KJ, Tutschek B, Willers R, *et al.* The G20210A prothrombin-gene mutation and the plasminogen activator inhibitor (PAI-1) 5G/5G genotype are associated with early onset of severe preeclampsia. *J Thromb Haemost* 2005;3:686-91.
117. Grandone E, Margaglione M, Colaizzo D, Cappucci G, Scianname N, Montanaro S, *et al.* Prothrombotic genetic risk factors and the occurrence of gestational hypertension with or without proteinuria. *Thromb Haemost*

- 1999;81:349-52.
118. Higgins JR, Kaiser T, Moses EK, North R, Brennecke SP. Prothrombin G20210A mutation: is it associated with pre-eclampsia? *Gynecol Obstet Invest* 2000;50:254-57.
119. Hiltunen LM, Laivuori H, Rautanen A, Kaaja R, Kere J, Krusius T, *et al.* Blood group AB and factor V Leiden as risk factors for pre-eclampsia: a population-based nested case-control study. *Thromb Res* 2009;124:167-73.
120. Jarvenpää J, Pakkila M, Savolainen ER, Perheentupa A, Jarvela I, Ryyanen M. Evaluation of factor V Leiden, prothrombin and methylenetetrahydrofolate reductase gene mutations in patients with severe pregnancy complications in northern Finland. *Gynecol Obstet Invest* 2006;62:28-32.
121. Kankova K, Benes P, Unzeitig V, Izakovicova-Holla L, Znojil V, Vacha J. Lack of association between pregnancy-induced hypertension and familial thrombophilia in a Czech population. *Prenatal and Neonatal Medicine* 2000;5:223-29.
122. Karakantza M, Androustopoulos G, Mougiou A, Sakellaropoulos G, Kourounis G, Decavalas G. Inheritance and perinatal consequences of inherited thrombophilia in Greece. *International Journal of Gynecology and Obstetrics* 2008;100:124-29.
123. Kupferminc MJ, Eldor A, Steinman N, Many A, Bar-Am A, Jaffa A, *et al.* Increased frequency of genetic thrombophilia in women with complications of pregnancy. *N Engl J Med* 1999;340:9-13.
124. Kupferminc MJ, Fait G, Many A, Gordon D, Eldor A, Lessing JB. Severe preeclampsia and high frequency of genetic thrombophilic mutations. *Obstet Gynecol* 2000;96:45-49.
125. Kupferminc MJ, Peri H, Zwang E, Yaron Y, Wolman I, Eldor A. High prevalence of the prothrombin gene mutation in women with intrauterine growth retardation, abruptio placentae and second trimester loss. *Acta Obstet Gynecol Scand* 2000;79:963-67.
126. Larciprete G, Gioia S, Angelucci PA, Brosio F, Barbati G, Angelucci GP, *et al.* Single inherited thrombophilias and adverse pregnancy outcomes. *J Obstet Gynaecol Res* 2007;33:423-30.
127. Livingston JC, Barton JR, Park V, Haddad B, Phillips O, Sibai BM. Maternal and fetal inherited thrombophilias are not related to the development of severe preeclampsia. *Am J Obstet Gynecol* 2001;185:153-57.
128. Malek-Khosravi S, Rahimi Z, Jalilvand F, Parsian A. Thrombophilic mutations and susceptibility to preeclampsia in Western Iran. *J Thromb Thrombolysis* 2012;33:109-15.
129. Mello G, Parretti E, Marozio L, Pizzi C, Lojacono A, Frusca T, *et al.* Thrombophilia is significantly associated with severe preeclampsia: results of a large-scale, case-controlled study. *Hypertension* 2005;46:1270-74.
130. Mendilcioglu I, Bilgen T, Arıkan Y, Keser I, Simsek M, Timuragaoglu A. The association between inherited thrombophilias and pregnancy-related hypertension recurrence. *Archives of Gynecology and Obstetrics* 2011;284:837-41.
131. Morrison ER, Miedzybrodzka ZH, Campbell DM, Haïtes NE, Wilson BJ, Watson MS, *et al.* Prothrombotic genotypes are not associated with pre-eclampsia and gestational hypertension: results from a large population-based study and systematic review. *Thromb*

- Haemost* 2002;87:779-85.
132. O'Shaughnessy KM, Fu B, Downing S, Morris NH. Thrombophilic polymorphisms in pre-eclampsia: altered frequency of the functional 98C>T polymorphism of glycoprotein IIIa. *J Med Genet* 2001;38:775-77.
 133. Seremak-Mrozikiewicz A, Drews K, Wender-Ozegowska E, Mrozikiewicz PM. The significance of genetic polymorphisms of factor V Leiden and prothrombin in the preeclamptic Polish women. *J Thromb Thrombolysis* 2010;30:97-104.
 134. Yalinkaya A, Erdemoglu M, Akdeniz N, Kale A, Kale E. The relationship between thrombophilic mutations and preeclampsia: a prospective case-control study. *Ann Saudi Med* 2006;26:105-09.
 135. Faisel F, Romppanen EL, Hiltunen M, Helisalmi S, Laasanen J, Punnonen K, et al. Susceptibility to pre-eclampsia in Finnish women is associated with R485K polymorphism in the factor V gene, not with Leiden mutation. *Eur J Hum Genet* 2004;12:187-91.
 136. Watanabe H, Hamada H, Yamakawa-Kobayashi K, Yoshikawa H, Arinami T. Evidence for an association of the R485K polymorphism in the coagulation factor V gene with severe preeclampsia from screening 35 polymorphisms in 27 candidate genes. *Thromb Haemost* 2001;86:1594-95.
 137. Currie L, Peek M, McNiven M, Prosser I, Mansour J, Ridgway J. Is there an increased maternal-infant prevalence of Factor V Leiden in association with severe pre-eclampsia? *BJOG* 2002;109:191-96.
 138. Davalos IP, Moran MC, Martinez-Abundis E, Gonzalez-Ortiz M, Flores-Martinez SE, Machorro V, et al. Methylenetetrahydrofolate reductase C677T polymorphism and Factor V Leiden variant in Mexican women with preeclampsia/eclampsia. *Blood Cells Mol Dis* 2005;35:66-69.
 139. Dizon-Townson DS, Nelson LM, Easton K, Ward K. The factor V Leiden mutation may predispose women to severe preeclampsia. *Am J Obstet Gynecol* 1996;175:902-05.
 140. Karimi S, Yavarian M, Azinfar A, Rajaei M, Kootenae MA. Evaluation the frequency of factor V Leiden mutation in pregnant women with preeclampsia syndrome in an Iranian population. *Iranian Journal of Reproductive Medicine* 2012;10:59-66.
 141. Kim YJ, Williamson RA, Murray JC, Andrews J, Pietscher JJ, Peraud PJ, et al. Genetic susceptibility to preeclampsia: roles of cytosine-to-thymine substitution at nucleotide 677 of the gene for methylenetetrahydrofolate reductase, 68-base pair insertion at nucleotide 844 of the gene for cystathionine beta-synthase, and factor V Leiden mutation. *Am J Obstet Gynecol* 2001;184:1211-17.
 142. Lindoff C, Ingemarsson I, Martinsson G, Segelmark M, Thysell H, Astedt B. Preeclampsia is associated with a reduced response to activated protein C. *Am J Obstet Gynecol* 1997;176:457-60.
 143. Mello G, Parretti E, Martini E, Mecacci F, La Torre P, Cioni R, et al. Usefulness of screening for congenital or acquired hemostatic abnormalities in women with previous complicated pregnancies. *Haemostasis* 1999;29:197-203.
 144. Murphy RP, Donoghue C, Nallen RJ, D'Mello M, Regan C, Whitehead AS, et al. Prospective evaluation of the risk conferred by factor V Leiden and

- thermolabile methylenetetrahydrofolate reductase polymorphisms in pregnancy. *Arterioscler Thromb Vasc Biol* 2000;20:266-70.
145. Omar SZ, Qvist R, Khaing SL, Muniandy S, Bhalla S. Thrombophilic mutations in pre-eclampsia and pregnancy-induced hypertension. *J Obstet Gynaecol Res* 2008;34:174-78.
146. O'Shaughnessy KM, Fu B, Ferraro F, Lewis I, Downing S, Morris NH. Factor V Leiden and thermolabile methylenetetrahydrofolate reductase gene variants in an East Anglian preeclampsia cohort. *Hypertension* 1999;33:1338-41.
147. Prasmusinto D, Skrablin S, Fimmers R, van der Ven K. Ethnic differences in the association of factor V Leiden mutation and the C677T methylenetetrahydrofolate reductase gene polymorphism with preeclampsia. *Eur J Obstet Gynecol Reprod Biol* 2004;112:162-69.
148. Rigo J, Jr., Nagy B, Fintor L, Tanyi J, Beke A, Karadi I, *et al.* Maternal and neonatal outcome of preeclamptic pregnancies: the potential roles of factor V Leiden mutation and 5,10 methylenetetrahydrofolate reductase. *Hypertens Pregnancy* 2000;19:163-72.
149. von Tempelhoff GF, Heilmann L, Spanuth E, Kunzmann E, Hommel G. Incidence of the factor V Leiden-mutation, coagulation inhibitor deficiency, and elevated antiphospholipid-antibodies in patients with preeclampsia or HELLP-syndrome. Hemolysis, elevated liver-enzymes, low platelets. *Thromb Res* 2000;100:363-65.
150. Daher S, Sass N, Oliveira LG, Mattar R. Cytokine genotyping in preeclampsia. *Am J Reprod Immunol* 2006;55:130-35.
151. de Groot CJ, Jansen MW, Bertina RM, Schonkeren JJ, Helmerhorst FM, Huizinga TW. Interleukin 10-2849AA genotype protects against pre-eclampsia. *Genes Immun* 2004;5:313-14.
152. de Lima TH, Sass N, Mattar R, Moron AF, Torloni MR, Franchim CS, *et al.* Cytokine gene polymorphisms in preeclampsia and eclampsia. *Hypertens Res* 2009;32:565-69.
153. Haggerty CL, Ferrell RE, Hubel CA, Markovic N, Harger G, Ness RB. Association between allelic variants in cytokine genes and preeclampsia. *Am J Obstet Gynecol* 2005;193:209-15.
154. Kamali-Sarvestani E, Kiany S, Gharresi-Fard B, Robati M. Association study of IL-10 and IFN-gamma gene polymorphisms in Iranian women with preeclampsia. *J Reprod Immunol* 2006;72:118-26.
155. Mirahmadian M, Kalantar F, Heidari G, Safdarian L, Mansouri R, Amirzargar AA. Association of tumor necrosis factor-alpha and interleukin-10 gene polymorphisms in Iranian patients with pre-eclampsia. *Am J Reprod Immunol* 2008;60:179-85.
156. Stonek F, Metzenbauer M, Hafner E, Philipp K, Tempfer C. Interleukin-10 -1082 G/A promoter polymorphism and pregnancy complications: results of a prospective cohort study in 1,616 pregnant women. *Acta Obstet Gynecol Scand* 2008;87:430-33.
157. Vural P, Degirmencioglu S, Saral NY, Demirkan A, Akgul C, Yildirim G, *et al.* Tumor necrosis factor alpha, interleukin-6 and interleukin-10 polymorphisms in preeclampsia. *J Obstet Gynaecol Res* 2010;36:64-71.
158. Hubel CA, Roberts JM, Ferrell RE. Association of pre-eclampsia with common coding sequence variations in the lipoprotein lipase gene. *Clin Genet*

- 1999;56:289-96.
159. Kim YJ, Williamson RA, Chen K, Smith JL, Murray JC, Merrill DC. Lipoprotein lipase gene mutations and the genetic susceptibility of preeclampsia. *Hypertension* 2001;38:992-96.
160. Pappa KI, Roubelakis M, Vlachos G, Marinopoulos S, Zissou A, Anagnou NP, *et al.* Variable effects of maternal and paternal-fetal contribution to the risk for preeclampsia combining GSTP1, eNOS, and LPL gene polymorphisms. *J Matern Fetal Neonatal Med* 2011;24:628-35.
161. Zhang C, Austin MA, Edwards KL, Farin FM, Li N, Hsu L, *et al.* Functional variants of the lipoprotein lipase gene and the risk of preeclampsia among non-Hispanic Caucasian women. *Clin Genet* 2006;69:33-39.
162. Also-Rallo E, Lopez-Quesada E, Urreizti R, Vilaseca MA, Lailla JM, Balcells S, *et al.* Polymorphisms of genes involved in homocysteine metabolism in preeclampsia and in uncomplicated pregnancies. *Eur J Obstet Gynecol Reprod Biol* 2005;120:45-52.
163. Canto P, Canto-Cetina T, Juarez-Velazquez R, Rosas-Vargas H, Rangel-Villalobos H, Canizales-Quinteros S, *et al.* Methylenetetrahydrofolate reductase C677T and glutathione S-transferase P1 A313G are associated with a reduced risk of preeclampsia in Maya-Mestizo women. *Hypertens Res* 2008;31:1015-19.
164. Chikosi AB, Moodley J, Pegoraro RJ, Lanning PA, Rom L. 5,10 methylenetetrahydrofolate reductase polymorphism in black South African women with pre-eclampsia. *Br J Obstet Gynaecol* 1999;106:1219-20.
165. De Maat MP, Jansen MW, Hille ET, Vos HL, Bloemenkamp KW, Buitendijk S, *et al.* Preeclampsia and its interaction with common variants in thrombophilia genes. *J Thromb Haemost* 2004;2:1588-93.
166. Hill LD, York TP, Kusanovic JP, Gomez R, Eaves LJ, Romero R, *et al.* Epistasis between COMT and MTHFR in maternal-fetal dyads increases risk for preeclampsia. *PLoS One* 2011;6:e16681.
167. Jaaskelainen E, Keski-Nisula L, Toivonen S, Romppanen EL, Helisalmi S, Punnonen K, *et al.* MTHFR C677T polymorphism is not associated with placental abruption or preeclampsia in Finnish women. *Hypertens Pregnancy* 2006;25:73-80.
168. Kaiser T, Brennecke SP, Moses EK. C677T methylenetetrahydrofolate reductase polymorphism is not a risk factor for pre-eclampsia/eclampsia among Australian women. *Hum Hered* 2001;51:20-22.
169. Klai S, Fekih-Mrissa N, El HS, Kaabecchi N, Nsiri B, Rachdi R, *et al.* Association of MTHFR A1298C polymorphism (but not of MTHFR C677T) with elevated homocysteine levels and placental vasculopathies. *Blood Coagul Fibrinolysis* 2011;22:374-78.
170. Kobashi G, Yamada H, Asano T, Nagano S, Hata A, Kishi R, *et al.* Absence of association between a common mutation in the methylenetetrahydrofolate reductase gene and preeclampsia in Japanese women. *Am J Med Genet* 2000;93:122-25.
171. Laivuori H, Kaaja R, Ylikorkala O, Hiltunen T, Kontula K. 677 C-->T polymorphism of the methylenetetrahydrofolate reductase gene and preeclampsia. *Obstet Gynecol* 2000;96:277-80.
172. Nagy B, Hupuczi P, Papp Z. High

- frequency of methylenetetrahydrofolate reductase 677TT genotype in Hungarian HELLP syndrome patients determined by quantitative real-time PCR. *Journal of Human Hypertension* 2007;21:154-58.
173. Pegoraro RJ, Chikosi A, Rom L, Roberts C, Moodley J. Methylenetetrahydrofolate reductase gene polymorphisms in black South Africans and the association with preeclampsia. *Acta Obstet Gynecol Scand* 2004;83:449-54.
 174. Perez-Mutul J, Gonzalez-Herrera L, Sosa-Cabrera T, Martinez-Olivares R. A mutation in the 5,10-methylenetetrahydrofolate reductase gene is not associated with preeclampsia in women of southeast Mexico. *Arch Med Res* 2004;35:231-34.
 175. Powers RW, Minich LA, Lykins DL, Ness RB, Crombleholme WR, Roberts JM. Methylenetetrahydrofolate reductase polymorphism, folate, and susceptibility to preeclampsia. *J Soc Gynecol Invest* 1999;6:74-79.
 176. Prasmusinto D, Skrablin S, Hofstaetter C, Fimmers R, van der Ven K. The methylenetetrahydrofolate reductase 677 C-->T polymorphism and preeclampsia in two populations. *Obstet Gynecol* 2002;99:1085-92.
 177. Procopciuc LM, Caracostea G, Zaharie G, Puscas M, Iordache G, Olteanu I, et al. Mutant Maternal and Fetal Thrombophilic Genotypes as a Risk Factor for Preeclampsia. *Gineco Ro* 2010;6:74-81.
 178. Rajkovic A, Mahomed K, Rozen R, Malinow MR, King IB, Williams MA. Methylenetetrahydrofolate reductase 677 C --> T polymorphism, plasma folate, vitamin B(12) concentrations, and risk of preeclampsia among black African women from Zimbabwe. *Mol Genet Metab* 2000;69:33-39.
 179. Sohda S, Arinami T, Hamada H, Yamada N, Hamaguchi H, Kubo T. Methylenetetrahydrofolate reductase polymorphism and pre-eclampsia. *J Med Genet* 1997;34:525-26.
 180. Williams MA, Sanchez SE, Zhang C, Bazul V. Methylenetetrahydrofolate reductase 677 C-->T polymorphism and plasma folate in relation to pre-eclampsia risk among Peruvian women. *J Matern Fetal Neonatal Med* 2004;15:337-44.
 181. Yilmaz H, Unlucerci Y, Gurdol F, Isbilen E, Isbir T. Association of pre-eclampsia with hyperhomocysteinaemia and methylenetetrahydrofolate reductase gene C677T polymorphism in a Turkish population. *Aust N Z J Obstet Gynaecol* 2004;44:423-27.
 182. Chen LK, Huang CH, Yeh HM, Lee CN, Shyu MK, Hsieh FJ, et al. Polymorphisms in the endothelial nitric oxide synthase gene may be protective against preeclampsia in a Chinese population. *Reprod Sci* 2007;14:175-81.
 183. Diaz-Olguin L, Coral-Vazquez RM, Canto-Cetina T, Canizales-Quinteros S, Ramirez RB, Fernandez G, et al. Endothelial nitric oxide synthase haplotypes are associated with preeclampsia in Maya mestizo women. *Dis Markers* 2011;31:83-89.
 184. Fatini C, Sticchi E, Gensini F, Genuardi M, Tondi F, Gensini GF, et al. Endothelial nitric oxide synthase gene influences the risk of pre-eclampsia, the recurrence of negative pregnancy events, and the maternal-fetal flow. *J Hypertens* 2006;24:1823-29.
 185. Grandone E, Colaizzo D, Martinelli P, Pavone G, Errico M, Vecchione G, et al. Does endothelial nitric oxide synthase gene variation play a role

- in the occurrence of hypertension in pregnancy? *Hypertens Pregnancy* 2003;22:149-55.
186. Ozturk E, Balat O, Pehlivan S, Ugur MG, Ozcan C, Sever T, *et al.* Endothelial nitric oxide synthase gene polymorphisms in preeclampsia with or without eclampsia in a Turkish population. *J Obstet Gynaecol Res* 2011;37:1778-83.
 187. Salimi S, Naghavi A, Mokhtari M, Noora M, Yaghmaei M. Lack of relationship between endothelial nitric oxide synthase gene 4b/a and T-786C polymorphisms with preeclampsia in southeast of Iran. *Arch Gynecol Obstet* 2012;285:405-9.
 188. Sandrim VC, Palei AC, Cavalli RC, Araujo FM, Ramos ES, Duarte G, *et al.* eNOS haplotypes associated with gestational hypertension or preeclampsia. *Pharmacogenomics* 2008;9:1467-73.
 189. Sandrim VC, Palei AC, Sertorio JT, Cavalli RC, Duarte G, Tanus-Santos JE. Effects of eNOS polymorphisms on nitric oxide formation in healthy pregnancy and in pre-eclampsia. *Mol Hum Reprod* 2010;16:506-10.
 190. Serrano NC, Casas JP, Diaz LA, Paez C, Mesa CM, Cifuentes R, *et al.* Endothelial NO synthase genotype and risk of preeclampsia: a multi-center case-control study. *Hypertension* 2004;44:702-07.
 191. Tempfer CB, Dorman K, Deter RL, O'Brien WE, Gregg AR. An endothelial nitric oxide synthase gene polymorphism is associated with preeclampsia. *Hypertens Pregnancy* 2001;20:107-18.
 192. Zdoukopoulos N, Doxani C, Messinis IE, Stefanidis I, Zintzaras E. Polymorphisms of the endothelial nitric oxide synthase (NOS3) gene in preeclampsia: a candidate-gene association study. *BMC Pregnancy Childbirth* 2011;11:89.
 193. Kim YJ, Park BH, Park H, Jung SC, Pang MG, Ryu HM, *et al.* No association of the genetic polymorphisms of endothelial nitric oxide synthase, dimethylarginine dimethylaminohydrolase, and vascular endothelial growth factor with preeclampsia in Korean populations. *Twin Res Hum Genet* 2008;11:77-83.
 194. Seremak-Mrozikiewicz A, Drews K, Barlik M, Sieroszewski P, Grzeskowiak E, Mrozikiewicz P. The significance of -786T > C polymorphism of endothelial NO synthase (eNOS) gene in severe preeclampsia. *J Matern Fetal Neonatal Med* 2011;24:432-36.
 195. Best LG, Nadeau M, Bercier S, Dauphinais S, Davis J, Davis K, *et al.* Genetic Variants, Endothelial Function, and Risk of Preeclampsia Among American Indians. *Hypertens Pregnancy* 2012;31:1-10.
 196. Hakli T, Romppanen EL, Hiltunen M, Helisalmi S, Punnonen K, Heinonen S. Endothelial nitric oxide synthase polymorphism in preeclampsia. *J Soc Gynecol Investig* 2003;10:154-57.
 197. Kim YJ, Park HS, Lee HY, Ha EH, Suh SH, Oh SK, *et al.* Reduced L-arginine level and decreased placental eNOS activity in preeclampsia. *Placenta* 2006;27:438-44.
 198. Kobashi G, Yamada H, Ohta K, Kato E, Ebina Y, Fujimoto S. Endothelial nitric oxide synthase gene (NOS3) variant and hypertension in pregnancy. *Am J Med Genet* 2001;103:241-44.
 199. Landau R, Xie HG, Dishy V, Wood AJ, Stein CM, Smiley RM. No association of the Asp298 variant of the endothelial nitric oxide synthase gene with preeclampsia. *Am J Hypertens* 2004;17:391-94.

200. Nishizawa H, Pryor-Koishi K, Suzuki M, Kato T, Sekiya T, Tada S, *et al.* Analysis of nitric oxide metabolism as a placental or maternal factor underlying the etiology of pre-eclampsia. *Gynecol Obstet Invest* 2009;68:239-47.
201. Sharma D, Trivedi SS, Bhattacharjee J. Oxidative stress and eNOS (Glu298Asp) gene polymorphism in preeclampsia in Indian population. *Mol Cell Biochem* 2011;353:189-93.
202. Singh A, Sharma D, Raghunandan C, Bhattacharjee J. Role of inflammatory cytokines and eNOS gene polymorphism in pathophysiology of pre-eclampsia. *Am J Reprod Immunol* 2010;63:244-51.
203. Turan F, Ilhan N, Kaman D, Ates K, Kafkasli A. Glu298Asp polymorphism of the endothelial nitric oxide synthase gene and plasma concentrations of asymmetric dimethylarginine in Turkish pre-eclamptic women without fetal growth retardation. *J Obstet Gynaecol Res* 2010;36:495-501.
204. Yaghmaei M, Salimi S, Mokhtari M, Naghavi A, Saravani M, Farajian-Mashadi F. Endothelial nitric oxide synthase gene Glu298Asp polymorphism and risk of preeclampsia in South East of Iran. *African Journal of Biotechnology* 2011;10:10712-17.
205. Yoshimura T, Yoshimura M, Tabata A, Shimasaki Y, Nakayama M, Miyamoto Y, *et al.* Association of the missense Glu298Asp variant of the endothelial nitric oxide synthase gene with severe preeclampsia. *J Soc Gynecol Investig* 2000;7:238-41.
206. Yoshimura T, Chowdhury FA, Yoshimura M, Okamura H. Genetic and environmental contributions to severe preeclampsia: lack of association with the endothelial nitric oxide synthase Glu298Asp variant in a developing country. *Gynecol Obstet Invest* 2003;56:10-13.
207. Pegoraro RJ, Hira B, Rom L, Moodley J. Plasminogen activator inhibitor type 1 (PAI1) and platelet glycoprotein IIIa (PGIIIa) polymorphisms in Black South Africans with pre-eclampsia. *Acta Obstet Gynecol Scand* 2003;82:313-17.
208. Yamada N, Arinami T, Yamakawa-Kobayashi K, Watanabe H, Sohda S, Hamada H, *et al.* The 4G/5G polymorphism of the plasminogen activator inhibitor-1 gene is associated with severe preeclampsia. *J Hum Genet* 2000;45:138-41.
209. Franchim CS, Sass N, Mattar R, Pendelosi KP, Lin LH, Torloni MR, *et al.* Inflammatory mediators gene polymorphisms in preeclampsia. *Hypertens Pregnancy* 2011;30:338-46.
210. Molvarec A, Jeremendy A, Kovacs M, Prohaszka Z, Rigo J, Jr. Toll-like receptor 4 gene polymorphisms and preeclampsia: lack of association in a Caucasian population. *Hypertens Res* 2008;31:859-64.
211. van Rijn BB, Franx A, Steegers EA, de Groot CJ, Bertina RM, Pasterkamp G, *et al.* Maternal TLR4 and NOD2 gene variants, pro-inflammatory phenotype and susceptibility to early-onset preeclampsia and HELLP syndrome. *PLoS One* 2008;3:e1865.
212. Xie F, Hu Y, Speert DP, Turvey SE, Peng G, Money DM, *et al.* Toll-like receptor gene polymorphisms and preeclampsia risk: a case-control study and data synthesis. *Hypertens Pregnancy* 2010;29:390-98.
213. Canto-Cetina T, Canizales-Quinteros S, de la Chesnaye E, Coral-Vazquez R, Mendez JP, Canto P. Analysis of

- C-850T and G-308A polymorphisms of the tumor necrosis factor-alpha gene in Maya-Mestizo women with preeclampsia. *Hypertens Pregnancy* 2007;26:283-91.
214. Chen G, Wilson R, Wang SH, Zheng HZ, Walker JJ, McKillop JH. Tumour necrosis factor-alpha (TNF-alpha) gene polymorphism and expression in pre-eclampsia. *Clin Exp Immunol* 1996;104:154-59.
 215. Dizon-Townson DS, Major H, Ward K. A promoter mutation in the tumor necrosis factor alpha gene is not associated with preeclampsia. *J Reprod Immunol* 1998;38:55-61.
 216. Freeman DJ, McManus F, Brown EA, Cherry L, Norrie J, Ramsay JE, *et al.* Short- and long-term changes in plasma inflammatory markers associated with preeclampsia. *Hypertension* 2004;44:708-14.
 217. Kaiser T, Grehan M, Brennecke SP, Moses EK. Association of the TNF2 allele with eclampsia. *Gynecol Obstet Invest* 2004;57:204-09.
 218. Levesque S, Moutquin JM, Lindsay C, Roy MC, Rousseau F. Implication of an AGT haplotype in a multigene association study with pregnancy hypertension. *Hypertension* 2004;43:71-78.
 219. Molvarec A, Jermendy A, Nagy B, Kovacs M, Varkonyi T, Hupuczi P, *et al.* Association between tumor necrosis factor (TNF)-alpha G-308A gene polymorphism and preeclampsia complicated by severe fetal growth restriction. *Clin Chim Acta* 2008;392:52-57.
 220. Pazarbasi A, Kasap M, Guzel AI, Kasap H, Onbasioglu M, Ozbakir B, *et al.* Polymorphisms in the tumor necrosis factor-alpha gene in Turkish women with pre-eclampsia and eclampsia. *Acta Med Okayama* 2007;61:153-60.
 221. Saarela T, Hiltunen M, Helisalmi S, Heinonen S, Laakso M. Tumour necrosis factor-alpha gene haplotype is associated with pre-eclampsia. *Mol Hum Reprod* 2005;11:437-40.
 222. Stonek F, Hafner E, Metzzenbauer M, Katharina S, Stumpflen I, Schneeberger C, *et al.* Absence of an association of tumor necrosis factor (TNF)-alpha G308A, interleukin-6 (IL-6) G174C and interleukin-10 (IL-10) G1082A polymorphism in women with preeclampsia. *J Reprod Immunol* 2008;77:85-90.
 223. Heiskanen J, Romppanen EL, Hiltunen M, Iivonen S, Mannermaa A, Punnonen K, *et al.* Polymorphism in the tumor necrosis factor-alpha gene in women with preeclampsia. *J Assist Reprod Genet* 2002;19:220-23.
 224. Papazoglou D, Galazios G, Koukourakis MI, Panagopoulos I, Kontomanolis EN, Papatheodorou K, *et al.* Vascular endothelial growth factor gene polymorphisms and pre-eclampsia. *Mol Hum Reprod* 2004;10:321-24.
 225. Shim JY, Jun JK, Jung BK, Kim SH, Won HS, Lee PR, *et al.* Vascular endothelial growth factor gene +936 C/T polymorphism is associated with preeclampsia in Korean women. *Am J Obstet Gynecol* 2007;197:271-74.

CHAPTER III

**CLASSICAL COMPLEMENT
ACTIVATION AS A FOOTPRINT
FOR MURINE AND HUMAN
ANTIPHOSPHOLIPID ANTIBODY-
INDUCED FETAL LOSS**

DANIELLE COHEN • ALETTA BUURMA • NATASCHA GOEMAERE
GUILLERMINA GIRARDI • SASKIA LE CESSIE • SICCO SCHERJON
KITTY BLOEMENKAMP • EMILE DE HEER • JAN ANTHONIE BRUIJN
INGEBORG BAJEMA

JOURNAL OF PATHOLOGY

Abstract

BACKGROUND Recurrent miscarriage, fetal growth restriction and intrauterine fetal death are frequently occurring complications of pregnancy in patients with systemic lupus erythematosis (SLE) and antiphospholipid syndrome (APS). Murine models show that complement activation plays a pivotal role in antiphospholipid antibody-mediated pregnancy morbidity, but the exact pathways of complement activation and their potential role in human pregnancy are insufficiently understood. We hypothesized that the classical pathway would play a major role in inducing fetal loss.

METHODS Pregnant C57BL/6 mice and mice deficient in C1q and factor D were injected with antiphospholipid antibodies or normal human IgG. Mouse placentas were subsequently stained with an anti-C4 antibody and anti-normal human IgG to determine the presence of classical complement activation and IgG binding. Findings in mice were validated in 88 human placentae from 83 women (SLE and APS cases versus controls), which were immunohistochemically stained for C4d, C1q, properdin and MBL. Staining patterns were compared to pregnancy outcome.

RESULTS In murine placentae of mice pretreated with antiphospholipid antibodies, increased C4 deposition was observed, which was associated with adverse fetal outcome but not with IgG binding. In humans, diffuse C4d staining at the feto–maternal interface was present almost exclusively in patients with SLE and/or APS ($p < 0.001$) and was related to intrauterine fetal death ($p = 0.03$).

CONCLUSIONS Our data show that presence of C4d in murine and human placentae is strongly related to adverse fetal outcome in the setting of SLE and APS. The excessive deposition of C4d supports the concept of severe autoantibody-mediated injury at the fetal–maternal interface. We suggest C4d as a potential biomarker of autoantibody-mediated fetal loss in SLE and APS.

Introduction

Recurrent miscarriage, fetal growth restriction and intrauterine fetal death (IUFD) are devastating complications of pregnancy that occur 20-40 times more often in patients with systemic lupus erythematosus (SLE) and antiphospholipid syndrome (APS) than in healthy pregnant women.^{1;2} The presence of circulating antiphospholipid antibodies (aPL), which is a prerequisite for APS and occurs in 40% of SLE patients, is strongly associated with thrombosis and fetal loss.³

The increased risk of thrombosis in the presence of aPL suggests that thrombosis of the uteroplacental vasculature could be an important cause of pregnancy morbidity. However, thrombotic lesions are not significant and even not always detectable in placentas of patients with aPL-mediated fetal loss.⁴ Recent studies in complement-deficient murine models⁵⁻⁸ suggested that complement activation plays a pivotal role in aPL-mediated fetal loss, shifting the focus more towards inflammation as the primary causative mechanism.^{9;10} However, the exact pathways of complement activation and their potential role in human pregnancy are insufficiently understood. We hypothesized that classical complement activation is the main responsible pathway in SLE- and APS-related pregnancy morbidity, based on an antibody-mediated allo-response at the fetal-maternal interface. We therefore investigated the role of classical, alternative and mannose binding lectin (MBL) pathway activation in a murine model of aPL-mediated fetal injury and in human placentae of pregnancies affected by SLE and APS. As a marker for classical pathway activation we used C4d, a marker for classical complement activation.

In transplantation pathology, C4d is widely used as a biomarker to diagnose antibody-mediated allograft rejection.¹¹ As a degradation product of C4, one of the main components of the classical complement cascade, C4d has the ability to bind covalently to cell surfaces and basement membranes near sites of C4 activation.

Covalently bound C4d is anchored to the tissue, and remains attached much longer than the antibodies that originally activated the classical pathway. This makes C4d a highly stable marker, and has led to C4d being referred to as a ‘footprint’ of antibody-mediated tissue injury.¹²

In a mouse model of aPL-mediated fetal loss, we first studied murine placentae with an anti-C4 antibody to determine presence of classical complement activation. Observations in mice were validated by investigating the presence C4d deposits in placentae of patients with SLE and/or APS in relation to pregnancy outcome, compared to healthy and disease controls. In additional studies we confirmed that placental C4d depositions reflect specific involvement of classical pathway activation, and are not derived from lectin or alternative pathway activation.

Materials and methods

MOUSE MODEL OF ANTIPHOSPHOLIPID ANTIBODY-INDUCED

FETAL LOSS Adult mice (6-8 weeks), C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME, USA) and mice deficient in C1q and factor D (C1qfDKO) mice¹³ (generously provided by Greg Stahl, Harvard University) were used in all experiments. On days 8 and 12 of pregnancy, females were treated with intraperitoneal (i.p.) injections of human IgG-containing aPL (aPL-IgG; 10 mg) or normal human IgG (NH-IgG; 10 mg) as previously described.^{5;14} APL-IgGs were obtained from patients with APS (characterized by high titer aPL antibodies, >140 GPL units); NH-IgG was obtained from healthy non-autoimmune individuals.

The mice (C57BL/6 and C1qfDKO) were sacrificed on day 8, 2h after aPL-IgG injection (cases, n=5) or NH-IgG injection (controls, n=5), the uteri were dissected and deciduae and placentae were harvested. Another group of cases (n=5) and controls (n=5) were euthanized on day 15 of pregnancy to harvest placentae. Resorption sites that result from loss of a previously viable embryo

were counted as previously described.^{5;14}

For immunohistochemical studies, deciduae from day 8 and placentae from day 15 of pregnancy were fixed in 4% paraformaldehyde, frozen in OCT compound and cut into 10 µm sections. The sections were stained for C4d with a rat monoclonal anti-C4 antibody (C4 antibody, 16D2, ab11863; Abcam, Cambridge, MA, USA) at a dilution of 1/50. This antibody reacts with murine C4, C4b and C4d. As a positive control we used a kidney of a mouse injected with FB1 (mouse monoclonal-aPL that activates complement). An HRP-labelled secondary antibody and DAB as substrate or a FITC-labeled secondary antibody were used to develop the reaction. For fluorescence staining, gold antifade reagent with DAPI (Invitrogen, Carlsbad, CA, USA) was used. Day 15 placentae were also stained with anti-human IgG (Sigma-Aldrich, St Louis, MO) at a dilution of 1/150. All animal studies were approved by the institutional Animal Care and Use Committee of the Hospital for Special Surgery or Weill Medical College of Cornell University and York College, City University of New York, New York.

PATIENTS AND PLACENTAE All tissue samples were handled in a coded fashion, according to Dutch national ethical guidelines (Code for Proper Secondary Use of Human Tissue, Dutch Federation of Medical Scientific Societies). We studied 88 placentae from 83 women who delivered at the Obstetrical Department of the Leiden University Medical Centre (LUMC) between 1995 and 2009. The women were subdivided into three groups. The case-group consisted of 21 patients with a confirmed diagnosis of SLE and/or APS, from whom we selected all available placentae (26 placentae of 21 patients). Multiple placentae were available from four patients in the case group; for statistical analysis, only the placenta of the first pregnancy was taken into account. All patients in the case group were tested for antiphospholipid antibodies (lupus anticoagulant and anticardiolipin antibodies) according to the most recent guidelines.^{15;16}

For a first control group (live-birth controls), we included 40 placentae of pregnancies that resulted in live births in the same period as above. These placentae represented pregnancies ranging from completely normal to relatively complicated, including both maternal and fetal morbidity. None of the patients had preeclampsia or Hemolysis Elevated Liver enzymes and Low Platelets (HELLP) syndrome. For a second control group (IUFD-controls), we included 22 placentae of pregnancies that resulted in IUFD by various causes other than SLE or APS. Fetal losses in this group were mainly caused by fetal chromosomal abnormalities. An overview of the clinical characteristics of all patients, derived from the case-records of the LUMC, is given in Table 1.

ROUTINE HISTOPATHOLOGY AND IMMUNOHISTOCHEMISTRY

Placentae were fixed in 4% buffered formalin and embedded in paraffin. Paraffin sections were routinely stained with haematoxylin and eosin (H&E). To study complement activation, immunohistochemical staining was performed for C4d (BI-RC4d, Biomedica Gruppe, Austria), C1q (Dako Cytomation, Denmark), MBL (Sigma-Aldrich Biotechnology) and properdin (primary antibody kindly provided by the department of Nephrology, Leiden, the Netherlands). Optimal antibody dilutions and incubation times for the different antibodies were pre-determined by means of titration on positive control sections.

C4D Endogenous peroxidase activity was blocked. Antigen retrieval was performed with 10 mM citrate buffer (pH 6.0). A polyclonal rabbit anti-human c4d antibody (Biomedica Gruppe, Austria), was applied at a dilution of 1:80 in 1% bsa/pbs, and slides were incubated for one hour at room temperature. The slides were then incubated with a secondary antibody (anti-rabbit EnVision, K5007, Dako Cytomation, Denmark) for 30 minutes. Staining was visualized with diaminobenzidine (Dako Cytomation, Denmark) and counterstained with Haematoxylin. A tissue sample from a renal biopsy of a patient

with humoral rejection with c4d-positive staining served as a positive control.

C1Q Endogenous peroxidase activity was blocked. Antigen retrieval was performed in edta-tris (pH 9.0). A polyclonal rabbit antihuman polyclonal c1q antibody (Dako Cytomation, Denmark) was applied at a dilution of 1:700 in 1%bsa/pbs, and slides were incubated for one hour at 37 C. The slides were then incubated with a secondary antibody (anti-rabbit EnVision, K5007, Dako Cytomation, Denmark) for 30 minutes. Staining was visualized with diaminobenzidine (Dako Cytomation, Denmark) and counterstained with Haematoxylin. Tonsil tissue served as a positive control.

MBL Endogenous peroxidase activity was blocked. Antigen retrieval was performed with 10 mM citrate buffer (pH 6.0). A polyclonal rabbit anti-human mbl antibody (Sigma-Aldrich Biotechnology) was applied at a dilution of 1:250 in 1% bsa/pbs, and slides were incubated for one hour at room temperature. The slides were then incubated with a secondary antibody (anti-rabbit EnVision, K5007, Dako Cytomation, Denmark) for 30 minutes. Staining was visualized with diaminobenzidine (Dako Cytomation, Denmark) and counterstained with Haematoxylin. Liver tissue served as a positive control.

PROPERDIN Endogenous peroxidase activity was blocked. Antigen retrieval was performed with 10 mM citrate buffer (pH 6.0). The slides were subsequently blocked with 5% heat-inactivated normal human serum in 1% bovine serum albumin/phosphate-buffered saline (bsa/pbs) for 45 min at room temperature. A polyclonal rabbit anti-human properdin antibody (kindly provided by the department Nephrology, Leiden, the Netherlands) was applied at a dilution of 1:800 in 1%bsa/pbs overnight. The slides were then incubated with a secondary antibody (anti-rabbit EnVision, K5007, Dako Cytomation, Denmark) for 30 minutes. Staining was visualized with

diaminobenzidine (DakoCytomation, Denmark) and counterstained with Haematoxylin. A kidney with membranous nephropathy was used as a positive control.

QUANTIFICATION OF PLACENTAL MORPHOLOGY AND

IMMUNOHISTOPATHOLOGY Sections were evaluated by two experienced pathologists, who scored the slides blinded to the patients' clinical data. Differences in scorings were resolved by re-reviewing the sections and coming to consensus. Each placenta was scored separately for the presence or absence of histopathological changes associated with decreased uteroplacental perfusion: acute and/or chronic deciduitis, decidual necrosis, increased syncytial knots, accelerated villous maturity, accelerated maturity, avascular villi, villous infarcts, retroplacental hematomas, intervillous thrombi and decidual vasculopathy.^{17;18}

Positivity for immunohistochemical stainings was scored semi-quantitatively. A random area of 1 x 1 cm was selected from each tissue sample for scoring. Staining intensity around syncytiotrophoblast was scored as 0, 1, or 2, with 0 representing the total absence of staining, 1 representing focal positive staining, and 2 representing diffuse staining of all syncytiotrophoblast cell and basement membranes within this area. Intravillous endothelial staining was scored on a 0, 1, 2 scale, with similar definitions as above.

STATISTICAL ANALYSIS Categorical variables were compared using the Chi-square test. Differences in quantitative parameters between groups were assessed using One-way ANOVA (for data normally distributed) or Kruskal Wallis H one-way analysis (for data not normally distributed). All analyses were performed using SPSS statistical software package (version 16.0; Chicago, IL). A p-value less than 0.05 was considered statistically significant.

Results

MOUSE MODEL OF APL-IGG TREATED MICE

C4 DEPOSITION IN APL-IGG TREATED MICE VERSUS

CONTROLS Placentae from the surviving fetuses in aPL-treated mice showed increased C4 deposition in the labyrinth (Figure 1Bi) when compared to placentae from NH-IgG treated mice (Figure 1Ai). The labyrinth is the area of active fetal-maternal exchange in the murine placenta, equivalent to chorionic villi in humans.

Specifically, C4 deposition was observed on the trophoblast giant cells (TGC) (Figure 1Bii,iii). These cells are crucial for implantation and invasion of the conceptus into maternal decidua of the uterus. Abnormalities in these cells can cause placental defects and compromised pregnancies^{19;20} and can explain the high resorption frequency observed in embryos from aPL-treated mice.^{5;14}

C4 DEPOSITION IN C1Q- AND FACTOR D-DEFICIENT MICE

To rule out the possibility that C4d positivity was caused by lectin pathway activation, we studied pregnancy outcomes in mice that were deficient for complement component C1q and factor D (Figure 1C). Mice deficient in the classical pathway component C1q and the alternative pathway component factor D were protected from aPL-induced fetal injury. The fetal resorption frequency in these mice was not different from that calculated in NH-IgG treated mice with uneventful pregnancies (Figure 1C). No C4 deposition was observed, either in deciduae nor in placentae from C1qfDKO mice treated with aPL (data not shown).

IGG DEPOSITION To study whether complement deposition coincides with aPL-binding in aPL-treated mice with increased fetal loss, we stained for human IgG in day 15 placentae from surviving fetuses. In contrast with the robust C4a deposition observed in these placentae, no IgG staining was found (Figure 1Biv). IgG was also negative in placentae from NH-IgG controls (Figure 1Aiv).

CLINICAL DATA Patients in all groups were of similar age. In the case group of patients with SLE and/or APS, 15 patients met the American College of Rheumatology criteria for SLE²¹, 12 patients met the classification criteria for APS²², and 6 patients had both SLE and APS (see Table 1). None of the patients in the live-born control group had been tested for aPL. In the IUFD-group all patients with an IUFD of unknown etiology were tested for aPL. None of the patients met the laboratory criteria for APS. Other patient and pregnancy characteristics are shown in Table 1.

HISTOPATHOLOGICAL FINDINGS IN CASES VERSUS CONTROLS

Retroplacental hematomas and intervillous thrombi were infrequent and not increased either in placentae from patients with SLE and/or APS, compared to both control groups. Villous infarction and accelerated maturity occurred more often in placentae from patients with SLE/APS than in both control groups. Figure 2 shows the incidence of various histopathological findings in all placentae.

DETECTION OF COMPLEMENT COMPONENTS AND THEIR ASSOCIATION WITH CLINICAL PARAMETERS

C4D When present, C4d showed positivity at the fetal-maternal interface, on the maternal side of the syncytiotrophoblast, in either in a focal or a diffuse staining pattern. Typical examples of C4d staining patterns are shown in figure 3A-C. A diffuse C4d staining pattern in the placenta was strongly associated with SLE and/or APS ($p < 0.001$; Figure 3M). Within the case group, diffuse C4d staining was

associated with IUFD ($p < 0.03$; Figure 3N). Detailed information on C4d staining in the cases versus controls is shown in Table 2.

Diffuse C4d staining around all syncytiotrophoblast cell and basement membranes (figure 3C) was found in 12 placentae: ten were from patients with SLE and/or APS, of whom five had a pregnancy resulting in IUFD, four pregnancies were characterized by severe intrauterine growth retardation and/or preeclampsia and one of the patients had an uncomplicated pregnancy, which was her first live born child after four late miscarriages. Two of the 12 placentae that were diffusely positive for C4d were from patients from the control group with IUFD; both patients had an unexplained IUFD. One patient was negative for aPL, the other appeared to have circulating aPL (anticardiolipin IgG antibodies) when she was tested three months after delivery. A diffuse or focal positive C4d staining pattern was never found in the control group with live-born children ($p < 0.001$). Detailed information on focal and absent C4d staining patterns is given in Table 2.

C1Q C1q was present in both intravillous endothelial cells and around the syncytiotrophoblast cells (Figure 3D-F) and was never completely negative, either in cases or controls (data not shown). In cases of diffuse C4d deposition around syncytiotrophoblast cells, C1q clearly co-localized with C4d depositions.

PROPERDIN Properdin was almost uniquely positive in intravillous endothelial cells, and showed minor positivity around syncytiotrophoblast cells in only two cases of live birth controls. It did not co-localize with C4d deposition (figure 3G-I). Diffuse properdin staining occurred almost at a similar rate in live born placentas and in placentas in the SLE/APS group (33% and 28%, respectively). Properdin positivity was negatively associated with IUFD: in only two out of 22 IUFD cases a diffuse staining pattern was observed.

MBL In all cases MBL deposition was absent (figure 3J-L), whereas liver tissue that was used as a positive control was evidently positive. MBL also did not occur in C4d positive placentae, or in placentae of patients with prolonged rupture of membranes.

PATIENTS OF WHICH MULTIPLE PLACENTAS OF SUBSEQUENT PREGNANCIES WERE AVAILABLE Multiple placentae were available from four patients in the case group. All placentae showed a consistent pattern of C4d staining (consistently positive or consistently negative), except for one patient who had a diffuse staining in one and a focal staining pattern in a second placenta. A detailed description of these patients and their pregnancies is given in Table 3.

Discussion

APS and SLE are autoantibody-mediated autoimmune diseases in which severe pregnancy morbidity is pertinent. The present study demonstrated that C4d deposits were present in areas of active fetal-maternal exchange in the murine placenta of mice pretreated with human aPL-IgG and absent in those treated with NH-IgG. C4d deposits in murine placentae were associated with an increased fetal absorption rate (Figure 1). Observations in mice were validated in human placentae, where placental C4d deposition was frequently and almost exclusively present around syncytiotrophoblast cells of placentae from patients with SLE and/or APS. Its presence in human placentae was strongly related to adverse pregnancy outcome in SLE and APS patients. In patients without SLE or APS but with IUFDs, diffuse C4d staining was rare. C4d staining was always negative in placentae from patients with live births (Figure 3, Table 2). Our data support the concept of a severe autoantibody-mediated immune response at the fetal-maternal interface, leading to impaired fetal outcome.

To rule out that C4d deposition is a reflection of lectin pathway activation, we studied the deposition patterns of MBL. In the mouse experiments, mice deficient in the classical pathway component C1q and the alternative pathway component factor D were protected from aPL-induced fetal injury. No C4 deposition was observed in placentae from these mice. MBL was not detectable in human placentae and did not co-localize with C4d. These observations indicate that it is very unlikely that the lectin pathway is involved in the pathogenesis of fetal losses induced by aPL.

Direct exposure of trophoblast to the maternal blood puts these cells at risk of being attacked by complement activation products, both in the setting of maternal autoimmune disease and in normal pregnancies. Our data indicate that deposition of the early classical pathway component C1q is most likely a non-pathological phenomenon. The observed presence of C1q in placentae of controls could reflect the physiological presence of maternal IgG and IgM. It is not surprising that this usually does not lead to fullblown activation of the complement cascade, because of the many complement-regulatory mechanisms normally present at the level of the syncytiotrophoblast.⁶ Alternatively, it was previously shown that decidual endothelial cells can synthesize C1q and express it on their surfaces in physiological situations.^{23;24} Bulla et al showed that deposition of C1q in the placenta was not necessarily associated with the presence of IgG, IgM or C4.²³ The clear association of C4d (a complement split-product more downstream than C1q) with SLE- and APS-related pregnancy morbidity, as presented in this study, may be regarded as evidence of a severe antibody-mediated allo-response, where the complement inhibitory mechanisms are surpassed and fail to suppress the evolving events in the complement cascade.

Evidence from mouse models suggested that a large part of the aPL-mediated placental damage is caused by amplification of the classical pathway by the alternative pathway.²⁵ However, we found properdin, a membrane bound marker of alternative pathway activation, in placentae of both patients with SLE and APS as well

as in live-birth controls, making no distinction between a good or bad pregnancy outcome. Interestingly, properdin depositions are found within the vessels of the villi, and did not co-localize with C4d deposits at the maternal side of the syncytiotrophoblast. This means that properdin must originate from the fetal complement system²⁶ and may reflect a general state of fetal distress, rather than being directly related to the C4d depositions.

Although the clinical implications of aPL are well known, testing for their presence remains difficult and only highly specialized laboratories can provide reliable test results.^{27;28} C4d stainings are performed in most clinical pathology laboratories and a diffuse C4d staining pattern is easy to recognize (Figure 3C). We therefore propose that placental C4d staining could be a useful additional tool to further strengthen the diagnosis of aPL-mediated fetal loss, for instance in the work-up of a first late miscarriage or fetal death. This might even have implications for treatment with heparin, as it was shown in animal models that heparin prevents aPL-induced fetal loss by inhibiting complement activation.²⁹ Alternatively, it would be interesting to explore whether targeted inhibition of complement activation would be beneficial for pregnancy outcome, similar to the findings in mice models.^{5;7}

The analogy between pregnancy and transplantation was made as early as 1953, when Peter Medawar introduced the concept of ‘the fetal allograft’.³⁰ In transplantation, humoral allograft rejection has gained much attention since the discovery of C4d.¹¹ Our data demonstrate that complicated pregnancies of patients with autoimmune diseases, such as APS, share several pathophysiological aspects with humoral rejection. Both aPL and donor-specific antibodies to donor-HLA bind at the frontier where cells from the one individual (mother or host) meet the other (fetus or graft).

Interestingly, both in pregnancy and in transplantation, we find no histological evidence for the binding of the antibodies themselves, whereas C4d remains attached and is easily detectable both in mice placentae at day 8 and 15 and in human placentae of various

gestational ages. In day 15 placentae of aPL-treated mice, we did not find NH-IgG deposition, whereas it was previously published that NH-IgG can be detected in day 8 placentas of similarly treated mice.³¹ This shows that these antibodies do bind at the fetal-maternal interface initially, but do not remain attached longer than a few days, whereas C4d is detectable throughout the whole pregnancy. From the transplant setting, it is known that this phenomenon can be explained by the relatively weak binding capacity of antibodies, compared to the covalent binding of C4d, which anchors to the damaged tissue.¹¹ In this study a parallel between the humoral rejection and pregnancy is now found in the form of C4d deposition, indicating that C4d might be a more reliable and long-lasting marker of placental antibody-mediated tissue injury.

In human placentae, we demonstrated that 62% of patients with SLE/APS have a focal or a diffuse C4d staining pattern. However, 38% of these patients do not show any deposition of C4d, while their pregnancy outcomes are also impaired by either growth restriction or preeclampsia or even fetal death. There are two important explanations for this situation. First, as we take only a small part of the placenta for analysis, C4d positivity present in a focal, patchy pattern through the whole placenta can be missed due to sampling error. In future studies, the effect of more extensive tissue sampling could be investigated. Secondly, it is evident that in any pregnancy, many more than C4d-related causes can lead to IUFD or impaired pregnancy outcome. For instance, anti-SSA or anti-SSB antibodies can cause a congenital heart block and, subsequently, fetal death, by traveling through the placenta into the fetal circulation. This is a totally different mechanism for SLE-related IUFD in which no trophoblastic C4d deposition is expected. Furthermore, there is always a chance for fetal chromosomal abnormalities, and in such cases no C4d deposition is expected, as we have illustrated in our IUFD control group.

In conclusion, we have shown that classical complement activation plays a major role in aPL-mediated fetal injury, and that placental

C4d deposition is a reflection of classical complement activation. C4d is strongly associated with impaired fetal outcome, both in a mouse model of aPL-mediated fetal loss and in human pregnancy affected by SLE and APS. Especially in women with a first pregnancy resulting in IUFD, placental C4d staining has potential as a diagnostical tool to detect aPL-mediated fetal loss. Further prospective studies need to confirm whether C4d positivity in a previous IUFD or late miscarriage can be considered as a biomarker of a future complicated pregnancy.

**FIG 1A-B INCREASED C4D DEPOSITION IN APL-IGG TREATED MICE
VERSUS NH-IGG CONTROLS** (*full colour version inside cover*)

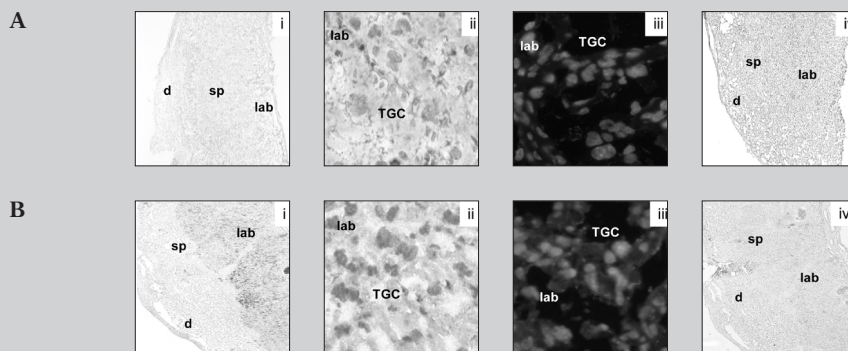
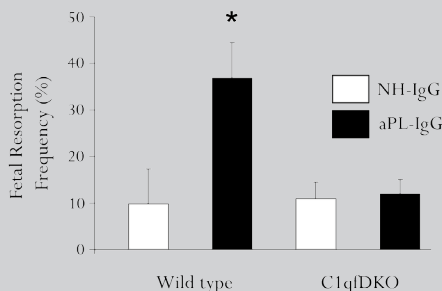


Figure BI, II and III show increased C4 deposition in the labyrinth (lab) compared to figure AI, II and III. The labyrinth is the area of active feto-maternal exchange in the murine placenta. The equivalent to this area in humans is the chorionic villi. C4 deposition was observed on the trophoblast giant cells (TGC) of aPL-IgG treated mice (Figure BII and III). In contrast, minimal C4d staining was found in placentas from NH-IgG-treated mice (Figure AI, II, III). C4d deposition in placentas was not associated with APL-IgG binding. Negative staining for human IgG was observed in day 15 placentas from APL-treated mice (Fig BIV). IgG deposition was also absent in placentas from NH-IgG-treated mice (Fig AIV).

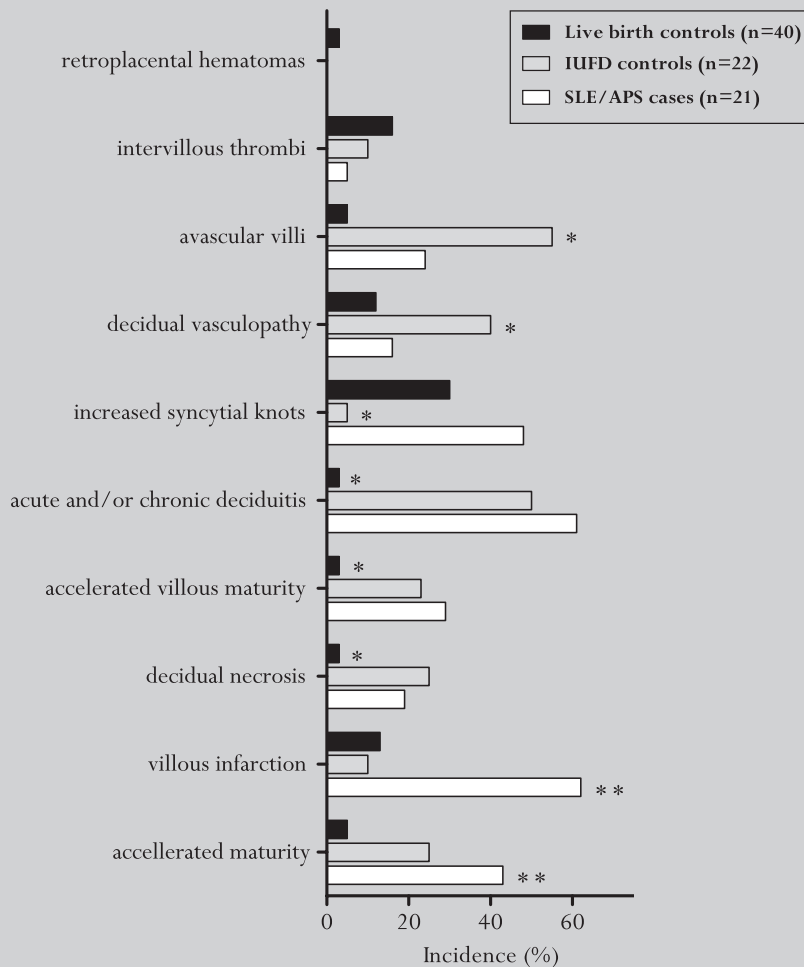
**FIG 1C FETAL RESORPTION FREQUENCY IN C1Q- AND FACTOR D
DEFICIENT MICE**



Mice deficient in the classical pathway component C1q and the alternative pathway component factor D are protected from aPL-induced fetal injury. The fetal resorption frequency in these mice was not different from that calculated in NH-IgG treated mice with uneventful pregnancies. No C4 deposition was observed neither in deciduas nor in placentas from C1qDKO mice treated with antiphospholipid antibodies (data not shown).

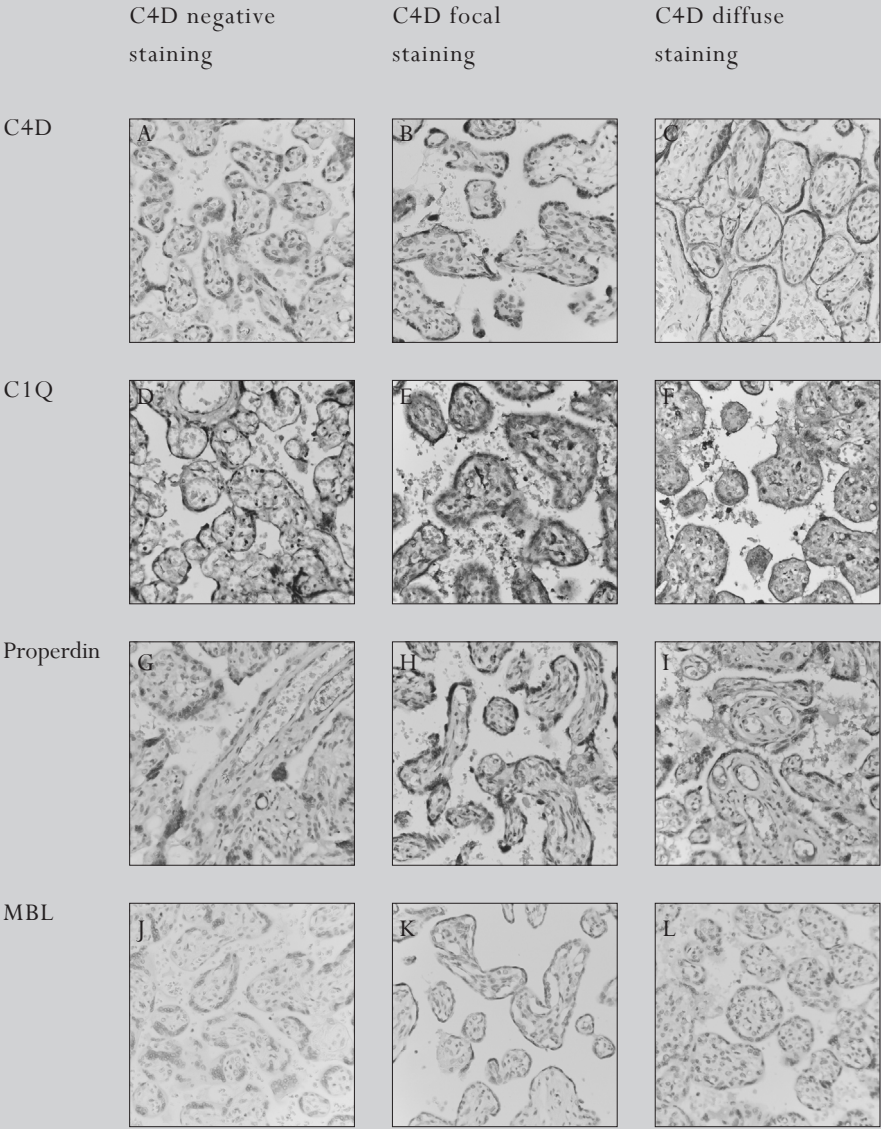
PLACENTAL HISTOLOGICAL PARAMETERS IN CASES VS CONTROLS

FIG 2



Bar graph of specific placental histological scores as described in Results. The figure gives an indication of the abundance of various lesions in different groups. The differences between groups are represented by (*) indicating a p-value of <0.05 and (**) indicating a p-value of <0.01 .

FIG 3 **IMMUNOHISTOCHEMICAL STAINING PATTERNS IN HUMAN PLACENTAS** *(full colour version inside cover)*



Panels 3A-L: Typical examples of immunohistochemical staining patterns of C4d, C1q, Properdin and MBL of placentas. Vertically the panels are organized in such a way that each column represents the same placenta. Horizontally the different immunohistochemical stainings are shown. The first column represents a C4d negative placenta of a patient with a live birth. The middle column represents a placenta of a patient with an IUFD, with a focal C4d staining pattern. In the third column a placenta of a patient with SLE and secondary APS is shown which is diffusely positive for C4d. The pregnancy was accompanied by severe maternal preeclampsia and severe fetal growth retardation.

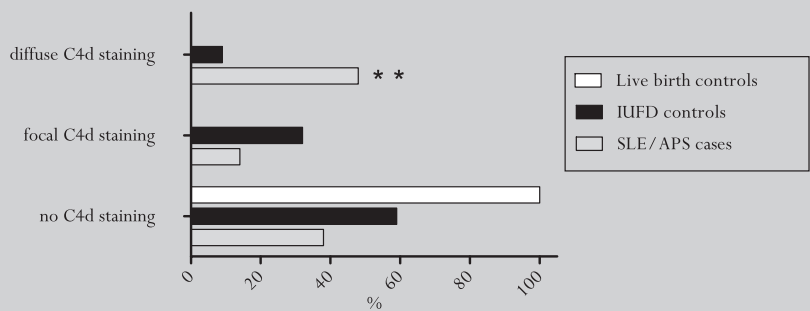
Panels 3A-C: Typical examples of different C4d staining intensities of villous syncytiotrophoblast cell and basement membranes observed in patients with SLE and/or APS compared to controls. Panels demonstrate the different staining intensities by which the placentas were scored: (A) ‘no placental C4d staining’, (B) ‘focal placental C4d staining’, and (C) ‘diffuse placental C4d staining’. In (C) it is clearly shown that C4d depositions are found on the maternal side of the placental syncytiotrophoblast, and not within the fetal vasculature.

Panels 3D-F: Typical examples of placental C1q staining. Interestingly, C1q was not unique for C4d positive placentas, but was observed frequently in non-C4d positive placentas too. This phenomenon is illustrated in figure (D), where a C4d negative placenta is evidently C1q positive. However, panel (F) shows that C1q does co-localization with C4d in a C4d positive placenta, confirming that C4d originates from classical pathway activity.

Panels 3G-I: Typical examples of properdin staining, in which properdin deposits on endothelial cells of the fetal vessels. Properdin deposition is not corresponding with the sites of C4d deposition, suggesting that classical complement activation does not necessarily lead to properdin deposition – i.e. alternative pathway activation.

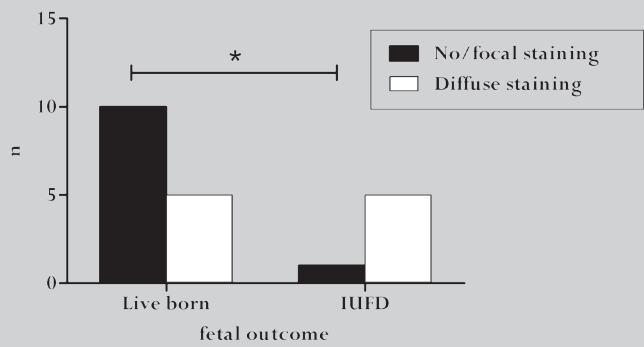
Panels J-L: In all cases MBL deposition was absent, further confirming that the MBL pathway does not contribute to the deposition of C4d in the setting of SLE and aPL-mediated fetal loss.

FIG 3M PLACENTAL C4D IN CASES VERSUS CONTROLS



The association of diffuse C4d staining with IUFD in SLE/APS cases.
The difference between the groups is represented by (**) indicating a p-value of < 0.01.

FIG 3N DIFFUSE C4D STAINING IN SLE/APS PATIENTS



Bar graph of C4d staining in SLE/APS cases versus control groups. The difference between the groups is represented by (*) indicating a p-value of <0.05. Figure 3 gives an indication of the abundance of diffuse C4d staining in the SLE/APS case group and the striking absence of diffuse C4d in the live birth controls.

TABLE 1 PATIENT CHARACTERISTICS

	SLE/APS* CASES (N=21)	IUFD** CONTROLS (N=22)	LIVE BIRTH CONTROLS (N=40)
Mean maternal age in years (SD)	29,7(4)	30,5 (6)	31,5 (6)
Mean gravidity (SD)	2,3 (1,5)	2,6 (1,6)	2,2 (0,87)
Mean parity (SD)	0,6 (1,1)	1,3 (1,4)	0,8 (0,8)
Previous live birth (%)	5 (24)	11 (50)	15 (38)
Previous miscarriage or fetal loss (%)	8 (38)	6 (27)	14 (35)
Gestational age at delivery (wk+day) (SD in days)	32+6 (4)	25+3 (8)	37+1 (7)
Delivery at <24 wk (%)	4 (19)	14 (64)	0
Delivery at 24-38 wk (%)	17 (81)	6 (27)	20 (50)
Delivery at 38-42 (%)	5 (24)	2 (9)	20 (50)
Intrauterine fetal death (%)	6(29)	22(100)	0
Fetal distress (%)	6(29)	0	11(27)
Mean birth weight (grams) (SD)	1639,3 (1060)	901,3 (1203)	2534,5 (981)
Placental weight (grams) (SD)	306,8 (173)	230,2 (205)	472,9 (139)
Heparin therapy during pregnancy (%)	8 (38)	0	0
Comorbidity			
SLE (%)	15 (71)	0	0
SLE and APS (secondary APS) (%)	6 (28)	0	0
APS, no SLE (primary APS) (%)	6 (28)	0	0
Lupus anticoagulant (%)	8 (38)	0	0
Anticardiolipin antibodies, IgG (%)	10 (48)	0	0
Preeclampsia (%)	5 (24)	0	0
HELLP syndrome (%)	2 (10)	0	0
Normal pregnancy and delivery (%)	2 (10)	0	14 (35)
Unexplained intrauterine fetal death (%)	0	5 (23)	0
Infant congenital abnormalities (%)	0	12 (55)	3 (8)

* Systemic Lupus Erythematosus and antiphospholipid syndrome

** Intrauterine fetal death

TABLE 2 C4D AND PROPERDIN IN SLE AND APS PLACENTAS VERSUS CONTROLS

	SLE/APS CASES * (N=21)	IUFD** CONTROLS (N=22)	LIVE BORN CONTROLS (N=40)	P-VALUE SLE/APS VS CONTROLS
no C4d deposition (%)	8 (38)	13 (59)	40(100)	P < 0.001
focal C4d deposition (%)	3 (14)	7 (32)	0	
diffuse C4d deposition (%)	10 (48)	2 (9)	0	
no properdin deposition (%)	8 (38)	19 (86)	17 (43)	P < 0.918
focal properdin deposition (%)	6 (29)	2 (9)	12 (30)	
diffuse properdin deposition (%)	7 (33)	1 (5)	11 (28)	

** Systemic Lupus Erythematosus and APS

* IUFD control group

TABLE 3 PATIENTS WITH MULTIPLE PLACENTAS

CASE NO	PATIENT DIAGNOSIS	GRAVIDITY	C4d STAINING	FETAL OUTCOME	WEEKS & DAYS
1	SLE*	G1	no tissue available	early miscarriage	< 10
		G2	no C4d staining	IUFD*** of unknown etiology	18
		G3	no C4d staining	IUFD, congenital heart block in the presence of anti-SSA and anti-SSB antibodies	31
		G4	no C4d staining	Live birth	38+1
2	SLE + APS**	G1	no tissue available	Live birth, IUGR†	37+2
		G2	diffuse C4d staining	Live birth, IUGR and severe Preeclampsia	38+4
		G3	focal C4d staining	Live birth, IUGR	38+3
3	Primary APS	G1	diffuse C4d staining	IUFD, severe IUGR and maternal HELLP ‡	23+2
		G2	no tissue available	live birth, HELLP syndrome and maternal liver infarction	37
		G3	diffuse C4d staining	IUFD, severe HELLP syndrome	28+2
4	Primary APS	G2	no tissue available	IUFD of unknown etiology	21+4
		G3	diffuse C4d staining	IUFD, severe IUGR	18+1
		G4	diffuse C4d staining	IUFD, severe IUGR	26+1

* Systemic lupus erythematosus

** Antiphospholipid syndrome

*** Intrauterine fetal death

† Intrauterine growth retardation

‡ Hemolysis Elevated Liver enzymes and Low Platelets

REFERENCES

1. Opatrny L, David M, Kahn SR, Shrier I, Rey E. Association between antiphospholipid antibodies and recurrent fetal loss in women without autoimmune disease: a metaanalysis. *J Rheumatol* 2006; 33:2214-2221.
2. Cohen D, Berger SP, Steup-Beekman GM, Bloemenkamp KW, Bajema IM. Diagnosis and management of the antiphospholipid syndrome. *BMJ* 2010; 340:c2541.
3. Ruiz-Irastorza G, Crowther M, Branch W, Khamashta MA. Antiphospholipid syndrome. *Lancet* 2010; 376:1498-509.
4. Sebire NJ, Backos M, El Gaddal S, Goldin RD, Regan L. Placental pathology, antiphospholipid antibodies, and pregnancy outcome in recurrent miscarriage patients. *Obstet Gynecol* 2003; 101(2):258-263.
5. Girardi G, Berman J, Redecha P, Spruce L, Thurman JM, Kraus D *et al*. Complement c5a receptors and neutrophils mediate fetal injury in the antiphospholipid syndrome. *J Clin Invest* 2003; 112:1644-1654.
6. Girardi G, Bulla R, Salmon JE, Tedesco F. The complement system in the pathophysiology of pregnancy. *Mol Immunol* 2006; 43:68-77.
7. Holers VM, Girardi G, Mo L, Guthridge JM, Molina H, Pierangeli SS *et al*. Complement c3 activation is required for antiphospholipid antibody-induced fetal loss. *J Exp Med* 2002; 195:211-220.
8. Di Simone N, Meroni PL, de Papa N, Raschi E, Caliandro D, De Carolis CS *et al*. Antiphospholipid antibodies affect trophoblast gonadotropin secretion and invasiveness by binding directly and through adhered beta2-glycoprotein I. *Arthritis Rheum* 2000; 43:140-150.
9. Cavazzana I, Manuela N, Irene C, Barbara A, Sara S, Orietta BM *et al*. Complement activation in anti-phospholipid syndrome: a clue for an inflammatory process? *J Autoimmun* 2007; 28:160-164.
10. Salmon JE, Girardi G. Antiphospholipid antibodies and pregnancy loss: a disorder of inflammation. *J Reprod Immunol* 2008; 77:51-56.
11. Colvin RB. Antibody-mediated renal allograft rejection: diagnosis and pathogenesis. *J Am Soc Nephrol* 2007; 18:1046-1056.
12. Nickleit V, Mihatsch MJ. Kidney transplants, antibodies and rejection: is c4d a magic marker? *Nephrol Dial Transplant* 2003; 18:2232-2239.
13. Shannon JG, Cockrell DC, Takahashi K, Stahl GL, Heinzen RA. Antibody-mediated immunity to the obligate intracellular bacterial pathogen *Coxiella burnetii* is Fc receptor- and complement-independent. *BMC Immunol* 2009; 10:26.
14. Redecha P, Franzke CW, Ruf W, Mackman N, Girardi G. Neutrophil activation by the tissue factor/Factor viia/ PAR2 axis mediates fetal death in a mouse model of antiphospholipid syndrome. *J Clin Invest* 2008; 118:3453-3461.
15. Pengo V, Tripodi A, Reber G, Rand JH, Ortel TL, Galli M *et al*. Update of the guidelines for lupus anticoagulant detection. Subcommittee on Lupus Anticoagulant/ Antiphospholipid Antibody of the Scientific and Standardisation Committee of the International Society on Thrombosis and Haemostasis. *J Thromb Haemost* 2009; 7:1737-1740.
16. Pierangeli SS, Harris EN. A protocol

- for determination of anticardiolipin antibodies by elisa. *Nat Protoc* 2008; 3:840-848.
17. Magid MS, Kaplan C, Sammaritano LR, Peterson M, Druzin ML, Lockshin MD. Placental pathology in systemic lupus erythematosus: a prospective study. *Am J Obstet Gynecol* 1998; 179:226-234.
18. Out HJ, Kooijman CD, Bruinse HW, Derksen RH. Histopathological findings in placentae from patients with intra-uterine fetal death and anti-phospholipid antibodies. *Eur J Obstet Gynecol Reprod Biol* 1991; 41:179-186.
19. Cross JC. How to make a placenta: mechanisms of trophoblast cell differentiation in mice--a review. *Placenta* 2005; 26 Suppl A:S3-S9.
20. Kraut N, Snider L, Chen CM, Tapscott SJ, Groudine M. Requirement of the mouse *I-mfa* gene for placental development and skeletal patterning. *EMBO J* 1998; 17:6276-6288.
21. Hochberg MC. Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus. *Arthritis Rheum* 1997; 40:1725.
22. Miyakis S, Lockshin MD, Atsumi T, Branch DW, Brey RL, Cervera R *et al.* International consensus statement on an update of the classification criteria for definite antiphospholipid syndrome (aps). *J Thromb Haemost* 2006; 4:295-306.
23. Bulla R, Agostinis C, Bossi F, Rizzi L, Debeus A, Tripodo C *et al.* Decidual endothelial cells express surface-bound c1q as a molecular bridge between endovascular trophoblast and decidual endothelium. *Mol Immunol* 2008; 45:2629-2640.
24. Bulla R, Bossi F, Agostinis C, Radillo O, Colombo F, De Seta F *et al.* Complement production by trophoblast cells at the feto-maternal interface. *J Reprod Immunol* 2009; 82:119-125.
25. Thurman JM, Kraus DM, Girardi G, Hourcade D, Kang HJ, Royer PA *et al.* A novel inhibitor of the alternative complement pathway prevents antiphospholipid antibody-induced pregnancy loss in mice. *Mol Immunol* 2005; 42:87-97.
26. Kohler PF. Maturation of the human complement system. I. Onset time and sites of fetal c1q, c4, c3, and c5 synthesis. *J Clin Invest* 1973; 52:671-677.
27. Devreese K, Hoylaerts MF. Challenges in the diagnosis of the antiphospholipid syndrome. *Clin Chem* 2010; 56:930-940.
28. Pengo V, Biasiolo A, Gesele P, Marongiu F, Erba N, Veschi F *et al.* Survey of lupus anticoagulant diagnosis by central evaluation of positive plasma samples. *J Thromb Haemost* 2007; 5:925-930.
29. Girardi G, Redecha P, Salmon JE. Heparin prevents antiphospholipid antibody-induced fetal loss by inhibiting complement activation. *Nat Med* 2004; 10:1222-1226.
30. Billingham RE, Brent I, Medawar PB. Actively acquired tolerance of foreign cells. *Nature* 1953; 172:603-606.
31. Berman J, Girardi G, Salmon JE. *tnf-alpha* is a critical effector and a target for therapy in antiphospholipid antibody-induced pregnancy loss. *J Immunol* 2005; 174:485-490.

CHAPTER IV

**PREECLAMPSIA IS
CHARACTERIZED BY
PLACENTAL COMPLEMENT
DYSREGULATION**

ALETTA BUURMA • DANIELLE COHEN • KIMBERLEY VERAAR
DORRITH SCHONKEREN • FRANS CLAAS • JAN ANTHONIE BRUIJN
KITTY BLOEMENKAMP • HANS BAELEDE

HYPERTENSION

Abstract

BACKGROUND Increasing evidence suggests that preeclampsia is associated with complement dysregulation. The origin of complement dysregulation in preeclampsia is unknown, and further unraveling this mechanism could provide both diagnostic tools and therapeutic targets. Because the placenta is believed to play a crucial role in the pathogenesis of preeclampsia, we investigated placentas from preeclamptic women (n=28) and controls (n=44) for the presence of complement activation products.

METHODS Immunohistochemistry was performed for C1q, mannose-binding lectin, properdin and C4d. Staining patterns were related to pregnancy outcome. Possible causes of complement activation were investigated, including the presence of immune deposits at the syncytiotrophoblast and changes in the placental mRNA expression of complement regulatory proteins.

RESULTS C4d was rarely present in placentas from healthy controls (3%), whereas it was observed in 50% of placentas obtained from preeclamptic women ($p=0.001$). In these placentas, C4d was observed in a focal (9/14) or diffuse (5/14) staining pattern at the syncytiotrophoblast. With respect to C1q, mannose-binding lectin and properdin, no differences were observed between cases and controls. In preeclamptic women, diffuse placental C4d was associated with a significantly lower gestational age at delivery. Furthermore, the mRNA expression of the complement regulatory proteins CD55 and CD59 was significantly upregulated in preeclampsia.

CONCLUSIONS In conclusion, there is evidence for increased classical pathway activation and altered complement regulation in preeclampsia. The relation between C4d and lower gestational age at birth suggests that the extent of complement dysregulation is associated with the severity of preeclampsia. Inhibiting excessive complement activation may be a promising therapeutic approach in the management of preeclampsia.

Introduction

Preeclampsia (PE) is a devastating pregnancy-specific syndrome, complicating 2 to 8% of pregnancies and contributing significantly to maternal and fetal morbidity and mortality.¹ The syndrome is characterized by endothelial dysfunction, presenting clinically as maternal hypertension and proteinuria after 20 weeks of gestation.²

Since the late 1980s, it became apparent that PE is relatively common among pregnant women with autoimmune diseases such as systemic lupus erythematosus (SLE) and the antiphospholipid syndrome (APS).^{3,4} Antiphospholipid antibodies can directly bind to trophoblast and activate the classical complement cascade, causing placental dysfunction, trophoblast-injury and impaired pregnancy outcome.^{5,6} In human placentas of SLE and APS patients, activation of the classical pathway of complement at the fetal-maternal interface can be detected in up to 70% of patients and is related to intra-uterine fetal loss and PE.⁷

The association between PE and SLE/ APS, combined with the observation that complement activation mediates pregnancy complications in these autoimmune diseases, suggests that the complement system could also be involved in the pathogenesis of PE in women without underlying autoimmune diseases. Indeed, preeclamptic women have elevated levels of circulating complement activation products and increased quantities of complement components in their placentas.⁸⁻¹⁰ Additionally, mutations in complement regulatory proteins predispose to PE.¹¹ Altogether, there is increasing evidence indicating that dysregulation of the complement system might play a role in the development of PE. However, it is unknown what triggers complement activation at the fetal-maternal interface and which of the complement pathways is activated during PE. More insight in this mechanism is essential in order to develop better prevention and treatment strategies aimed at the immunological aspects of this serious pregnancy complication.

The placenta is believed to play a crucial role in the pathogenesis

of PE. Therefore, we studied complement activation in placentas obtained from preeclamptic women and control subjects, using markers covering all complement activation-pathways. Furthermore, possible associations between placental complement activation and the clinical manifestations of PE were investigated, as well as possible causes of placental complement dysregulation, including changes in the mRNA expression of complement regulatory proteins and the presence of immune deposits at the fetal-maternal interface.

Methods

PATIENT SELECTION AND PLACENTA COLLECTION We studied 72 placentas obtained from women who delivered at the Department of Obstetrics of the Leiden University Medical Center between 2002 and 2011. For the case group, 28 placentas were obtained from women with PE.¹² This group contained several women with superimposed PE (n=8).¹²

For the first control group we included 30 placentas obtained from healthy women with uncomplicated pregnancies that resulted in live births. As a second control group, placentas were obtained from women whose pregnancy was affected by intra-uterine growth restriction (IUGR, defined as birth weight below fifth percentile for gestational age) but not by PE. All preeclamptic women in this study delivered by cesarean section. As it cannot be excluded that lengthy delivery may affect the extent and distribution of complement deposits, we aimed to mainly include controls who also delivered by cesarean section (63%). Detailed information on patient characteristics in each group can be found in Table 1.

Tissue samples from a central part of the placenta were collected immediately after delivery. The study was approved by the ethics committee of the Leiden University Medical Center, and informed consent was obtained from all patients.

IMMUNOHISTOCHEMISTRY To investigate complement activation, immunohistochemistry was performed for the following complement components: C4d (a component of both the classical and mannose-binding lectin [MBL] pathway), C1q (specific for classical pathway activation), MBL (representing mannose-binding lectin pathway activation), properdin (specific for alternative pathway activation), C3d, and the neo-antigen of the membrane attack complex or MAC (can both be formed by activation of any of the three pathways). Sections were deparaffinized and antigen retrieval was performed. After blocking for endogenous peroxidase, sections were incubated with antibodies to C1q (DakoCytomation, Denmark, 1:800), C4d (Biomedica Gruppe, Austria, 1:50), MBL (Sigma-Aldrich Biotechnology, 1:500), properdin (1:800), C3d (Abcam, 1:800) and SC5b-9 (Quidel, San Diego, CA, 1:150). Binding of the primary antibody was visualized with appropriate secondary antibodies and diaminobenzidine as a chromogen. Sections were counterstained with Haematoxylin. The IUGR group was included to determine how specific C4d is for PE. Therefore, these placentas were only stained for C4d.

IMMUNOFLUORESCENCE To investigate the presence of immune deposits at the syncytiotrophoblast, double immunofluorescent staining was performed to identify cytokeratin-7 (KRT7, specifically staining the syncytiotrophoblast) concomitant with human immunoglobulins. Frozen sections of placenta were washed and fixed in a mixture of acetone and alcohol. Sections were incubated for 1 hour with rabbit anti-human IgG/IgM/IgA (DakoCytomation, Denmark, 1:100) and mouse anti-human KRT7 (Dako Cytomation, Denmark, 1:100). Subsequently, sections were incubated for 30 minutes with FITC-labeled goat anti-rabbit (Sigma-Aldrich Biotechnology, 1:200) and TRITC-labeled goat anti-mouse (Sigma-Aldrich Biotechnology, 1:100) antibodies. For negative controls, the primary antibodies were substituted with normal mouse serum (DakoCytomation, Denmark) and normal rabbit immunoglobulin

(DakoCytomation, Denmark) in the same concentrations as the primary antibodies. When immune deposits were observed in the combined IgA/IgM/IgG staining, immunofluorescence was performed separately for IgA, IgM and IgG to determine which isotype was predominantly present.

QUANTIFICATION OF IMMUNOHISTOCHEMICAL

AND IMMUNOFLOUORESCENT STAINING Positivity for immunohistochemical staining was scored semi-quantitatively by two independent observers. The staining intensity at the surface of the syncytiotrophoblast was scored as absent (<10%), focal (10-50%) or diffuse (>50%). For immunofluorescence, slides were blindly scored by two independent observers for the absence or presence of immune deposits on the syncytiotrophoblast using a fluorescent microscope (DM5500B, Leica Instruments). When immune deposits were observed, we scored which immunoglobulin isotype was most prominently present.

RNA EXTRACTION AND QUANTITATIVE PCR ANALYSIS

Quantitative PCR was performed to quantify the placental mRNA expression of the membrane-associated complement regulatory proteins CD55 (Decay Accelerating Factor), CD46 (Membrane Cofactor Protein) and CD59. RNA was isolated and reversed to cDNA using an AMV cDNA synthesis kit (Roche, Indianapolis, IN). SYBR Green quantitative PCR was performed according to the manufacturer's protocol. Expression of complement regulatory proteins was measured by the comparative threshold cycle method and normalized to hypoxanthinephosphoribosyltransferase (HPRT) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression. The primer pairs (Table S1) were designed to span at least one intron to avoid amplification of genomic DNA along with cDNA. To verify the accuracy of amplification, a melting curve analysis was performed. All cDNA samples were treated in duplicate.

STATISTICAL ANALYSIS Categorical variables were compared using either the Chi-square test or the Fisher exact test. Differences in quantitative parameters between groups were assessed using one-way ANOVA (for data normally distributed) or Kruskal-Wallis H one-way analysis (for data not normally distributed). Differences in means were compared using either the unpaired t-test or the Mann-Whitney U test. All analyses were performed using SPSS statistical software package (version 17.0; Chicago, IL). A p -value < 0.05 was considered statistically significant.

Results

CLINICAL DATA Maternal ages were comparable between PE and control subjects. As expected, blood pressure and proteinuria were significantly higher in the PE group. Additionally, the average gestational age at delivery, birth weight and placenta weight were significantly lower as compared to the controls. These and additional clinical characteristics are provided in Table 1.

IMMUNOHISTOCHEMISTRY C4d was observed in 14 (50%) out of 28 placentas obtained from women with PE, whereas it was rarely present in placentas from healthy subjects (1 out of 30, 3%). In the IUGR group, 3 (21%) out of 14 subjects showed placental C4d. Of these 3 women, 2 suffered from gestational hypertension. When present, C4d was observed at the syncytiotrophoblast in either a focal or a diffuse staining pattern (Figure 1). Table 2 shows the incidence of the different C4d staining patterns; this is visualized in Figure 2. Chi-squared analysis indicated a strong association between C4d and PE ($p=0.001$). No relation was observed between the moment of onset of preeclampsia and placental C4d staining patterns. Among women with superimposed PE ($n=8$), three did not show placental C4d, four had focal and one had diffuse placental C4d.

C1q was observed at the syncytiotrophoblast and in intravillous endothelial cells (Figure 1). C1q was never completely negative, neither in cases nor in control subjects (data not shown). In cases of diffuse C4d, C1q and C4d co-localized. Properdin was not observed at the syncytiotrophoblast but occasionally in intravillous endothelial cells (Figure 1), both in cases and healthy subjects (no statistically significant difference, data not shown). When subdividing women with PE according to their C4d staining pattern, no differences were observed in the amount and distribution of properdin deposits. MBL was absent in all placentas, whereas liver tissue that served as a positive control was clearly positive. C3d was frequently observed at the syncytiotrophoblast in a focal staining pattern. However, no relation was observed between the presence of C4d and the presence of C3d and no significant differences were observed between cases and controls (data not shown). Staining for MAC was observed in areas of villous injury. It was rarely observed at the fetal-maternal interface, and as a consequence, co-localization with C4d was infrequent. Images of C3d and MAC staining are provided in Figure S1.

IMMUNOFLUORESCENCE In placentas obtained from preeclamptic women, immune deposits were observed at the syncytiotrophoblast in 10 out of 27 placentas. Staining for KRT7 confirmed that immune deposits were located at the syncytiotrophoblast (Figure 3). In 23% of the placentas with no or minimal C4d (n=13), immune deposits were present. Of the placentas with focal C4d (n=9), 44% showed immune deposits. Placentas with diffuse C4d (n=5) showed immune deposits in 60%. Although a trend was observed between the presence of immune deposits and C4d, this was not statistically significant (Figure 4). In most placentas that showed immune deposits, the predominant isotype was IgM (Figure S2 and Table S2).

PLACENTAL MRNA EXPRESSION OF COMPLEMENT

REGULATORY PROTEINS The mRNA levels of CD59 were on average 4-fold increased in preeclamptic women as compared to controls ($p<0.01$). The placental mRNA expression of CD55 was also significantly higher in preeclamptic women, with on average a nearly 2-fold increase as compared to healthy subjects ($p<0.05$). The mRNA expression levels of CD46 were comparable between groups. The relative mRNA expression levels are illustrated in Figure 5.

RELATION BETWEEN C4d AND CLINICAL MANIFESTATIONS

The different C4d staining patterns were not associated with the height of blood pressure or the amount of urinary protein (Table S3). Within the PE group, diffuse placental C4d was associated with a significantly ($p=0.03$) lower gestational age at delivery, as compared to cases with focal or no C4d (Figure 6). All preeclamptic women delivered by cesarian section. Therefore, gestational age depended on a clinical decision rather than on a spontaneous onset of labor. Overall, no relationships were observed between the indications to end pregnancy and C4d staining patterns. Among preeclamptic women, diffuse C4d was also associated with lower birth weight ($p=0.04$). However, with respect to birth weight percentiles, differences between groups were not statistically significant.

Discussion

Increasing evidence suggests that PE is associated with complement activation but it remains unknown what triggers complement activation during PE and which complement pathways are involved. This study demonstrates that classical complement activation is present in placentas from women with PE. C4d, the most important marker of classical complement activation, is present in placentas from a substantial subset of women with PE whereas it was observed in only one out of 30 placentas obtained from healthy subjects. In

IUGR placentas, C4d was almost exclusively found in cases which were also affected by gestational hypertension. No differences between PE patients and healthy subjects were observed with respect to the intensity and distribution of C1q, MBL and properdin. Furthermore, this study demonstrates that PE is associated with a significantly higher placental mRNA expression of the complement regulatory proteins CD59 and CD55. In conclusion, placental classical pathway activation may be a novel diagnostic tool and therapeutic target in PE.

In the present study, C4d was observed at the fetal-maternal interface in approximately half of the placentas obtained from women with PE. In contrast, C4d was rarely present in control placentas. C4d appears to be relatively specific for hypertensive disorders of pregnancy, as it was infrequently observed in placentas from normotensive women with IUGR (1/11). When it was observed in IUGR placentas, these pregnancies were often (2/3) also complicated by gestational hypertension. As C4d is a component of both the classical and mannose-binding lectin pathway, we investigated the presence of C1q and MBL in order to determine which pathway is responsible for C4d deposits. MBL was never observed, making it very unlikely that C4d deposits are a result of mannose-binding lectin pathway activation. In accordance with previous reports, C1q was observed at the syncytiotrophoblast both in physiological and pathological pregnancies.^{7,13} When C4d was present in a diffuse staining pattern, it co-localized with C1q, indicating that C4d deposits are most likely the result of classical pathway activation.

Evidence of classical complement activation in a subset of women with PE raises several questions. First, one may wonder why not all preeclamptic women develop placental complement deposits. PE is considered a complex disease, in which many different variables affect the risk to develop the disorder. Consequently, attempts have been made to subdivide cases of PE into different phenotypical and etiological subgroups.² Although speculative, women with placental C4d may represent such a specific etiological subgroup, which

raises the question: what causes classical pathway activation in these placentas?

Generally, complement deposits can be the consequence of either excessive activation or inadequate regulation of the complement system. In the case of PE, classical pathway activation could result from the binding of circulating antibodies, comparable to pregnancy complications in patients with APS, in which antiphospholipid antibodies bind to the syncytiotrophoblast and thereby activate the classical pathway.^{5,6} However, classical pathway activation is not exclusively triggered by immune complexes; it can also be induced by binding of C1q to apoptotic cells.¹⁴ In the current study, a trend was observed between placental C4d and the presence of immune deposits. This difference was not statistically significant. However, our groups were probably too small to reach statistical significance and therefore, the difference could be statistically significant when investigating larger samples. Importantly, in these immune deposits, IgM was the predominant isotype. IgM is known to bind to damaged tissue in the setting of ischemia-reperfusion injury, thereby activating the complement system.¹⁵ Therefore, the predominant presence of IgM suggests that immune deposits on the syncytiotrophoblast may represent a non-specific reaction to injury, rather than a specific antibody-mediated response against fetal antigens.

Because mutations in complement regulatory proteins predispose to PE¹¹, apparently not only excessive activation but also inadequate inhibition of the complement system may be involved in the development of PE. Deficient complement inhibition could possibly explain why in controls the classical pathway stops at the level of C1q, while in a subset of women with PE the classical pathway progresses beyond this level, resulting in the deposition of C4d. Because both C1 inhibitor and Factor H can inhibit classical pathway activation, a (relative) shortage of these complement inhibitors could be responsible for classical pathway activation beyond the level of C1q.^{16;17} To investigate whether placental complement deposits in case of PE could be due to insufficient local regulation, we measured

the placental mRNA expression of the complement regulatory proteins that are widely expressed within the placenta. A significant upregulation of CD55 and CD59 mRNA expression levels was observed. This suggests the presence of a fetal feedback mechanism to maintain trophoblast integrity in the face of complement activation. The importance of complement regulation at the fetal-maternal interface is confirmed by our finding that C4d infrequently co-localized with MAC. This finding suggests that within the placenta, regulatory mechanisms prevent downstream activation of the complement system. This is in line with the observed upregulation of CD59 mRNA, which may prevent the formation of the MAC on the syncytiotrophoblast. Importantly, no relationships were observed between the presence of placental C4d and C3d deposits. Furthermore, the amount of placental C3d was not increased in PE as compared to healthy control subjects. Within the complement system, C3 is downstream to C4d. One may speculate that within the placenta, complement regulatory mechanisms prevent progression of complement activation beyond the level of C4d.

Clinically, the presence of placental C4d in a diffuse staining pattern was associated with a significantly lower mean gestational age (27+1 weeks) at delivery. Because all PE pregnancies were ended by cesarian section, the initiation of birth was based on a clinical decision rather than on a spontaneous onset of labor. Apparently, in the cases with diffuse C4d, the fetal or maternal condition was severely threatened and did not allow for longer expectant management. This excessive placental complement activation consequently provides a lead for therapeutic intervention. Indeed, murine models have shown that complement inhibition is effective in abrogating PE manifestations^{18;19}. Additionally, heparin, which is known to inhibit complement activation²⁰, is effective in preventing recurrent early-onset PE in women with inherited thrombophilia²¹.

In conclusion, we have demonstrated that PE is associated with the presence of placental C4d and with an upregulation of the mRNA expression of complement regulatory proteins. Placental

C4d deposits are likely a result of excessive classical complement activation. Importantly, the presence of placental C4d is associated with the severity of PE. Altogether, the current data suggest that the complement system plays an essential role in the pathogenesis of PE and may be a novel therapeutic target in the management of PE.

FIG 1 **DIFFERENT IMMUNOHISTOCHEMICAL STAININGS**
(full colour version inside cover)

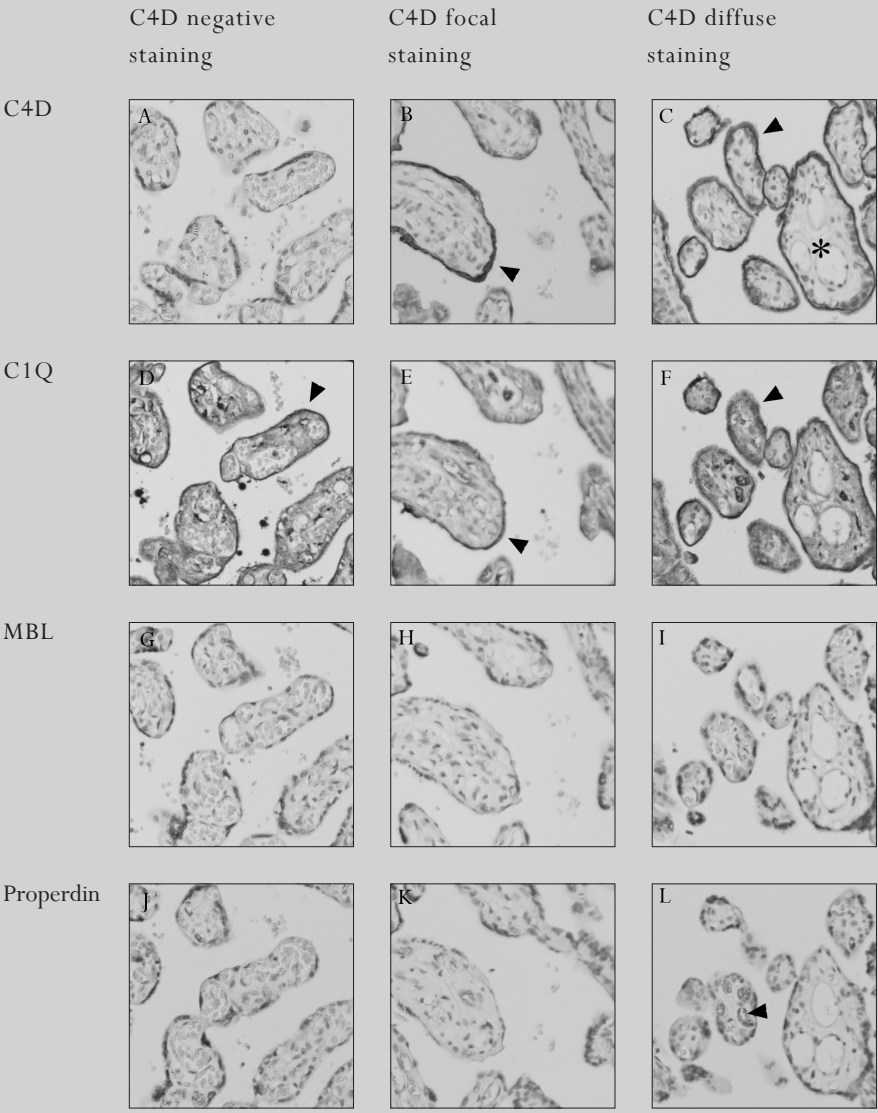


Figure 1 shows sequential sections of placentas stained immunohistochemically for C4d, C1q, MBL and properdin. Each column represents an individual placenta, the immunohistochemical stainings are shown horizontally. The first column shows a C4d-negative placenta obtained from a woman whose pregnancy was uncomplicated. The middle column shows a placenta obtained from a woman with PE, showing a focal C4d staining pattern. In the third column, a placenta of a patient with PE is shown, which is diffusely positive for C4d.

Panels A to C show the different C4d staining patterns. B and C illustrate that C4d is present at the surface of the syncytiotrophoblast (arrowheads), and not within villi (*).

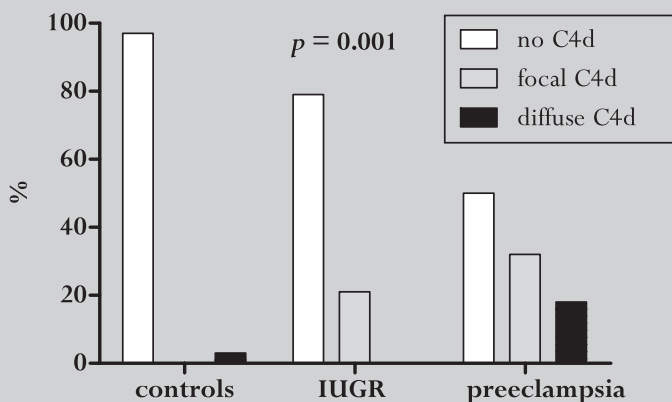
Panels D to F show examples of placental C1q staining. C1q was not exclusively present in C4d-positive placentas but also in placentas in which C4d was absent. This phenomenon is illustrated in D, which shows a C4d-negative placenta that is evidently C1q positive (arrowhead). However, E and F demonstrate that C1q (arrowheads) does co-localize with C4d in C4d-positive placentas.

Panels G to I show that MBL was never observed.

Panels J to L show typical examples of properdin staining, demonstrating that properdin is exclusively localized on endothelial cells of the fetal vessels (arrowhead).

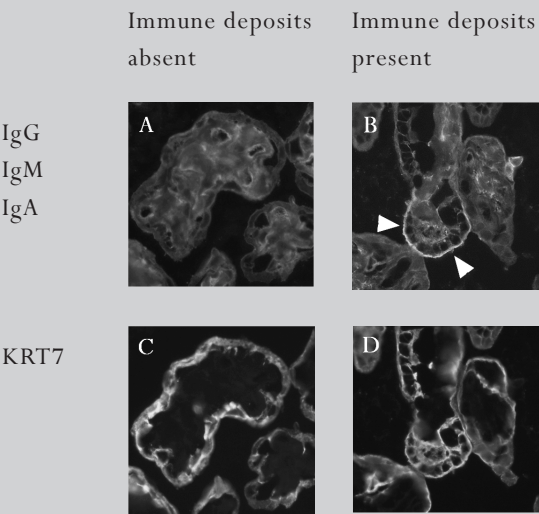
THE ASSOCIATION BETWEEN C4D AND PREECLAMPSIA

FIG 2



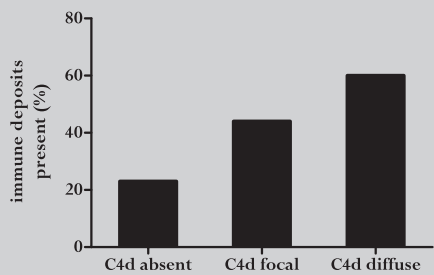
The figure illustrates the abundance of C4d in the PE group as compared to the control groups ($p = 0.001$).

FIG 3 **IMMUNOFLUORESCENT STAINING PATTERNS**
(full colour version inside cover)



Typical immunofluorescent staining patterns of immunoglobulins and cytokeratin-7 (KRT7) in preeclamptic placentas. Each column represents an individual placenta; the different immunofluorescent stainings are shown horizontally. A and B show immunoglobulin staining patterns. Staining was scored as ‘absence of immunoglobulins on syncytiotrophoblast’ (A) or ‘presence of immunoglobulins on syncytiotrophoblast’ (B, see arrowheads). C and D show KRT7 staining, demonstrating the syncytiotrophoblast.

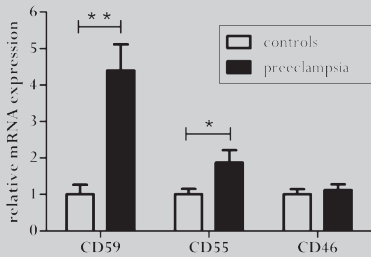
FIG 4 **INCIDENCE OF IMMUNE DEPOSITS**



Incidence of immune deposits at the syncytiotrophoblast in preeclamptic women with no, focal or diffuse placental C4d.

MRNA EXPRESSION OF COMPLEMENT REGULATORY PROTEINS

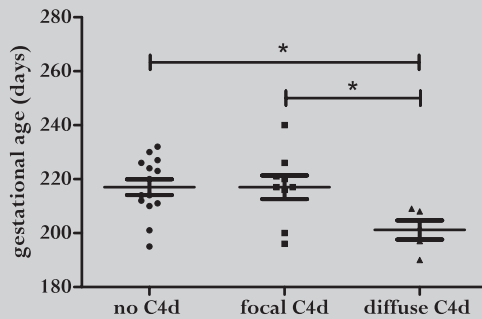
FIG 5



Relative placental mRNA expression levels of the different complement regulatory proteins in cases versus controls, expressed as mean $\pm$ SEM. *p < 0.05 and **p < 0.01.

GESTATIONAL AGE IN PREECLAMPTIC WOMEN

FIG 6



The figure shows the gestational age (in days) in preeclamptic women with no, focal or diffuse placental C4d. *p < 0.05.

TABLE 1 PATIENT CHARACTERISTICS

CHARACTERISTICS	PRE- ECLAMPSIA (N=28)	HEALTHY CONTROLS (N=30)	IUGR CONTROLS (N=14)
Mean maternal age in years (SD)	31.6 (5.9)	33.9 (4.0)	32.7 (6.5)
Mean maternal BMI (kg/m ²) (SD)	26.8 (7.1)	25.3 (4.3)	24.3 (5.3)
Mean gravidity (SD)	2.1 (1.9)*	2.9 (1.2)	1.7 (0.7)*
Mean parity (SD)	0.8 (1.2)*	1.3 (1.0)	0.6 (0.8)*
Highest diastole (mmHg) (SD)	107.5 (13.9)* †	76.4 (5.1)	81.1 (9.2)
Proteinuria (g/24h) (SD)	5.2 (5.1)* †	0.0 (0.0)	0.0 (0.0)
Gestational age at delivery (weeks + days; SD in days)	30 + 4 (9)* †	39 + 4 (12)	35 + 5 (33)*
Birth weight (g) (SD)	1167 (320)* †	3609 (429)	1782 (769)*
Birth weight percentile ‡			
<5 (%)	4 (14.3)	1 (3.3)	14 (100)
5-10 (%)	2 (7.1)	1 (3.3)	0 (0)
10-20 (%)	8 (28.6)	0 (0)	0 (0)
20-50 (%)	9 (32.1)	10 (33.3)	0 (0)
50-80 (%)	5 (17.9)	12 (40.0)	0 (0)
>80 (%)	0 (0)	6 (20.0)	0 (0)
Placenta weight (g) (SD)	261 (90)*	646 (137)	304 (118)*
Mode of delivery ‡			
Caesarean section (%)	28 (100)	19 (63.3)	6 (42.9)
Vaginal delivery (%)	0 (0)	11 (36.7)	8 (57.1)
Complications			
HELLP syndrome (%) ‡	5 (18)	0 (0)	0 (0)
Eclampsia (%)	3 (11)	0 (0)	0 (0)
Comorbidity			
Pre-existent hypertension (%) ‡	8 (27)	0 (0)	1 (7)
Type 1 diabetes (%)	1 (4)	0 (0)	0 (0)
Inherited thrombophilia (%)	2 (7)	1 (3)	1 (7)

* denotes a statistically significant difference compared to the healthy controls

† denotes a statistically significant difference when compared to the IUGR controls

‡ denotes a statistically significant in the overall comparison (Chi-square)

C4D IN PE PLACENTAS VERSUS CONTROLS ***TABLE 2**

C4D STAINING PATTERN	PREECLAMPSIA (N = 28)	HEALTHY CONTROLS (N = 30)	IUGR CONTROLS (N = 14)
No C4d (%)	14 (50)	29 (97)	11 (79)
Focal C4d (%)	9 (32)	0 (0)	3 (21)
Diffuse C4d (%)	5 (18)	1 (3)	0 (0)

* Chi-squared test (overall comparison): $p = 0.001$

TABLE S1 PRIMERS USED FOR QUANTITATIVE PCR

GENE	PRIMER SEQUENCES (FORWARD, REVERSE)	PRODUCT SIZE
CD46	F: TTCCTGGCGCTTTCCTGGGT R: GTTGGTGGCTCCTCACAGGC	84
CD55	F: TCCTGGCGAGAAGGACTCAGTGA R: AGCCTTGTTGGCACCTCGCA	96
CD59	F: TGTCCCTTCCTTCCAGGTTCTGT R: TGACGGCTGTTTTCAGTCAGC	161
HPRT	F: AGATGGTCAAGGTCGCAAGC R: TCAAGGGCATATCCTACAACAAAC	115
GAPDH	F: TTCCAGGAGCGAGATCCCT R: CACCCATGACGAACATGGG	175

**TABLE S2 PREDOMINANT ISOTYPE IN PLACENTAL
IMMUNOGLOBULIN DEPOSITS**

PREDOMINANT ISOTYPE	NUMBER OF PLACENTAS
IgM (%)	9 (90)
IgA (%)	1 (10)
IgG (%)	0 (0)

**CLINICAL CHARACTERISTICS OF WOMEN WITH
PREECLAMPSIA AND NO, FOCAL OR DIFFUSE PLACENTAL C4D
DEPOSITS**

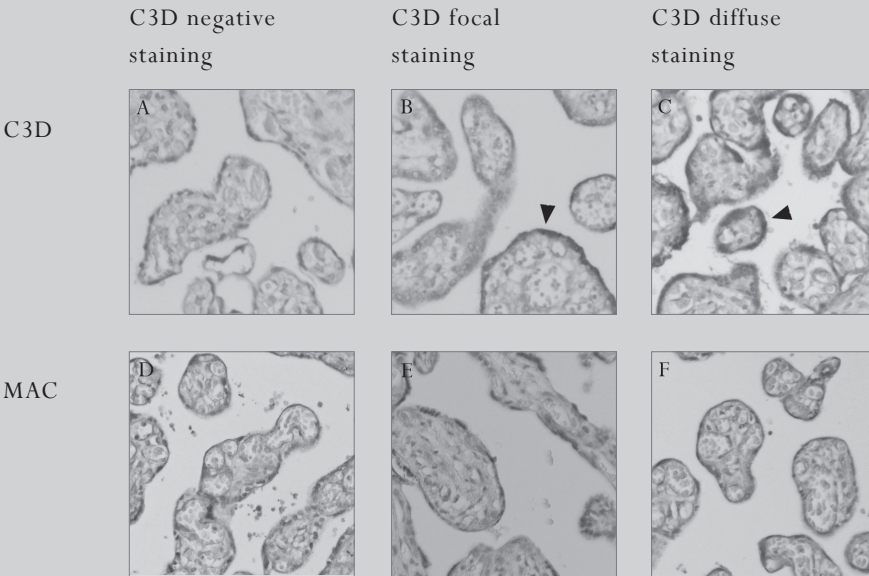
TABLE S1

CHARACTERISTICS	C4D ABSENT (N = 14)	FOCAL C4D (N = 9)	DIFFUSE C4D (N = 5)
Mean maternal age in years (SD)	32.2 (5)	32.2 (4)	28.8 (9.7)
Mean maternal BMI (kg/m ²) (SD)	27.7 (5.9)	24.0 (11)	28.1 (6.2)
Mean gravidity (SD)	1.9 (1.2)	2.1 (1.6)	2.8 (3.5)
Mean parity (SD)	0.6 (1.1)	0.8 (0.8)	1.0 (2.2)
Highest diastole (mmHg) (SD)	110 (17)	104 (10)	106 (8)
Proteinuria (g/24h) (SD)	6.5 (5.8)	3.5 (3.8)	4.6 (5.3)
Mean gestational age at delivery (weeks + days; SD in days)	31+0 (11)	31+0 (13)	27+1 (8) *†
Birth weight (g) (SD)	1278 (296)	1150 (326)	887 (226) *
Birth weight percentile			
<5 (%)	0 (0)	3 (33.3)	1 (20.0)
5-10 (%)	1 (7.1)	1 (11.1)	0 (0)
10-20 (%)	5 (35.7)	1 (11.1)	2 (40.0)
20-50 (%)	5 (35.7)	2 (22.2)	2 (40.0)
50-80 (%)	3 (21.4)	2 (22.2)	0 (0)
Gender			
Female (%)	9 (64)	4 (44)	1 (20)
Male (%)	5 (36)	5 (56)	4 (80)
Indication to end pregnancy			
Fetal	5 (36)	3 (33)	3 (60)
Maternal	6 (43)	4 (44)	1 (20)
Combination	3 (21)	2 (22)	1 (20)

* denotes a statistically significant difference when compared with C4d absent

† denotes a statistically significant difference when compared with focal C4d

FIG S1 **EXAMPLE IMAGES OF C3D AND MAC STAINING PATTERNS**
(full colour version inside cover)



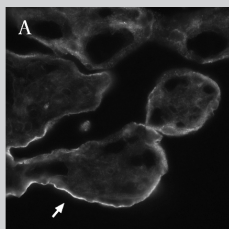
A to C show C3d staining patterns. In case C3d was present, it was observed on the syncytiotrophoblast (arrowheads). This observation suggests that placental complement activation progresses beyond the level of C4d. D to F show typical MAC staining patterns. MAC was rarely observed on the syncytiotrophoblast. Therefore, co-localization with C4d was infrequent. This observation indicates that classical pathway activation does not necessarily result in activation of the entire complement cascade, confirming that complement regulatory mechanisms may prevent placental complement-mediated damage.

IMAGES OF SEPARATE IGM, IGA AND IGG STAINING ON PLACENTAS OBTAINED FROM WOMEN WITH PREECLAMPSIA

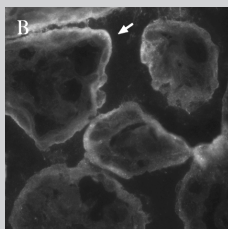
FIG S2

(full colour version inside cover)

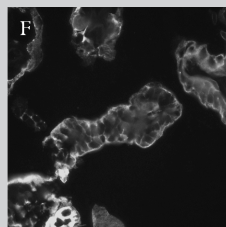
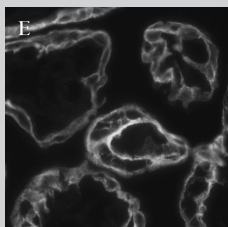
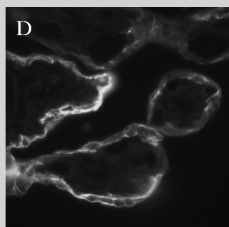
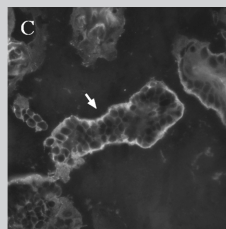
IgM



IgA



IgG



KRT7

The upper row shows the different immunoglobulin stainings. Arrows indicate immunoglobulin deposits at the syncytiotrophoblast. The lower row demonstrates cytokeratin-7 staining, indicating the location of the syncytiotrophoblast. Magnification 400x.

REFERENCES

1. Khan KS, Wojdyla D, Say L, Gulmezoglu AM, Van Look PF. WHO analysis of causes of maternal death: a systematic review. *Lancet*. 2006;367:1066-1074.
2. Steegers EA, von Dadelszen P, Duvekot JJ, Pijnenborg R. Pre-eclampsia. *Lancet*. 2010;376:631-644.
3. Cohen D, Berger SP, Steup-Beekman GM, Bloemenkamp KW, Bajema IM. Diagnosis and management of the antiphospholipid syndrome. *BMJ*. 2010;340:c2541.
4. Duckitt K, Harrington D. Risk factors for pre-eclampsia at antenatal booking: systematic review of controlled studies. *BMJ*. 2005;330:565.
5. Girardi G, Berman J, Redecha P, Spruce L, Thurman JM, Kraus D *et al*. Complement C5a receptors and neutrophils mediate fetal injury in the antiphospholipid syndrome. *J Clin Invest*. 2003;112:1644-1654.
6. Holers VM, Girardi G, Mo L, Guthridge JM, Molina H, Pierangeli SS *et al*. Complement C3 activation is required for antiphospholipid antibody-induced fetal loss. *J Exp Med*. 2002;195:211-220.
7. Cohen D, Buurma A, Goemaere NN, Girardi G, le Cessie S, Scherjon S, *et al*. Classical complement activation as a footprint for murine and human antiphospholipid antibody-induced fetal loss. *J Pathol*. 2011;225:502-511.
8. Lynch AM, Murphy JR, Byers T, Gibbs RS, Neville MC, Giclas PC *et al*. Alternative complement pathway activation fragment Bb in early pregnancy as a predictor of preeclampsia. *Am J Obstet Gynecol*. 2008;198:385-389.
9. Sinha D, Wells M, Faulk WP. Immunological studies of human placen-
tae: complement components in pre-eclamptic chorionic villi. *Clin Exp Immunol*. 1984;56:175-184.
10. Tedesco F, Radillo O, Candussi G, Nazzaro A, Mollnes TE, Pecorari D. Immunohistochemical detection of terminal complement complex and S protein in normal and pre-eclamptic placentae. *Clin Exp Immunol*. 1990;80:236-240.
11. Salmon JE, Heuser C, Triebwasser M, Liszewski MK, Kavanagh D, Roumenina L *et al*. Mutations in complement regulatory proteins predispose to preeclampsia: a genetic analysis of the PROMISSE cohort. *PLoS Med*. 2011;8:e1001013.
12. Brown MA, Lindheimer MD, de Swiet, Van Assche A, Moutquin JM. The classification and diagnosis of the hypertensive disorders of pregnancy: statement from the International Society for the Study of Hypertension in Pregnancy (ISSHP). *Hypertens Pregnancy*. 2001;20:IX-XIV.
13. Bulla R, Agostinis C, Bossi F, Rizzi L, Debeus A, Tripodo C *et al*. Decidual endothelial cells express surface-bound C1q as a molecular bridge between endovascular trophoblast and decidual endothelium. *Mol Immunol*. 2008;45:2629-2640.
14. Nauta AJ, Trouw LA, Daha MR, Tijssma O, Nieuwland R, Schwaebble WJ *et al*. Direct binding of C1q to apoptotic cells and cell blebs induces complement activation. *Eur J Immunol*. 2002;32:1726-1736.
15. Weiser MR, Williams JP, Moore FD, Jr., Kobzik L, Ma M, Hechtman HB *et al*. Reperfusion injury of ischemic skeletal muscle is mediated by natural antibody and complement. *J Exp Med*. 1996;183:2343-2348.

16. Davis AE, III, Lu F, Mejia P. C1 inhibitor, a multi-functional serine protease inhibitor. *Thromb Haemost.* 2010;104:886-893.
17. Kishore U, Sim RB. Factor H as a regulator of the classical pathway activation. *Immunobiology.* 2012;217:162-168.
18. Girardi G, Yarilin D, Thurman JM, Holers VM, Salmon JE. Complement activation induces dysregulation of angiogenic factors and causes fetal rejection and growth restriction. *J Exp Med.* 2006;203:2165-2175.
19. Qing X, Redecha PB, Burmeister MA, Tomlinson S, D'Agati VD, Davisson RL *et al.* Targeted inhibition of complement activation prevents features of preeclampsia in mice. *Kidney Int.* 2011;79:331-339.
20. Girardi G, Redecha P, Salmon JE. Heparin prevents antiphospholipid antibody-induced fetal loss by inhibiting complement activation. *Nat Med.* 2004;10:1222-1226.
21. de Vries JI, van Pampus MG, Hague WM, Bezemer PD, Joosten JH. Low-Molecular-Weight Heparin Added to Aspirin in the Prevention of Recurrent Early-Onset Preeclampsia in women with Inheritable Thrombophilia: the FRUIT-RCT. *J Thromb Haemost.* 2012;10:64-72.

CHAPTER V

PLACENTAL COMPLEMENT ACTIVATION IS RELATED TO PLACENTAL DYSFUNCTION IN THE SETTING OF PREECLAMPSIA

ALETTA BUURMA • KIMBERLEY VERAAR • DANIELLE COHEN
NATASCHA GOEMAERE • JAN ANTHONIE BRUIJN
KITTY BLOEMENKAMP • HANS BAELE

Abstract

INTRODUCTION Increasing evidence suggests that the complement system is involved in the pathogenesis of preeclampsia. We have recently demonstrated a strong association between preeclampsia and placental complement activation. However, it remains unknown whether placental complement activation in the setting of preeclampsia is related to disturbed placental function and the different related clinical manifestations.

METHODS We collected 111 placentas from women whose pregnancy was complicated by preeclampsia. Placentas were stained immunohistochemically for C4d, a stable tissue marker for complement activation. C4d staining patterns were related to pregnancy outcomes and the clinical and histopathological manifestations of preeclampsia.

RESULTS C4d was absent in 43.3%, present in a focal staining pattern in 36.0% and in a diffuse staining pattern in 20.7%. The presence of diffuse C4d was associated with a significantly lower mean gestational age at delivery ($p < 0.05$). Importantly, the presence of placental C4d was also associated with intra-uterine fetal death ($p < 0.01$), significantly lower placenta weight ($p < 0.05$), and various histopathological manifestations of preeclampsia, including placental infarction ($p < 0.01$), increased syncytial knots ($p < 0.01$) and deciduitis ($p < 0.01$).

DISCUSSION The current study confirms that preeclampsia is frequently associated with placental C4d deposits. Importantly, a relation between preeclampsia-associated intra-uterine fetal death and placental C4d was established. The presence of placental C4d was also associated with significantly smaller placentas and with hallmarks of reduced placental perfusion, suggesting that complement activation is related to placental dysfunction in the setting of preeclampsia.

Introduction

Preeclampsia is a devastating pregnancy complication that is characterized—in its simplest form—by new-onset hypertension and proteinuria after 20 weeks of gestation. Maternal health can be further threatened by seizures (eclampsia) and the HELLP syndrome (hemolysis, elevated liver enzymes, and low platelets)¹, whereas fetal outcomes are frequently compromised by intra-uterine growth restriction, placental abruption and occasionally even intra-uterine fetal death.² Importantly, preeclampsia is associated with a risk for recurrence in subsequent pregnancies, particularly in women with severe, early-onset preeclampsia in their initial pregnancy.³ Preeclampsia remains a leading cause of maternal and perinatal mortality and morbidity, as no therapy is available other than (iatrogenic) delivery.⁴

Although the etiology of preeclampsia is probably as heterogeneous as its presentation, several lines of evidence suggest that the complement system is involved in the pathogenesis of preeclampsia. The complement system is an ancient part of innate immunity that helps to eradicate invading pathogens. However, the system can also damage host tissues if excessively activated or inadequately regulated. In animal models of preeclampsia, complement activation results in the development of preeclampsia symptoms, while complement inhibition prevents the manifestations of preeclampsia.^{5,6} Women with preeclampsia have higher levels of circulating complement components.^{7,8} In addition, mutations in complement regulatory proteins predispose to preeclampsia and of the preeclamptic women with these mutations, many have severe, early-onset preeclampsia and/or preeclampsia complicated by intra-uterine growth restriction or HELLP syndrome.⁸ Furthermore, increased quantities of several complement activation products are observed in placentas of women with preeclampsia.⁹⁻¹¹ Illustratively, our group has recently demonstrated that C4d—a stable tissue marker for complement activation—is frequently observed in

placentas from preeclamptic women, whereas it is rarely present in placentas from women with uneventful pregnancies.¹¹

In settings other than preeclampsia, such as antiphospholipid syndrome, placental complement activation is associated with profound placental injury, both in experimental models and human placentas.¹²⁻¹⁴ Experimental models of preeclampsia have demonstrated a similar relation between complement activation and placental injury and dysfunction.⁵ Therefore, we hypothesized that women with preeclampsia and placental complement activation have increased placental injury and dysfunction compared to preeclamptic women without placental complement activation. To test this hypothesis, we stained 111 placentas obtained from preeclamptic women for C4d and related the presence of placental C4d to the clinical manifestations and the histopathological lesions that characterize preeclampsia.

Methods

PATIENTS AND PLACENTAS We studied 111 placentas obtained from women who were diagnosed with preeclampsia and who delivered at the Obstetrical Department of the Leiden University Medical Center (LUMC) between 2001 and 2011. Preeclampsia was defined as the presence of hypertension and proteinuria after 20 weeks of gestation.¹⁵ All clinical data was derived from the case-records of the LUMC. We obtained clinical information on maternal age, gravidity, parity, height of blood pressure, amount of urinary protein, gestational age at delivery, birth- and placenta weight, mode of delivery and maternal and fetal morbidity (Table 1).

ETHICS All tissue samples were handled in a coded fashion, according to Dutch national ethical guidelines (*Code for Proper Secondary Use of Human Tissue*, Dutch Federation of Medical Scientific Societies).

PLACENTA PATHOLOGY AND IMMUNOHISTOCHEMISTRY

All placentas were formalin fixed and paraffin embedded.

Paraffin sections were routinely stained with haematoxylin and eosin (H&E). To relate placental complement activation to the clinical and histopathological manifestations of preeclampsia, immunohistochemical staining was performed for C4d. Sections were deparaffinized and rehydrated, after which heat induced antigen retrieval was performed in Tris-EDTA buffer (pH 9.0). After blocking for endogenous peroxidase, sections were incubated for 1 hour with polyclonal rabbit anti-human C4d (1:50, Biomedica Gruppe, Austria). Binding of the primary antibody was visualized with appropriate secondary antibodies and diaminobenzidine as a chromogen. Sections were counterstained with Haematoxylin. Renal tissue of a patient with humoral rejection served as a positive control. For the negative control, the primary antibody was omitted and replaced by Rabbit Immunoglobulin Fraction (DakoCytomation).

QUANTIFICATION OF PLACENTAL MORPHOLOGY AND IMMUNOHISTOPATHOLOGY

Sections were evaluated by an experienced pathologist, who scored the slides blinded to the patients' clinical data. Each placenta was scored separately for the presence or absence of histopathological changes associated with decreased uteroplacental perfusion: acute and/or chronic deciduitis; decidual necrosis; increased syncytial knots; accelerated villous maturity; accelerated maturity; avascular villi; villous infarcts; retroplacental haematomas; intervillous thrombi; and decidual vasculopathy.

Positivity for immunohistochemical staining was scored semi-quantitatively by two independent observers. Staining intensity around syncytiotrophoblast was scored as 0, 1 or 2, with 0 representing absence of staining (<10%), 1 representing focal staining (10-50%) and 2 representing diffuse staining (>50%).¹⁶

STATISTICAL ANALYSES Categorical variables were compared using the Chi-square test. When comparing numerical data from >2 independent groups, a one-way ANOVA was performed. All analyses were performed using SPSS statistical software package (version 20; Chicago, IL). A p -value < 0.05 was considered statistically significant.

Results

C4d is frequently observed in placentas of women with preeclampsia. C4d was observed in 63 out of 111 (56.7%) placentas. When present, C4d was observed at the syncytiotrophoblast in either a focal or a diffuse staining pattern. Focal C4d deposits were observed in 40 (36.0%) placentas, whereas 23 (20.7%) placentas showed a diffuse C4d staining pattern. Table 2 shows the incidence of the different C4d staining patterns among women with preeclampsia and Figure 1 shows representative examples of the different C4d staining patterns.

PLACENTAL C4D IS ASSOCIATED WITH PLACENTAL

DYSFUNCTION When subdividing women according to their C4d staining pattern (Table 3), women with placental C4d had a significantly lower mean gestational age at delivery, as compared to women without placental C4d. Furthermore, intra-uterine fetal death was significantly more common among women with placental C4d as compared to women without placental C4d ($p < 0.01$). In addition, the mean birth weight at delivery and the average placenta weight were significantly lower in women with placental C4d. To exclude the confounding effect of gestational age on birth weight, we looked up birth weight percentiles and placenta weight percentiles. Placenta weight percentiles were significantly lower among women with placental C4d ($p < 0.05$). No statistically significant difference was observed between the groups with respect to birth weight percentiles. To investigate whether placental complement activation is primarily related to placental dysfunction, or also to the maternal

manifestations of preeclampsia, we compared the height of blood pressure and the amount of urinary protein between preeclamptic women with and without placental C4d. A trend was observed between the amount of placental C4d and the amount of urinary protein (Table 3). No significant differences were observed with respect to the height of blood pressure, eclampsia and the prevalence of HELLP syndrome.

PLACENTAL C4D IS RELATED TO PLACENTAL INJURY

The histopathological changes associated with decreased uteroplacental perfusion were frequently observed in the placentas included in the study. When subdividing placentas according to their C4d staining pattern, a strong relation ($p<0.01$) was observed between the presence of C4d and the presence of placental infarction (Figure 2). Placental C4d was frequently observed around areas of infarction, of which a representative image is provided as supplementary data (Figure S1). The presence of placental C4d was also associated with a higher incidence of acute and/or chronic deciduitis ($p<0.01$). Furthermore, a higher incidence of increased syncytial knots was observed in placentas in which C4d was present ($p<0.01$). Additionally, the presence of C4d was related to the presence of decidual vasculopathy ($p<0.05$). Avascular villi were observed in 37% of preeclamptic women without IUFD and in 61% of women with both preeclampsia and IUFD. Its presence was significantly associated with C4d deposits ($p<0.05$).

PATIENTS OF WHICH PLACENTAS OF SUBSEQUENT

PREGNANCIES WERE AVAILABLE Of two patients included in the study, paraffin-embedded placenta material from a subsequent pregnancy was available. These two placentas were also stained for C4d to investigate whether the presence or absence of C4d is consistent among subsequent pregnancies. The first patient initially had a preeclamptic pregnancy for which a Caesarean Section was performed at 34+6 weeks. Macroscopically, the placenta consisted

of two lobes with a combined weight of 351 g. On macroscopic examination, no obvious signs of infarction were observed. Microscopically, micro-infarcts were observed, as well as pronounced syncytial knotting, which is frequently observed in the setting of placental hypoxia. No C4d was observed in this placenta. In the placenta of the subsequent pregnancy, there was no C4d either. The patient delivered at 41 weeks gestational age of a healthy son (3500 g). The placenta (460 g) was sent in for examination because of a suspected placenta accreta, which necessitated manual removal. Some placental infarction (<5%) but no signs of placenta accreta were observed. The second patient initially had a pregnancy complicated by fetal loss in the setting of severe preeclampsia, severe intra-uterine growth restriction (IUGR) and HELLP syndrome at 23 weeks of gestation. The placenta was removed manually and it was severely damaged. Microscopically, fibrosis of the villous stroma was observed, as well as fibrin deposition and some infarction. The C4d staining showed intense and diffuse C4d deposits along the syncytiotrophoblast of all villi. The subsequent pregnancy was also complicated by IUGR and hypertension for which the patient delivered by Caesarean section at 30+6 weeks gestational age. The placenta (184 g) showed increased syncytial knotting, as well as accelerated villous maturity; findings that are consistent with increased placental maturity and ischemia. Strikingly, this placenta also showed C4d deposits, although less prominent than in the placenta from the previous pregnancy.

Discussion

In this study, which includes 111 placentas obtained from preeclamptic women, we have shown that the presence of complement activation (C4d) is associated with a significantly lower gestational age at delivery and intra-uterine fetal death. Moreover, the presence of C4d is also associated with significantly

lower placenta weight percentiles and a significantly higher prevalence of several histopathological changes associated with reduced uteroplacental perfusion. These data suggest that placental complement activation is related to placental injury and dysfunction in the setting of preeclampsia.

We show a strong relation between placental classical complement activation and preeclampsia-associated intra-uterine fetal death (IUFD). Placental pathology plays a notable role in IUFD, both in developing nations and in high-income countries.^{17,18} However, the exact mechanisms underlying IUFD in women with preeclampsia remain largely unknown. Placental classical pathway activation has long been recognized as an important cause of pregnancy complications in women with the autoimmune disorders systemic lupus erythematosus and antiphospholipid syndrome. Adverse pregnancy complications in these women include frequent early and late fetal loss.^{19,20} In human placentas of women with antiphospholipid syndrome, particularly the classical pathway marker C4d is strongly related to IUFD. IUFD in the setting of antiphospholipid syndrome is associated with classical pathway activation at the fetal-maternal interface, in a similar pattern as observed in the current study.²¹ In a murine model of immune-mediated fetal loss, complement activation leads to increased fetal loss and complement inhibition prevents this.¹² Altogether, both murine and human studies have demonstrated that complement activation is involved in anti-phospholipid antibody induced fetal loss. The strong association between the presence of C4d and IUFD in this study suggests that IUFD in the setting of preeclampsia may also be complement mediated. It has indeed been observed that IUFD in the setting of preeclampsia is accompanied by activation of the complement system, as indicated by elevated maternal plasma concentrations of complement split products.²² However, this is the first study that reports an association between IUFD and complement activation at the placental level in women with preeclampsia. We also show a relation between placental complement

activation and placenta size. Importantly, preeclampsia is associated with significantly smaller placentas.²³ Our results suggest that among preeclamptic women, the smallest placentas are among the women with placental complement activation. This is evidenced by the fact that in the presence of placental C4d, the average placenta weight percentile is significantly lower compared to placentas from preeclamptic women without placental C4d. Complement activation could affect placental development and function, as previously shown in an animal model of preeclampsia⁶, thereby resulting in smaller placentas.

The current study also demonstrates a strong association between placental complement activation and various histopathological findings associated with reduced uteroplacental perfusion. The presence of C4d was strongly related to placental infarction. Placental infarction is a frequent finding in preeclampsia and the extent of placental infarction correlates with the severity of preeclampsia.²⁴ Complement activation could contribute to the development and extent of infarction, like previously demonstrated in the setting of experimental myocardial infarction.²⁵ The presence of C4d was also strongly correlated to the incidence of increased syncytial knots. Syncytial knots are the main placental production site of sFlt-1, suggesting that these knots play an important role in the pathogenesis of preeclampsia.²⁶ Because inflammatory cytokines stimulate the release of trophoblasts from the placenta²⁷, it is not unlikely that complement activation may also be involved in the formation and release of syncytial knots. Furthermore, C4d deposits were associated with decidual vasculopathy, which is in line with a previous report.²⁸ Like in the setting of atherosclerosis²⁹, excessive complement activation could contribute to the development of decidual vasculopathy. Placental complement activation is not only associated with injury to the maternal vasculature, as the presence of C4d was also related to avascular villi, which results from fetal vascular damage. Finally, placental C4d was associated with deciduitis; a placental inflammatory reaction. This is in line

with observations in a murine model of preeclampsia, in which complement activation is associated with placental inflammation.⁶ Of two patients included in the current study, placentas from subsequent pregnancies were available. The first patient showed no C4d in both placentas. The other patient, on the contrary, showed placental C4d deposits in both placentas, which were derived from pregnancies that were complicated by severe, early-onset hypertensive disorders of pregnancy as well as severe IUGR, in one case complicated by fetal loss. Placental C4d appears to be related to the extent of placental dysfunction in preeclampsia and these two cases suggest that the presence or absence of C4d may be consistent among subsequent pregnancies. Severe, early-onset preeclampsia is most likely to recur in subsequent pregnancies.³ Particularly those women with severe preeclampsia as well as placental C4d may have the highest chance of developing complications during later pregnancies. It would be interesting to investigate whether placental C4d can serve as a biomarker to predict adverse pregnancy outcomes in future pregnancies among women with preeclampsia in an initial pregnancy.

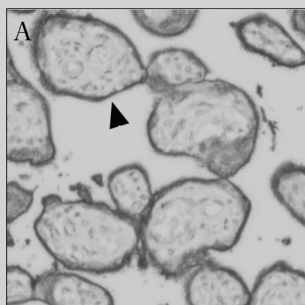
The findings in the current study confirm the association between preeclampsia and placental classical pathway activation, as previously reported by our group.¹¹ It must be acknowledged that it is difficult to determine whether complement activation is a cause or a consequence of preeclampsia. In the setting of IUFD, C4d deposits could be a late event, as severely damaged or apoptotic tissue can induce classical pathway activation.³⁰ However, placental C4d is frequently absent in cases of IUFD due to chromosomal abnormalities, indicating that IUFD does not necessarily result in complement activation.²¹ Furthermore, C4d is also observed in placentas of live-born children, indicating that C4d may have been present before the fetus died. Moreover, complement inhibition prevents fetal loss in a mouse model for preeclampsia, suggesting a causal relationship between complement activation and fetal loss.⁵ Therefore, complement activation could also precede IUFD.

Importantly, a rise in complement factor Bb precedes the onset of preeclampsia, suggesting that complement activation is an early event in the development of preeclampsia.⁷ Furthermore, complement activation in pregnant mice results in a variety of pregnancy complications and local blockade of complement activation at the fetal-maternal interface rescues both fetal loss and preeclampsia in mice.⁵ Although it remains uncertain whether placental C4d is a cause or a consequence of the pathophysiological processes taking place in preeclampsia, the current study does underline the importance of further investigating the role of the complement system in the pathogenesis of preeclampsia.

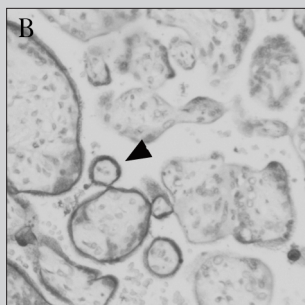
In conclusion, our results have demonstrated a strong relationship between placental complement activation and the prevalence of several placenta-related manifestations and complications of preeclampsia such as IUFD and small for gestational age placentas. In addition, we have shown that placental complement activation in preeclamptic women is associated with a higher prevalence of histopathological hallmarks of reduced placental perfusion. Altogether, placental classical pathway activation appears to be related to the extent of placental dysfunction and placental injury in women with preeclampsia. If complement activation contributes to the pathophysiology and/ or the severity of preeclampsia, it may be worth while exploring its possibilities as a disease marker or even a target for therapy.

FIG 1 DIFFERENT C4D STAINING PATTERNS

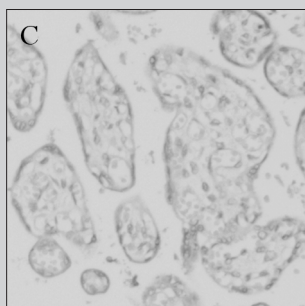
(full colour version inside cover)



diffuse C4D staining



focal C4D staining

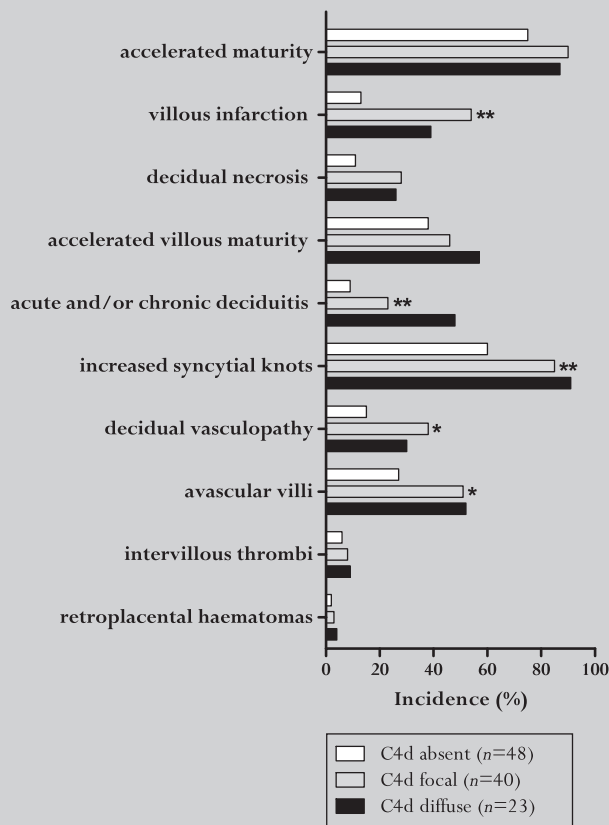


negative C4D staining

Overview showing representative images of the different C4d staining patterns. Staining was scored as 'diffuse placental C4d staining' (A), 'focal placental C4d staining' (B) or 'no or minimal placental C4d staining' (C). Images A and B clearly illustrate that C4d deposits are present at the surface of the syncytiotrophoblast (arrows), and not within villi.

BAR GRAPH OF SPECIFIC PLACENTAL HISTOLOGICAL SCORES AS DESCRIBED IN RESULTS

FIG 2



Bar graph of specific placental histological scores as described in Results, giving an indication of the abundance of various lesions in the different C4d groups. The differences between groups are represented by * $p < 0.05$ and ** $p < 0.01$.

TABLE 1 PATIENT CHARACTERISTICS

CHARACTERISTICS	
Mean maternal age in years (SD)	32.0 (6.5)
Mean gravidity (SD)	2.2 (1.6)
% gravida 1	44.1
Mean parity (SD)	0.6 (1.1)
Nulliparous (%)	68 (61.3)
Mean highest diastole (mmHg) (SD)	111.1 (11.4)
Mean proteinuria (g/24h) (SD)	6.5 (6.3)
Mean gestational age at delivery (weeks + days, SD in days)	30 + 2 (25.5)
Mean birth weight (g) (SD)	1166.7 (573.1)
Birth weight percentile	
<5 (%)	14 (13.5)
5-10 (%)	11 (10.6)
10-20 (%)	31 (29.9)
20-50 (%)	32 (30.8)
>50 (%)	16 (15.4)
Mean trimmed placenta weight (g) (SD)	226.2 (95.7)
Mode of delivery	
Caesarean section (%)	83 (74.8)
Spontaneous delivery (%)	4 (3.6)
Induction (%)	24 (21.6)
Complications	
Intra-uterine fetal death	18 (16.2)
HELLP syndrome (%)	31 (27.9)
Eclampsia (%)	12 (10.9)

TABLE 2 DISTRIBUTION OF DIFFERENT C4D STAINING PATTERNS AMONG WOMEN WITH PREECLAMPSIA

C4D STAINING PATTERN	n = 111
No C4d (%)	48 (43.3)
Focal C4d (%)	40 (36.0)
Diffuse C4d (%)	23 (20.7)

**CLINICAL CHARACTERISTICS OF WOMEN WITH
PREECLAMPSIA AND NO, FOCAL OR DIFFUSE PLACENTAL C4D**

TABLE 3

	No C4d (n = 48)	Focal C4d (n = 40)	Diffuse (n = 23)
Mean maternal age in years (SD)	31.5 (6.1)	32.2 (7.3)	32.5 (6.1)
Mean gravidity (SD)	2.3 (1.5)	2.1 (1.4)	2.1 (2.1)
G1 (%)	19 (39.6)	16 (40.0)	14 (60.9)
Mean parity (SD)	0.5 (0.8)	0.7 (1.0)	0.8 (1.7)
Nulliparous (%)	30 (62.5)	22 (55.0)	16 (69.6)
Mean highest diastole (mmHg) (SD)	110.3 (12.0)	111.6 (10.2)	111.7 (12.7)
Mean proteinuria (g/24h) (SD)	5.3 (5.0)	6.5 (5.7)	8.8 (8.8)
Mean gestational age at delivery (weeks + days, SD in days)	31 + 1 (25.2)	29 + 5 (26.5)	29 + 1 (21.7)*
Birth weight (g) (SD)	1359.3 (595.9)	1067.3 (551.8)*	933.5 (433.1)*
Birth weight percentile			
<5 (%)	6 (12.8)	5 (14.3)	3 (13.6)
5-10 (%)	2 (4.3)	5 (14.3)	4 (18.2)
10-20 (%)	15 (31.9)	10 (28.6)	6 (27.2)
20-50 (%)	19 (40.4)	9 (25.7)	4 (18.2)
>50 (%)	5 (10.6)	6 (17.1)	5 (22.7)
Placenta weight (g) (SD)	261.7 (98.3)	207.1 (91.4)*	181.1 (68.0)*
Placenta weight percentile †			
<3 (%)	14 (32.6)	24 (63.2)	10 (43.5)
3-10 (%)	12 (27.9)	10 (26.3)	7 (30.4)
>10 (%)	17 (39.5)	4 (10.5)	6 (26.1)
unknown (%)	5	2	0
Mode of delivery			
Caesarean section (%)	42 (87.5)	24 (60.0)	17 (73.9)
Spontaneous delivery (%)	2 (4.2)	2 (5.0)	0 (0.0)
Induction (%)	4 (8.3)	14 (35.0)	6 (26.1)
Maternal complications			
HELLP (%)	10 (20.8)	12 (30)	9 (39.1)
Eclampsia (%)	5 (10.4)	6 (15.4)	1 (4.3)
Fetal complications			
Intra-uterine fetal death (%) †	0 (0)	12 (30)	6 (26.1)

* denotes a statistically significant difference when compared with no C4d

† denotes a statistically significant difference in overall comparison

REFERENCES

1. Steegers EA, von Dadelszen P, Duvekot JJ, Pijnenborg R. Pre-eclampsia. *Lancet* 2010; 376:631-644.
2. Sibai BM. Diagnosis and management of gestational hypertension and preeclampsia. *Obstet Gynecol* 2003; 102:181-192.
3. Barton JR, Sibai BM. Prediction and prevention of recurrent preeclampsia. *Obstet Gynecol* 2008; 112:359-372.
4. Khan KS, Wojdyla D, Say L, Gulmezoglu AM, Van Look PF. WHO analysis of causes of maternal death: a systematic review. *Lancet* 2006; 367:1066-1074.
5. Qing X, Redecha PB, Burmeister MA, Tomlinson S, D'Agati VD, Davisson RL *et al.* Targeted inhibition of complement activation prevents features of preeclampsia in mice. *Kidney Int* 2011; 79:331-339.
6. Girardi G, Yarilin D, Thurman JM, Holers VM, Salmon JE. Complement activation induces dysregulation of angiogenic factors and causes fetal rejection and growth restriction. *J Exp Med* 2006; 203:2165-2175.
7. Lynch AM, Murphy JR, Byers T, Gibbs RS, Neville MC, Giclas PC *et al.* Alternative complement pathway activation fragment Bb in early pregnancy as a predictor of preeclampsia. *Am J Obstet Gynecol* 2008; 198:385-389.
8. Salmon JE, Heuser C, Triebwasser M, Liszewski MK, Kavanagh D, Roumenina L *et al.* Mutations in complement regulatory proteins predispose to preeclampsia: a genetic analysis of the PROMISSE cohort. *PLoS Med* 2011; 8:e1001013.
9. Faulk WP, Jarret R, Keane M, Johnson PM, Boackle RJ. Immunological studies of human placenta: complement components in immature and mature chorionic villi. *Clin Exp Immunol* 1980; 40:299-305.
10. Sinha D, Wells M, Faulk WP. Immunological studies of human placenta: complement components in pre-eclamptic chorionic villi. *Clin Exp Immunol* 1984; 56:175-184.
11. Buurma A, Cohen D, Veraar K, Schonkeren D, Claas FH, Bruijn JA *et al.* Preeclampsia Is Characterized by Placental Complement Dysregulation. *Hypertension* 2012; 60:1332-7.
12. Girardi G, Berman J, Redecha P, Spruce L, Thurman JM, Kraus D *et al.* Complement C5a receptors and neutrophils mediate fetal injury in the antiphospholipid syndrome. *J Clin Invest* 2003; 112:1644-1654.
13. Piona A, La RL, Tincani A, Faden D, Magro G, Grasso S *et al.* Placental thrombosis and fetal loss after passive transfer of mouse lupus monoclonal or human polyclonal anti-cardiolipin antibodies in pregnant naive BALB/c mice. *Scand J Immunol* 1995; 41:427-432.
14. Shamoni JM, Salmon JE, Hyjek E, Baergen RN. Excessive complement activation is associated with placental injury in patients with antiphospholipid antibodies. *Am J Obstet Gynecol* 2007; 196:167-5.
15. Brown MA, Lindheimer MD, de Swiet M, Van Assche A, Moutquin JM. The classification and diagnosis of the hypertensive disorders of pregnancy: statement from the International Society for the Study of Hypertension in Pregnancy (ISSHP). *Hypertens Pregnancy* 2001; 20:IX-XIV.
16. Solez K, Colvin RB, Racusen LC, Haas M, Sis B, Mengel M *et al.* Banff 07 classification of renal allograft pathology

- gy: updates and future directions. *Am J Transplant* 2008; 8:753-760.
17. Flenady V, Koopmans L, Middleton P, Froen JF, Smith GC, Gibbons K *et al*. Major risk factors for stillbirth in high-income countries: a systematic review and meta-analysis. *Lancet* 2011; 377:1331-1340.
18. McClure EM, Nalubamba-Phiri M, Goldenberg RL. Stillbirth in developing countries. *Int J Gynaecol Obstet* 2006; 94:82-90.
19. Cohen D, Berger SP, Steup-Beekman GM, Bloemenkamp KW, Bajema IM. Diagnosis and management of the antiphospholipid syndrome. *BMJ* 2010; 340:c2541.
20. Smyth A, Oliveira GH, Lahr BD, Bailey KR, Norby SM, Garovic VD. A systematic review and meta-analysis of pregnancy outcomes in patients with systemic lupus erythematosus and lupus nephritis. *Clin J Am Soc Nephrol* 2010; 5:2060-2068.
21. Cohen D, Buurma A, Goemaere NN, Girardi G, le Cessie S, Scherjon S *et al*. Classical complement activation as a footprint for murine and human antiphospholipid antibody-induced fetal loss. *J Pathol* 2011; 225:502-11.
22. Richani K, Romero R, Soto E, Espinoza J, Nien JK, Chaiworapongsa T *et al*. Unexplained intrauterine fetal death is accompanied by activation of complement. *J Perinat Med* 2005; 33:296-305.
23. Dahlstrom B, Romundstad P, Oian P, Vatten LJ, Eskild A. Placenta weight in pre-eclampsia. *Acta Obstet Gynecol Scand* 2008; 87:608-611.
24. Vinnars MT, Nasiell J, Ghazi S, Westgren M, Papadogiannakis N. The severity of clinical manifestations in preeclampsia correlates with the amount of placental infarction. *Acta Obstet Gynecol Scand* 2011; 90:19-25.
25. Ito W, Schafer HJ, Bhakdi S, Klask R, Hansen S, Schaarschmidt S *et al*. Influence of the terminal complement-complex on reperfusion injury, no-reflow and arrhythmias: a comparison between C6-competent and C6-deficient rabbits. *Cardiovasc Res* 1996; 32:294-305.
26. Rajakumar A, Cerdeira AS, Rana S, Zsengeller Z, Edmunds L, Jayabalan A *et al*. Transcriptionally active syncytial aggregates in the maternal circulation may contribute to circulating soluble fms-like tyrosine kinase 1 in preeclampsia. *Hypertension* 2012; 59:256-264.
27. Chen LM, Liu B, Zhao HB, Stone P, Chen Q, Chamley L. IL-6, TNFalpha and TGFbeta promote nonapoptotic trophoblast deportation and subsequently causes endothelial cell activation. *Placenta* 2010; 31:75-80.
28. Hering L, Herse F, Verlohren S, Park JK, Wellner M, Qadri F *et al*. Trophoblasts reduce the vascular smooth muscle cell proatherogenic response. *Hypertension* 2008; 51:554-559.
29. Speidl WS, Kastl SP, Huber K, Wojta J. Complement in atherosclerosis: friend or foe? *J Thromb Haemost* 2011; 9:428-440.
30. Nauta AJ, Trouw LA, Daha MR, Tijsma O, Nieuwland R, Schwaebble WJ *et al*. Direct binding of C1q to apoptotic cells and cell blebs induces complement activation. *Eur J Immunol* 2002; 32:1726-1736.

CHAPTER VI

**PREECLAMPSIA AND PLACENTAL
FRAGMENTS IN THE MATERNAL
LUNG**

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

HYPERTENSION, IN PRESS

Summary

Preeclampsia is associated with an excessive shedding of placenta-derived multinucleated syncytial aggregates into the maternal circulation. However, it remains unclear whether these aggregates are transcriptionally active in the maternal organs and can therefore contribute to the systemic manifestations of preeclampsia. Using human placental tissue, we found that these syncytial aggregates are the principal site of expression of the anti-angiogenic factor sFlt-1 that has been pathogenically linked to the disease's signs and symptoms. In addition, in autopsy material obtained from women with preeclampsia (n=9), we observed significantly more placenta-derived syncytial aggregates in the lungs than in control subjects (n=26). Importantly, these placental aggregates still contained anti-angiogenic factors following their entrapment in the maternal lungs, suggesting that the transfer of syncytial aggregates to the maternal compartment may contribute to the systemic endothelial dysfunction that characterizes preeclampsia.

Introduction

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

The widespread endothelial dysfunction that characterizes preeclampsia is believed to be due to an imbalance between pro- and anti-angiogenic factors.^{2,3} The placenta is a major source of circulating anti-angiogenic factors during both normal and preeclamptic pregnancies.³⁻⁶ In preeclampsia in particular, the outermost layer of the placenta—the syncytiotrophoblast—forms

“knots” that contain high amounts of the anti-angiogenic protein sFlt-1.⁷ These syncytial knots are released into the maternal circulation, thereby becoming syncytial aggregates that can become lodged in maternal organs.⁸⁻¹⁰ Importantly, a recent study showed that upon their release, circulating syncytial aggregates remain transcriptionally active and likely serve as an autonomous source of sFlt-1 protein within the maternal circulation.⁷

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

Methods

PATIENT SELECTION AND TISSUE COLLECTION Placentas were obtained from preeclamptic¹¹ (n=32) and control (n=37) subjects who delivered at the Leiden University Medical Center (LUMC), the Netherlands from 2007 through 2010. All women gave written informed consent. Autopsy samples from women who died during pregnancy were obtained via a nationwide search using the Dutch PALGA system, a histopathology and cytopathology network and archive that includes all pathology laboratories within the Netherlands.¹² The paraffin-embedded lung samples obtained from 9 preeclampsia patients and 26 pregnant control subjects were provided by collaborating laboratories. The cause of death in each case was confirmed using the records of the National Maternal Mortality Committee of the Dutch Society of Obstetrics and Gynecology. All tissues were coded and handled anonymously in accordance with the Dutch National Ethics Guidelines (Code

for Proper Secondary Use of Human Tissue, Dutch Federation of Medical Scientific Societies). This study was approved by the ethics committee of the LUMC.

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

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

Y CHROMOSOME IN SITU HYBRIDIZATION A DIG-labeled DNA probe that specifically recognizes the Y chromosome¹³ was used to determine whether the putative syncytiotrophoblast aggregates in the maternal lungs were of fetal origin. Sections of lungs from

women who had carried a male fetus were incubated with the DIG-labeled probe. To visualize the probe, the sections were incubated first with a mouse-anti-DIG monoclonal antibody (Sigma-Aldrich) followed by goat-anti-mouse IgG Alexa-647 (Invitrogen).

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

Results

INCREASED PLACENTAL SFLT-1 MRNA EXPRESSION IN PREECLAMPSIA To compare the levels of sFlt-1 mRNA in the preeclamptic and control placentas, quantitative PCR was used to measure sFlt-1 mRNA. On average, the placental sFlt-1 mRNA levels were 6-fold higher in the preeclamptic placentas than in the placentas obtained from control subjects ($p < 0.001$, Mann-Whitney test). The preeclamptic placentas had more intense sFlt-1 staining (measured using *in situ* hybridization) than control placentas, particularly in the syncytial knots (Figure 1). In addition, the number of syncytial knots was significantly higher in the preeclamptic women than in the control subjects ($p < 0.05$, Figure 1). As expected, the sense control probe was negative in all samples (Figure 1).

THE PRESENCE OF HCG POSITIVE AGGREGATES IN MATERNAL LUNGS IS SIGNIFICANTLY ASSOCIATED WITH PREECLAMPSIA Because hCG was highly expressed within the syncytial knots, we considered hCG to be a suitable specific marker to study the presence of syncytiotrophoblast aggregates in maternal lungs. hCG-positive

multinucleated aggregates were observed in the lungs of six of the nine preeclamptic women. Following the observation that syncytial aggregates were present in the lungs of women with preeclampsia, we also stained control lung sections for hCG. Syncytial aggregates were observed in the lung samples of 6 of the 26 control subjects. Importantly, the women with preeclampsia had a significantly higher number of syncytial aggregates per 100 mm² lung tissue ($p < 0.05$, Mann-Whitney test, Figure 2). Syncytial aggregates were found in the pregnant control subjects whose gestational age was 10-40 weeks and in preeclamptic women with a gestational age of 32-39 weeks. Aggregates were observed in subjects who died up to 13 days after delivery.

SYNCYTIOTROPHOBLAST AGGREGATES IN THE MATERNAL LUNG RETAIN THE sFLT-1 PROTEIN To test our hypothesis that syncytial aggregates retain sFlt-1 protein after transferring to the maternal compartment and becoming entrapped in the maternal lung, we stained the hCG-positive aggregates in the maternal lung samples for Flt-1 protein. Staining sequential sections for Flt-1 and hCG revealed that these proteins were co-localized within the aggregates (Figure 2).

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

Discussion

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

Our finding that syncytial knots are the primary placental site of sFlt-1 mRNA synthesis is in agreement with the observations that syncytial knots have the highest placental levels of sFlt-1 protein and that these knots are more numerous in the setting of preeclampsia.^{7,14} Syncytial knots detach readily from the placenta, becoming syncytial aggregates that circulate in the maternal blood.⁷ It has long been known that circulating placental material—most likely trophoblast cells—can reach maternal organs, particularly the lungs.¹⁰ Using co-localization of hCG with the Y chromosome, we show that the placental multinucleated aggregates in the maternal lung were derived from the syncytiotrophoblast. Interestingly, these placenta-derived aggregates in the maternal lung still contained sFlt-1 protein. This observation supports the idea of circulating syncytial aggregates as a mechanism of sFlt-1 release into the maternal circulation. Importantly, we also found that preeclampsia was associated with a significantly higher number of syncytial aggregates within the maternal lung tissue. By releasing sFlt-1, these aggregates might contribute to the systemic endothelial dysfunction that is

characteristic of preeclampsia.

In addition, the presence of syncytial aggregates in maternal organs—particularly in the early stages of pregnancy—may play a key role in the development of immune tolerance. As early as gestational week 10, we observed syncytial aggregates in maternal lungs. Because preeclampsia rarely presents prior to 20 weeks of gestation,¹¹ we could not investigate the presence of syncytial aggregates in the lungs of preeclamptic women early in pregnancy. We did, however, observe syncytial aggregates in the lungs of preeclamptic women at gestational week 32 and later, and other groups have reported the presence of trophoblast fragments in maternal blood in earlier stages of preeclamptic pregnancy.¹⁵ Altogether, circulating syncytial aggregates are present early in pregnancy, and we and others¹⁶ have found a strong association between increased shedding of syncytial aggregates and preeclampsia. Thus, one may speculate that the release and transfer of syncytial aggregates to the maternal compartment is an early event in the pathogenesis of preeclampsia.

The presence and persistence of fetal cells in maternal organs may also have both short-term and long-term implications for postpartum maternal health. Syncytial aggregates that remain in the maternal lungs may undergo further disaggregation, forming smaller microparticles. These sFlt-1-loaded microparticles may—via their release into the systemic maternal circulation—contribute to endothelial dysfunction in maternal organs other than the lungs. We found that even 13 days after delivery, hCG-positive syncytial aggregates can be detected within the maternal lungs. This finding supports the idea that placenta-derived syncytial aggregates may be involved in the post-partum complications that are associated with preeclampsia. Preeclampsia usually resolves rapidly after delivery; however, in a subset of women, the symptoms and complications of preeclampsia can persist or present in the days following delivery. Because syncytial aggregates remain transcriptionally active up to 48 hours after delivery,⁷ we speculate that these aggregates may play a

role in the development of postpartum (pre)eclampsia.

Trophoblast cells are likely not the only fetal cell population that is present in the maternal lung. A previous study using mice suggested that fetal cells in the maternal lung are comprised of a mixture of cell types that includes trophoblasts, mesenchymal stem cells, and cells from the immune system.¹⁷ We have now confirmed the presence of trophoblast cells in the human maternal lung. In the long run, the release of vital cells from the placenta may result in chimerism, as fetal cells can be retained in the maternal blood and organs for decades after delivery.^{18,19} Because retained fetal cells have stem cell-like properties,²⁰ it can be speculated that these cells provide a mechanism through which maternal health can be affected for decades after pregnancy. Further studies are needed to determine the relevance and relative contribution of trophoblast cells—and other cell types—to maternal health. Likewise, understanding what drives the formation, detachment and transfer of syncytial knots to the maternal compartment—and why these knots produce sFlt-1—are important questions to be investigated. Nevertheless, this report highlights the importance of investigating further the role that syncytial aggregates play in preeclampsia and its complications.

PLACENTAL SFLT-1 MRNA LEVELS

FIG 1A

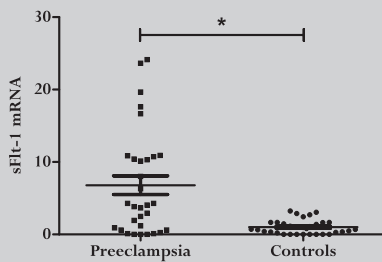


Figure 1A shows the relative sFlt-1 mRNA levels in the placentas of women with preeclampsia and control subjects with mean+SEM (* $p < 0.001$).

SFTL-1 MRNA IN SITU HYBRIDIZATION

FIG 1B

(full colour version inside cover)

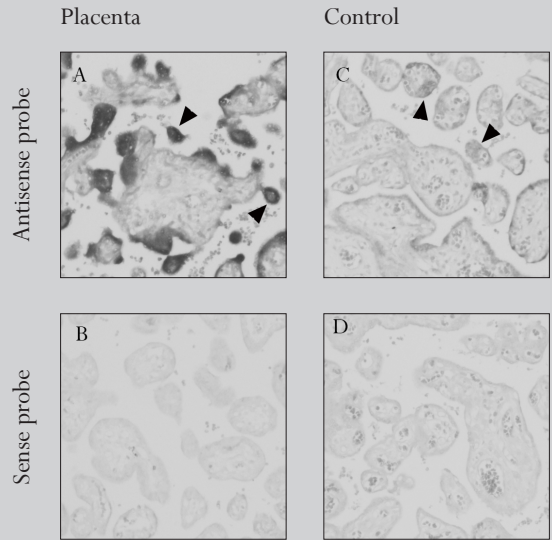


Figure 1B shows typical examples of sFlt-1 mRNA *in situ* hybridization in the placenta of a woman with preeclampsia (A and B) and a control subject (C and D). The term “syncytial knots” describes multinucleated structures that are loosely attached to the tips of placental villi *in situ*. Each column represents an individual placenta, and the various RNA probes are shown horizontally. The antisense probe (A and C) revealed that the syncytial knots (arrowheads) were the primary placental site of sFlt-1 mRNA production in both placentas. The sense probe (B and D) was used as a negative control.

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

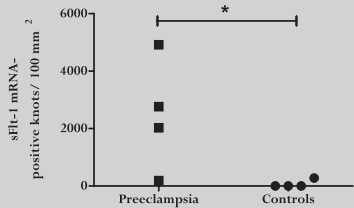


Figure 1C shows the density of sFLT-1 mRNA-positive syncytial knots in the placentas of preeclamptic women and control subjects. * $p < 0.05$.

FIG 2A STAININGS OF THE PLACENTA AND MATERNAL LUNGS

(full colour version inside cover)

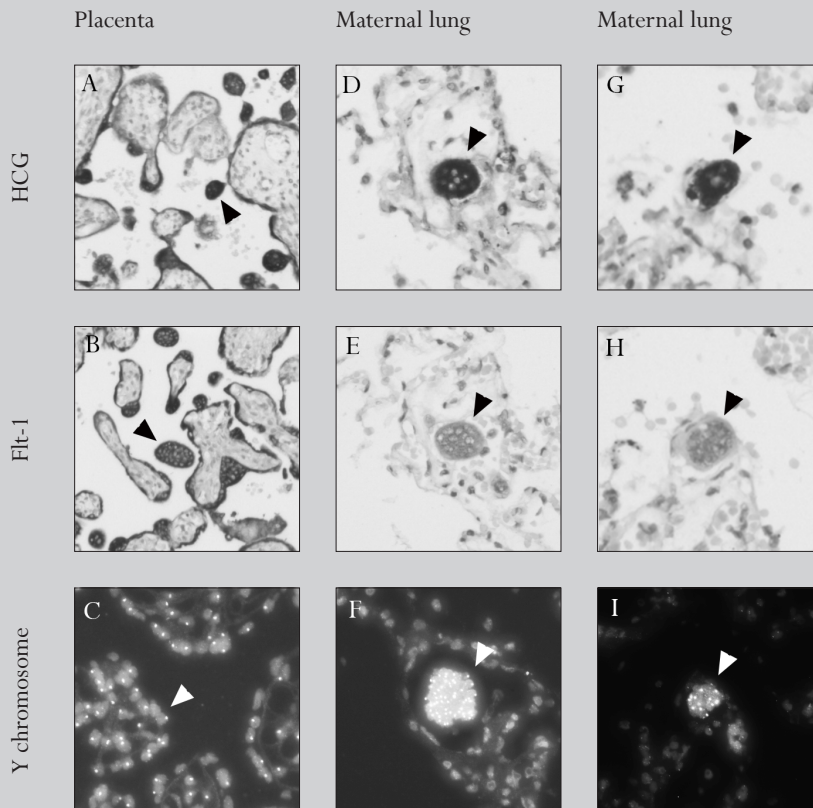


Figure 2A shows sections of placenta and sequential sections of maternal lungs that were stained for hCG or Flt-1 (using immunohistochemistry) or the Y chromosome (using *in situ* hybridization). To simplify the terminology, we use “syncytial knots” to describe multinucleated structures that are loosely attached to the tips of placental villi *in situ* and “syncytial aggregates” to describe detached multinuclear structures within the maternal lungs. The left column shows a placenta obtained from a preeclamptic patient, and the middle and right columns represent the lungs obtained from two women who were pregnant with a boy and died due to preeclampsia. The various stains are shown horizontally.

Subpanels A-C show that within the preeclamptic placenta, the hCG (A) and Flt-1 (B) proteins are most abundantly present within the syncytial knots (arrowheads). In addition, Y chromosome *in situ* hybridization (C) shows the presence of the Y chromosome in the nucleus (visible as red puncta).

Subpanels D and G show the presence of hCG-positive aggregates (arrowheads) in the lungs of two women who died due to preeclampsia.

Subpanels E and H show the Flt-1 staining patterns in sections that were sequential to the sections shown in panels d and g, respectively. These images demonstrate that within the maternal lungs, hCG-positive aggregates also contain Flt-1 protein.

The next sequential sections were used to perform Y chromosome *in situ* hybridization (subpanels F and I). These images show that the multinucleated aggregates contain Y chromosomes (arrowheads), indicating that it is very unlikely that these aggregates are not of fetal origin.

Altogether, the figure demonstrates co-localization of hCG, Flt-1 and the Y chromosome in the multinucleated aggregates (arrowheads).

FIG 2B **CO-LOCALIZATION OF HCG AND FLT-1**
(full colour version inside cover)

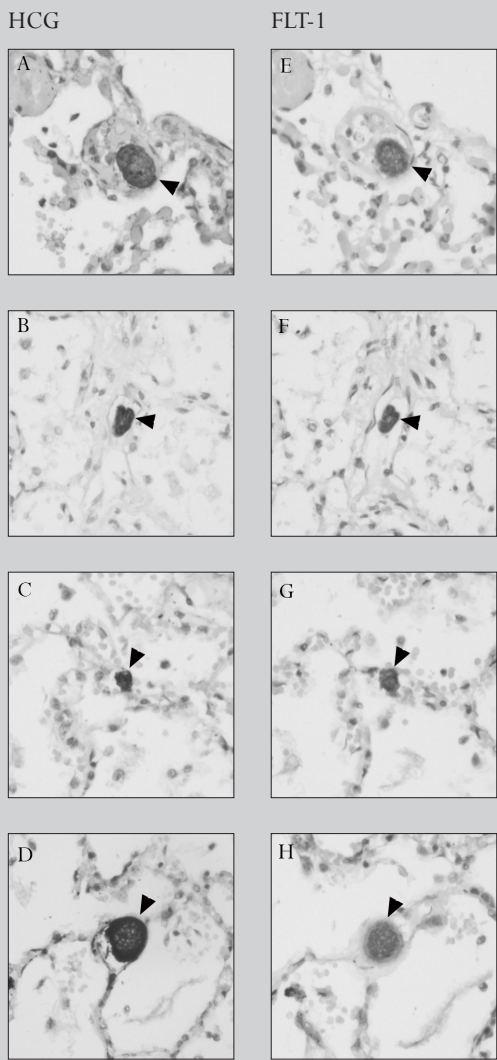


Figure 2B shows additional examples of multinucleate aggregates in maternal lungs, with co-localization of hCG (subpanels A-D) and Flt-1 (subpanels E-H). Each row shows matched sequential sections.

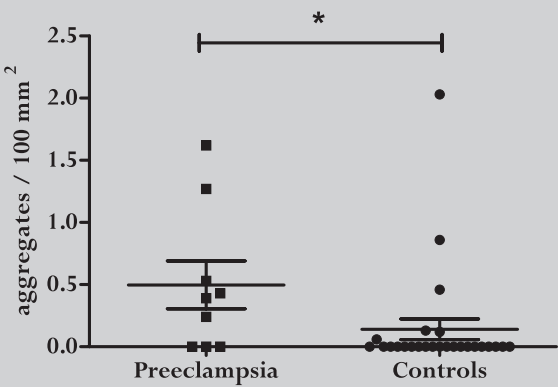


Figure 2C summarizes the number of hCG-positive syncytial aggregates within the maternal lungs of preeclamptic women (n=9) and control subjects (n=26). *p<0.05. The control subjects were women who died due to a cause other than a hypertensive disorder of pregnancy (e.g., pulmonary embolism, heart attack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

REFERENCES

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

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

CHAPTER VII

SUMMARY AND

GENERAL DISCUSSION

Summary

As outlined in the introduction, preeclampsia is a severe pregnancy-specific syndrome that originates in the placenta. The condition starts with abnormal placental development. The abnormally developed placenta produces excessive amounts of anti-angiogenic factors, which lead to endothelial dysfunction and thereby the clinical manifestations of preeclampsia. Although it is uncertain what precisely underlies placental dysfunction in the setting of preeclampsia, epidemiological data have pointed out that autoimmune diseases, a (family) history of preeclampsia and conditions characterized by endothelial dysfunction are important risk factors for the development of preeclampsia.¹ The wide variety of risk factors indicates that the etiology of preeclampsia is probably as heterogeneous as its presentation. This heterogeneity in risk factors is reflected in this thesis; it deals with immunologic, genetic and angiogenic aspects of preeclampsia.

CHAPTER 2 is focused on the genetic component of preeclampsia. Many genes have been investigated in the setting of preeclampsia, but it is uncertain which genetic variants are reproducibly associated with preeclampsia. In the study described in chapter 2, we selected all genetic variants that were significantly associated with preeclampsia in an initial study and were subsequently independently reproduced in at least one additional study. All studies that assessed these reproduced variants were combined in a random-effects meta-analysis. We identified 22 replicated genetic variants, of which seven remained significantly associated with preeclampsia following meta-analysis. These variants were in or near the following genes: angiotensin converting enzyme (*ACE*), cytotoxic T-lymphocyte associated protein 4 (*CTLA4*), coagulation factor 2 (*F2*), coagulation factor 5 (*FV*), lipoprotein lipase (*LPL*), and serine protease 1 (*SERPINE1*). These results suggest that the coagulation and fibrinolysis system as well as the renin-angiotensin system are

putative key players in the pathogenesis of preeclampsia. Functional studies remain to be performed to establish the precise role of these variants and pathways.

However, it seems wise not to focus exclusively on these variants and pathways. It is important to keep in mind that our meta-analysis only included genetic association studies. These studies investigated candidate genes—genes that are likely to be involved in the pathogenesis of preeclampsia, based on current knowledge. Hypothesis-free methods, such as genome-wide association studies, could identify new susceptibility genes.² In addition, next-generation sequencing—which allows the sequencing of DNA at unprecedented speeds—may identify rare causal variants that are associated with preeclampsia.

Importantly, the impact of genetic variants on the risk for developing preeclampsia should not be overestimated. Not all women with preeclampsia have mutations in their renin-angiotensin and coagulation and fibrinolysis systems. In addition, women that do carry such genetic variants frequently have completely normal, healthy pregnancies. These observations indicate that a second (or third, or fourth) hit is needed for developing the condition.

An additional hit in the setting of preeclampsia could include having an autoimmune disease. Preeclampsia as well as other pregnancy complications are highly prevalent among women with the autoimmune diseases antiphospholipid syndrome and systemic lupus erythematoses.^{1,3,4} **CHAPTER 3** is devoted to the mechanisms underlying pregnancy complications in women with these autoimmune diseases. Murine models show that complement activation plays a pivotal role in antiphospholipid antibody-mediated pregnancy morbidity.⁵ However, the exact pathways of complement activation and their potential role in human pregnancy are insufficiently understood. We hypothesized that the classical complement pathway plays a major role in pregnancy complications in women with the autoimmune diseases antiphospholipid syndrome

and systemic lupus erythematoses.

C4d, a stable marker of the classical- and mannose-binding lectin pathways, was used to study complement activation in murine as well as human placentas. In this study, we first demonstrated that C4d deposits are present in areas of fetal-maternal exchange in placentas of mice pretreated with human IgG containing antiphospholipid antibodies. On the contrary, C4d was absent in mice treated with normal human IgG. The presence of C4d in the murine placenta was associated with an increased fetal resorption rate. These observations in mice were subsequently validated in human placentas, in which placental C4d deposits were frequently and almost exclusively present around syncytiotrophoblast cells of placentas from patients with autoimmune diseases. In these patients, the presence of placental C4d was strongly associated with adverse pregnancy outcomes, including intra-uterine fetal death, preeclampsia and the HELLP syndrome. The presence of C1q (specific for the classical pathway) in the absence of MBL (indicating mannose-binding lectin pathway activation) suggests that the presence of C4d is most likely the result of classical pathway activation.

Altogether, the study described in chapter 3 underlines the importance of complement activation in mediating pregnancy complications in women with the autoimmune diseases antiphospholipid syndrome and systemic lupus erythematoses. At the same time, it confirms the relation between autoimmune disease and preeclampsia. The combination of these observations led to the hypothesis that placental complement activation may also be involved in the pathogenesis of preeclampsia. The experiments that were used to test this hypothesis are described in Chapters 4 and 5.

CHAPTER 4 describes the initial study that was set up to investigate the relation between preeclampsia and placental complement activation. Placentas from women with preeclampsia as well as controls were stained for C4d. In this study, C4d was frequently and almost exclusively observed in placentas of women with

preeclampsia. Importantly, the distribution of C4d in these preeclamptic placentas was highly similar to that in placentas from women with antiphospholipid-syndrome-associated pregnancy complications, suggesting a similar pathophysiological mechanism. However, in the setting of preeclampsia the underlying cause of placental complement activation is less clear than in women with autoimmune diseases, in which the presence of auto-antibodies is a likely cause of classical pathway activation. To further explore putative causes of placental complement activation, we measured the placental mRNA levels of several complement regulatory proteins by quantitative polymerase chain reaction. The placental mRNA levels of the complement regulatory proteins CD55 and CD59 were found to be significantly higher among women with preeclampsia as compared to healthy control subjects. This finding was not in line with our hypothesis that placental complement deposits could be the result of inadequate complement regulation. However, there may still be a relative shortage of complement inhibition. Another option is that the complement regulatory proteins are defective due to the presence of mutations.⁶ Altogether, this chapter has demonstrated that preeclampsia is strongly associated with placental complement dysregulation.

In addition, the results described in this chapter show that among women with preeclampsia the presence of C4d is related to a significantly lower gestational age at delivery. This finding suggests a relation between placental complement activation and the severity of preeclampsia. However, based on the limited number of placentas included in this study, relatively little can be concluded about the association between placental complement activation and the clinical manifestations of preeclampsia.

CHAPTER 5 further explores the relation between the extent of placental dysfunction in preeclampsia and placental complement activation. We collected a second series of placentas derived from preeclamptic pregnancies and linked the presence of placental C4d

to several clinical manifestations and placental histopathological hallmarks of preeclampsia. Among women with preeclampsia, the presence of placental C4d was associated with a significantly lower gestational age at delivery, increased rates of fetal loss, and significantly lower placenta weight percentiles. In addition, the presence of C4d was associated with a significantly higher incidence of several histopathological manifestations of reduced placental perfusion. Altogether, the presence of placental C4d appears to be related to placental injury and dysfunction in the setting of preeclampsia. However, it remains unknown whether placental complement activation is an early event in the pathogenesis of preeclampsia, or rather a late consequence or even an epiphenomenon.

Regardless the cause of preeclampsia, the end result is a dysfunctional placenta that releases excessive amounts of anti-angiogenic factors. Pregnancy—and preeclamptic pregnancy in particular—is characterized by a systemic increase in the levels of anti-angiogenic factors, including sFlt-1.⁷ The placenta is an important source of circulating sFlt-1. However, it remains disputable how sFlt-1 is released from the placenta, as its heparin-binding site causes sFlt-1 to have a strong avidity for extracellular matrix.⁸ A putative mechanism by which sFlt-1 reaches the maternal circulation is through the dissociation of placental material—called syncytial knots.⁸ **CHAPTER 6** explores this mechanism. The experiments described in this chapter show that syncytial knots are the main production site of sFlt-1. To study the systemic spread of this placental material, we investigated the presence of placental material in lungs of women who died during pregnancy. Syncytial aggregates were frequently observed in maternal lungs and importantly, preeclampsia was associated with a significantly higher number of syncytial aggregates within maternal lung tissue. In these syncytial aggregates, we demonstrated co-localization of the syncytiotrophoblast-specific marker hCG, the anti-angiogenic protein sFlt-1 as well as the Y-chromosome. These

observations make it very unlikely that putative placenta-derived multinucleated aggregates within the maternal lung are not of fetal origin. The observation that syncytial aggregates still contain anti-angiogenic proteins following entrapment in maternal lungs, suggests that syncytial aggregates may form an autonomous source of sFlt-1 within the maternal circulation. Through the release of sFlt-1, syncytial aggregates could contribute to the systemic endothelial dysfunction that characterizes preeclampsia. Further studies are warranted to investigate whether this shedding of placental material could also affect long-term maternal health, for example through the development of (micro-)chimerism. In addition, further research is needed to elucidate the mechanisms underlying sFlt-1 production and syncytial knot formation.

Questions arising from this thesis

The main questions that arise from this thesis are: How do the genetic, immunologic and angiogenic aspects of preeclampsia together contribute to the endothelial dysfunction that characterizes preeclampsia? What is cause and what is consequence? This general discussion will describe putative mechanisms by which the genetic, immunologic and angiogenic aspects of preeclampsia may be linked in the pathogenesis of preeclampsia.

CONSEQUENCES OF A GENETIC PREDISPOSITION As outlined in the introduction, early-onset preeclampsia is frequently associated with profound placental dysfunction, whereas late-onset preeclampsia is generally related to preexistent endothelial dysfunction in the mother. The meta-analysis described in Chapter 2 has indicated that genetic variants in the renin-angiotensin system and the coagulation and fibrinolysis system are significantly associated with preeclampsia. The observed association between preeclampsia and specific genetic variants raises the question: how do these genetic

variants contribute to the pathogenesis of preeclampsia?

Genetic variants could contribute to the maternal susceptibility related to late-onset preeclampsia. For instance, genetic variants in the renin-angiotensin system predispose to hypertension.^{9,10} Hypertension is in itself a risk factor for preeclampsia^{1,11} and therefore genetic variants in the renin angiotensin system could—through hypertension—increase the risk for developing preeclampsia.

Genetic variants could also predispose to placental dysfunction, thereby contributing to the development of early-onset preeclampsia. Genetic variants in the coagulation and fibrinolysis system may increase the risk for developing preeclampsia by affecting trophoblast growth and differentiation. This idea is supported by the observation that activated coagulation factors induce cell death and growth inhibition of placental trophoblast cells.¹² In a thrombomodulin knock-out mouse model, activation of the blood coagulation cascade at the fetal-maternal interface leads to embryonic lethality, indicating that a delicate balance between coagulation and fibrinolysis is essential for the maintenance of pregnancy.¹² These observations support the idea that genetic variants in the coagulation and fibrinolysis system can contribute to development of pregnancy complications, including preeclampsia. However, it remains largely unknown through which mechanism(s) excessive activation of the blood coagulation cascade causes pregnancy complications. Importantly, activation of the coagulation cascade has been linked to complement activation¹³, also in the setting of pregnancy complications. Mice injected with antiphospholipid-antibodies show increased fetal loss as well as excessive amounts of complement component C3b, neutrophil infiltration and increased tissue factor expression in their deciduas. However, no increases in fibrin and no thrombi are observed in these deciduas, suggesting that it is not thrombosis that mediates antiphospholipid-antibody induced fetal loss. In this murine model, binding of antiphospholipid-antibodies to trophoblast leads to the generation of the complement component

C5a. Binding of this potent anaphylatoxin to its receptor on neutrophils results in tissue factor expression on these neutrophils. Tissue factor expression contributes to oxidative burst, thereby potentiating neutrophil-mediated trophoblast injury. Importantly, blockade of tissue factor prevents antiphospholipid-antibody induced inflammation and fetal loss.^{14,15} These murine models suggest that the coagulation cascade as well as the complement system play important roles in the development of placenta-mediated pregnancy complications.

COMPLEMENT IN PREECLAMPSIA; CAUSE OR CONSEQUENCE?

It is certain that genetic variants in the renin-angiotensin system and the coagulation and fibrinolysis system are present before the onset of preeclampsia. In other words, these mutations can be involved in the pathogenesis of preeclampsia, but they can never be a consequence of the condition. The distinction between cause and consequence is more difficult in case of placental complement activation. Chapter 4 describes that preeclampsia is associated with increased complement activation as compared to healthy pregnant controls. However, it remains disputable whether complement activation is an early event in the pathogenesis of preeclampsia, or rather a consequence of the condition.

Complement activation could be a late event in the pathogenesis of preeclampsia as ischemia-reperfusion injury, as well as apoptotic tissue, can activate the classical pathway.^{16,17} Generalized endothelial dysfunction—for instance in the setting of diabetes mellitus, hypertension and obesity—may lead to episodes of placental underperfusion. Placental underperfusion and subsequent reperfusion may result in the generation of reactive oxygen species, consequently leading to placental injury and placental complement activation.

However, murine models of preeclampsia suggest that complement activation may also be an early event in the pathogenesis of preeclampsia. In the DBA/2 x CBA/J mouse model—which

spontaneously develops the key features of human preeclampsia, such as renal and placental manifestations as well as angiogenic dysregulation—complement inhibition prevents oxidative stress and placental dysfunction, as well as proteinuria and the renal pathologic features of preeclampsia.¹⁸ This indicates that complement has a crucial role in the development of preeclampsia-associated features in these mice. Importantly, inhibiting complement prevented placental abnormalities, suggesting that complement activation is involved in abnormal placentation. The idea that complement activation is an early event in the pathogenesis of human preeclampsia is further supported by an early rise in complement components in women destined to develop pregnancy complications.^{19,20}

PLACENTAL COMPLEMENT ACTIVATION: EXCESSIVE

ACTIVATION OR INADEQUATE REGULATION? A proposed mechanism that may underlie placental complement activation in the setting of preeclampsia is the binding of (auto-)antibodies to placental tissue. This concept is similar to pregnancy complications in women with the autoimmune diseases systemic lupus erythematosus and antiphospholipid syndrome, in which auto-antibodies bind directly to trophoblast, thereby activating the classical complement pathway. This leads to placental injury and complications like preeclampsia, IUGR and fetal loss. A putative cause of placental classical pathway activation in the setting of preeclampsia includes circulating AT1 auto-antibodies.²¹ These auto-antibodies are directed against the angiotensin II type I receptor, which is expressed at the syncytiotrophoblast. In a murine model of preeclampsia, administration of these antibodies leads to the typical manifestations of preeclampsia.²²

It is important to note that not all women with preeclampsia have circulating antibodies directed against the angiotensin II type I receptor. In these women, another putative cause of placental complement activation should be considered. Circulating anti-HLA antibodies could be involved in the pathogenesis of preeclampsia.

However, the presence of these antibodies is not always associated with adverse pregnancy outcomes.^{23,24} In women with circulating anti-HLA antibodies, classical pathway activation is possibly successfully repressed by regulatory mechanisms. It is likely that tissue damage and adverse pregnancy outcomes only occur when antibody-induced complement activation overwhelms the protective function of complement regulatory proteins.

Murine studies have underscored the importance of complement regulation during pregnancy. Embryos deficient in Crry—a murine complement regulator—are characterized by compromised survival, and their placentas show marked C3 depositions and inflammation. Mice that are deficient in both Crry (Crry-/-) and C3 (C3-/-) are rescued from lethality.²⁵ This elegantly demonstrates that complement activation at the fetal-maternal interface causes fetal loss. It also underlines the critical role of complement regulation in preventing complement-mediated damage and adverse pregnancy outcomes. In this light, the presence of mutations in complement regulatory proteins could play an important role in the pathogenesis of preeclampsia.

COMPLEMENT ACTIVATION IN RELATION TO ANGIOGENIC DYSREGULATION

Importantly, complement activation is also linked to angiogenic dysregulation. In murine models of preeclampsia, activation of the complement system results in increased levels of circulating sFlt-1, whereas complement inhibition prevents this.^{22,26} In addition, in vitro stimulation of monocytes with complement activation products triggers the release of sFlt-1.²⁶ These observations suggest that angiogenic dysregulation is a consequence of complement activation. However, it may also be the other way around. Circulating sFlt-1 sequesters VEGF so preeclampsia is associated with a shortage of VEGF. Reduced VEGF levels are associated with decreased levels of factor H, an important regulator of the complement system. Therefore, increased sFlt-1 levels may lead to excessive complement activation, as the complement system

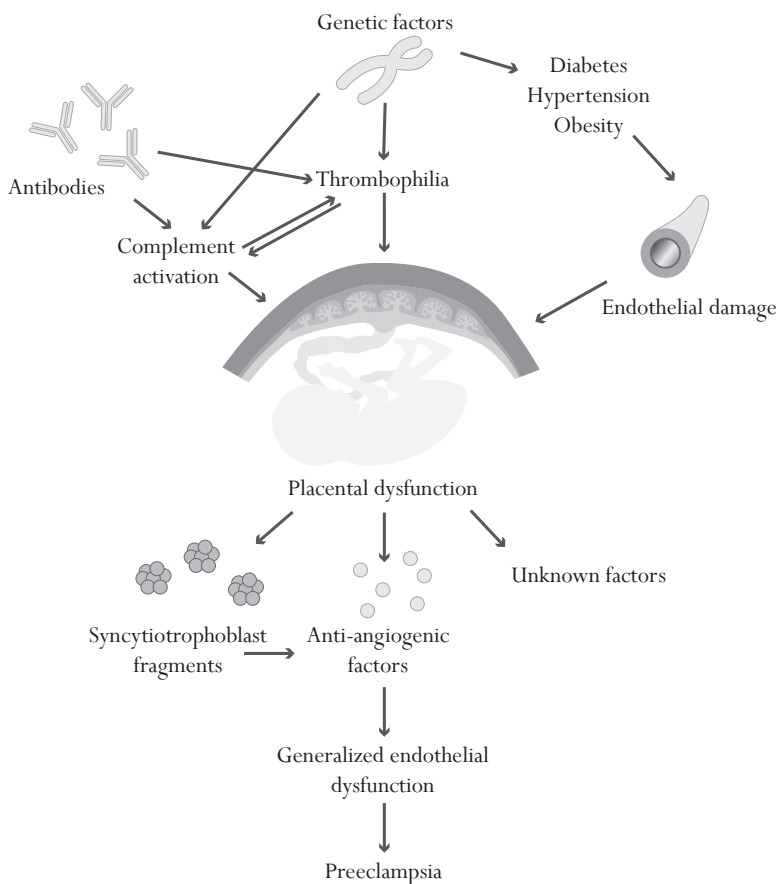
is inadequately regulated due to lower factor H levels.²⁷ In this thesis, an association between placental complement activation and the presence of increased syncytial knots was described. Syncytial knots are the main placental production site of sFlt-1.⁸ Although it remains uncertain how placental complement activation is related to the development of syncytial knots, the association indicates another link between the complement system and angiogenic dysregulation.

THE ROLE OF (PREEXISTENT) ENDOTHELIAL DYSFUNCTION

In addition to placental dysfunction, preexistent endothelial dysfunction also plays an important role in the development of preeclampsia. Importantly, there is overlap in sFlt-1 levels between preeclamptic women and healthy controls, and for this reason sFlt-1 levels in themselves are not very useful in predicting who will and who will not develop preeclampsia.⁷ Apparently, certain pregnant women remain perfectly healthy despite high sFlt-1 levels, whereas others are more sensitive to (even low) levels of sFlt-1 and develop preeclampsia. The same principle is observed in cancer patients who receive anti-VEGF therapy. A subgroup of patients develops hypertension and proteinuria, whereas others tolerate the systemic decrease in VEGF (which is functionally similar to increasing sFlt-1) well.²⁸ A small subset even develops a severe thrombotic microangiopathy in the kidney.²⁹ The question that arises is: why are some patients so sensitive to lower VEGF levels, whereas others tolerate it well? In the setting of preeclampsia, perhaps the women with most preexistent (vascular) damage are most sensitive to rises in sFlt-1 levels. Preexistent endothelial injury—such as in patients with diabetes mellitus, hypertension and obesity—predisposes to preeclampsia. Women with such preexistent endothelial injury may be more sensitive to the stress that pregnancy imposes on the body. The (physiological) rise in sFlt-1 may aggravate endothelial damage, thereby providing the additional hit that is necessary to develop preeclampsia. The idea that preexistent damage causes an excessive sensitivity to sFlt-1 is supported by experiments in a VEGF-knockout

mouse model. In this model, VEGF knockout in itself does not cause much damage, but extensive injury is observed when VEGF knockout is combined with the induction of diabetes.³⁰

PREECLAMPSIA: A MULTIPLE-HIT PATHOPHYSIOLOGY The mouse model described above elegantly illustrates that multiple hits are needed to cause injury. The same principle probably applies to preeclampsia, in which a combination of many different risk factors is needed to develop the condition. In this thesis, genetic, immunologic and angiogenic aspects of preeclampsia were investigated. These risk factors are individually not sufficient to cause preeclampsia; an interplay between different risk factors is needed to develop the condition. An important aspect in the development of preeclampsia could be the existence of positive feedback loops. For instance, complement activation induces sFlt-1 production, while at the same time an excess of sFlt-1 could lead to even more complement activation. Similarly, endothelial dysfunction could contribute to abnormal placental development, which in turn leads to excessive sFlt-1 production. Indeed, excessive amounts of circulating sFlt-1 aggravate endothelial damage, thereby further compromising the placental function. The possible interactions between the different risk factors for preeclampsia are shown in the figure alongside.



A proposed scheme on how the genetic, immunologic and angiogenic aspects could be linked in the pathogenesis of preeclampsia.

FUTURE PERSPECTIVES The links visualized in the proposed model are to be confirmed in further studies. In addition, little is known about the relevance and the relative contribution of the genetic and immunologic aspects in the pathogenesis of preeclampsia. Importantly, it is likely that the etiology of preeclampsia differs among patients. However, all women with preeclampsia have in common that they present with endothelial dysfunction and angiogenic dysregulation, and many are characterized by complement dysregulation. Therapeutic strategies may be targeted towards these aspects of the disease. Indeed, anti-sFlt-1 therapy has successfully been applied in a small number of women with preeclampsia, resulting in a prolongation of pregnancy.³¹ In addition, eculizumab—a targeted inhibitor of complement protein C5—has recently been administered to a woman with preeclampsia as well as the HELLP syndrome. This resulted in clinical improvement, complete normalization of lab parameters and prolongation of pregnancy.³² Further research is warranted to validate these findings in larger clinical studies.

REFERENCES

1. Duckitt K, Harrington D. Risk factors for pre-eclampsia at antenatal booking: systematic review of controlled studies. *BMJ* 2005; 330:565.
2. Roten LT, Johnson MP, Forsmo S, Fitzpatrick E, Dyer TD, Brennecke SP *et al.* Association between the candidate susceptibility gene ACVR2A on chromosome 2q22 and pre-eclampsia in a large Norwegian population-based study (the HUNT study). *Eur J Hum Genet* 2009; 17:250-257.
3. Cohen D, Berger SP, Steup-Beekman GM, Bloemenkamp KW, Bajema IM. Diagnosis and management of the antiphospholipid syndrome. *BMJ* 2010; 340:c2541.
4. Heilmann L, Schorsch M, Hahn T, Fareed J. Antiphospholipid syndrome and pre-eclampsia. *Semin Thromb Hemost* 2011; 37:141-145.
5. Girardi G, Berman J, Redecha P, Spruce L, Thurman JM, Kraus D *et al.* Complement C5a receptors and neutrophils mediate fetal injury in the antiphospholipid syndrome. *J Clin Invest* 2003; 112:1644-1654.
6. Salmon JE, Heuser C, Triebwasser M, Liszewski MK, Kavanagh D, Roumenina L *et al.* Mutations in complement regulatory proteins predispose to preeclampsia: a genetic analysis of the PROMISSE cohort. *PLoS Med* 2011; 8:e1001013.
7. Levine RJ, Maynard SE, Qian C, Lim KH, England LJ, Yu KF *et al.* Circulating angiogenic factors and the risk of preeclampsia. *N Engl J Med* 2004; 350:672-683.
8. Rajakumar A, Cerdeira AS, Rana S, Zsengeller Z, Edmunds L, Jeyabalan A *et al.* Transcriptionally active syncytial aggregates in the maternal circulation may contribute to circulating soluble fms-like tyrosine kinase 1 in preeclampsia. *Hypertension* 2012; 59:256-264.
9. Kunz R, Kreutz R, Beige J, Distler A, Sharma AM. Association between the angiotensinogen 235T-variant and essential hypertension in whites: a systematic review and methodological appraisal. *Hypertension* 1997; 30:1331-1337.
10. Sayed-Tabatabaei FA, Oostra BA, Isaacs A, van Duijn CM, Witterman JC. ACE polymorphisms. *Circ Res* 2006; 98:1123-1133.
11. Steegers EA, von Dadelszen P, Duvekot JJ, Pijnenborg R. Pre-eclampsia. *Lancet* 2010; 376:631-644.
12. Isermann B, Sood R, Pawlinski R, Zogg M, Kalloway S, Degen JL *et al.* The thrombomodulin-protein C system is essential for the maintenance of pregnancy. *Nat Med* 2003; 9:331-337.
13. Oikonomopoulou K, Ricklin D, Ward PA, Lambris JD. Interactions between coagulation and complement--their role in inflammation. *Semin Immunopathol* 2012; 34:151-165.
14. Redecha P, Tilley R, Tencati M, Salmon JE, Kirchhofer D, Mackman N *et al.* Tissue factor: a link between C5a and neutrophil activation in antiphospholipid antibody induced fetal injury. *Blood* 2007; 110:2423-2431.
15. Redecha P, van Rooijen N, Torry D, Girardi G. Pravastatin prevents miscarriages in mice: role of tissue factor in placental and fetal injury. *Blood* 2009; 113:4101-4109.
16. Nauta AJ, Trouw LA, Daha MR, Tijssma O, Nieuwland R, Schwaebler WJ *et al.* Direct binding of C1q to apoptotic cells and cell blebs induces comple-

- ment activation. *Eur J Immunol* 2002; 32:1726-1736.
17. Weiser MR, Williams JP, Moore FD, Jr., Kobzik L, Ma M, Hechtman HB *et al.* Reperfusion injury of ischemic skeletal muscle is mediated by natural antibody and complement. *J Exp Med* 1996; 183:2343-2348.
 18. Qing X, Redecha PB, Burmeister MA, Tomlinson S, D'Agati VD, Davison RL *et al.* Targeted inhibition of complement activation prevents features of preeclampsia in mice. *Kidney Int* 2011; 79:331-339.
 19. Lynch AM, Murphy JR, Byers T, Gibbs RS, Neville MC, Giclas PC *et al.* Alternative complement pathway activation fragment Bb in early pregnancy as a predictor of preeclampsia. *Am J Obstet Gynecol* 2008; 198:385-389.
 20. Lynch AM, Gibbs RS, Murphy JR, Giclas PC, Salmon JE, Holers VM. Early elevations of the complement activation fragment C3a and adverse pregnancy outcomes. *Obstet Gynecol* 2011; 117:75-83.
 21. Dechend R, Muller DN, Wallukat G, Homuth V, Krause M, Dudenhausen J *et al.* Activating auto-antibodies against the AT1 receptor in preeclampsia. *Autoimmun Rev* 2005; 4:61-65.
 22. Wang W, Irani RA, Zhang Y, Ramin SM, Blackwell SC, Tao L *et al.* Autoantibody-Mediated Complement C3a Receptor Activation Contributes to the Pathogenesis of Preeclampsia. *Hypertension* 2012; 60:712-21.
 23. Balasch J, Ercilla G, Vanrell JA, Vives J, Gonzalez-Merlo J. Effects of HLA antibodies on pregnancy. *Obstet Gynecol* 1981; 57:444-446.
 24. Regan L, Braude PR, Hill DP. A prospective study of the incidence, time of appearance and significance of anti-paternal lymphocytotoxic antibodies in human pregnancy. *Hum Reprod* 1991; 6:294-298.
 25. Xu C, Mao D, Holers VM, Palanca B, Cheng AM, Molina H. A critical role for murine complement regulator crry in fetomaternal tolerance. *Science* 2000; 287:498-501.
 26. Girardi G, Yarilin D, Thurman JM, Holers VM, Salmon JE. Complement activation induces dysregulation of angiogenic factors and causes fetal rejection and growth restriction. *J Exp Med* 2006; 203:2165-2175.
 27. Keir LS, Welsh G, Coward R, Richards A, Spooner RA, Saleem M. The Podocyte is the Initial Target in the Renal Pathogenesis of Diarrhoea-Associated Haemolytic Uraemic Syndrome. [abstract] SA-PO2344, ASN Kidney Week 2011.
 28. Eskens FA, Verweij J. The clinical toxicity profile of vascular endothelial growth factor (VEGF) and vascular endothelial growth factor receptor (VEGFR) targeting angiogenesis inhibitors; a review. *Eur J Cancer* 2006; 42:3127-3139.
 29. Eremina V, Jefferson JA, Kowalewska J, Hochster H, Haas M, Weisstuch J *et al.* VEGF inhibition and renal thrombotic microangiopathy. *N Engl J Med* 2008; 358:1129-1136.
 30. Sivaskandarajah GA, Jeansson M, Maezawa Y, Eremina V, Baelde HJ, Quaggin SE. Vegfa protects the glomerular microvasculature in diabetes. *Diabetes* 2012; 61:2958-2966.
 31. Thadhani R, Kisner T, Hagmann H, Bossung V, Noack S, Schaarschmidt W *et al.* Pilot study of extracorporeal removal of soluble fms-like tyrosine kinase 1 in preeclampsia. *Circulation* 2011; 124:940-950.

32. Burwick RM, Feinberg BB. Eculizumab for the treatment of preeclampsia/HELLP syndrome. *Placenta* Published Online First: 8 December 2012. doi: 10.1016/j.placenta.2012.11.014.

NEDERLANDSE SAMENVATTING

Preeclampsie & de placenta

Preeclampsie—in de volksmond bekend onder de naam zwangerschapsvergiftiging—is een aandoening die uitsluitend voorkomt bij zwangere vrouwen. De beginfase van preeclampsie is asymptomatisch; de zwangere vrouw merkt nog niks en ook haar bloeddruk is normaal. In dit stadium is bij sommige vrouwen al een afwijkende ontwikkeling van de placenta vast te stellen, terwijl dit bij anderen minder op de voorgrond staat. In een later stadium van de ziekte gaat de placenta anti-angiogenetische factoren produceren. Dit zijn stoffen die de groei en het onderhoud van bloedvaten remmen. Deze anti-angiogenetische factoren komen in het bloed van de moeder terecht en leiden daar tot schade aan de bloedvaten. Deze schade resulteert uiteindelijk in het ontstaan van de kenmerkende symptomen van preeclampsie; hoge bloeddruk en eiwitverlies in de urine. Het is onbekend wat precies ten grondslag ligt aan de afwijkende ontwikkeling van de placenta bij vrouwen met preeclampsie. Ook is niet geheel duidelijk wat de oorzaak is van preeclampsie bij vrouwen zonder uitgesproken placenta dysfunctie. Wel is uit epidemiologisch onderzoek gebleken dat er een aantal belangrijke risicofactoren bestaan voor preeclampsie, waaronder het hebben van een auto-immuunziekte, het voorkomen van preeclampsie in de familie- en/of voorgeschiedenis en de aanwezigheid van condities die geassocieerd zijn met vaatschade, zoals hoge bloeddruk, diabetes mellitus en obesitas. Deze diversiteit aan risicofactoren suggereert dat het waarschijnlijk niet mogelijk is om één oorzaak van preeclampsie aan te wijzen; het is veel waarschijnlijker dat een combinatie van verschillende factoren ten grondslag ligt aan het ziektebeeld. Deze diversiteit aan mogelijke oorzaken komt terug in dit proefschrift: de genetische, immunologische en angiogenetische aspecten van preeclampsie komen aan bod.

HOOFDSTUK 2 gaat in op de genetische aspecten van preeclampsie. Dit hoofdstuk beschrijft een meta-analyse van genetische associatie studies die zijn uitgevoerd in het kader van preeclampsie. Het feit dat preeclampsie vaker voorkomt in bepaalde families suggereert dat een genetische component bijdraagt aan het ontstaan van de ziekte. Om de genetische achtergrond van preeclampsie te ontrafelen, zijn een groot aantal genetische associatie studies uitgevoerd. In dit soort studies wordt onderzocht of een mutatie in een zogenaamd kandidaat gen—een gen dat op basis van de huidige kennis over preeclampsie mogelijk een rol zou kunnen spelen—vaker voorkomt bij vrouwen met preeclampsie dan bij gezonde controles. Veel van deze studies zijn echter uitgevoerd met relatief kleine aantallen patiënten en controles, waardoor de resultaten van de individuele studies soms onbetrouwbaar en lastig te interpreteren zijn. Een meta-analyse kan worden gebruikt om resultaten uit meerdere individuele studies samen te voegen om zo tot een meer robuust resultaat te komen. De vraag behorende bij dit hoofdstuk was: welke genetische varianten zijn gereproduceerd en significant geassocieerd met preeclampsie na meta-analyse? Uit de meta-analyse bleek dat vooral genetische varianten die te maken hebben met stolling en bloeddrukregulatie geassocieerd zijn met preeclampsie. Hieruit kan geconcludeerd worden dat deze systemen waarschijnlijk een rol spelen in het ontstaan van preeclampsie. Het blijft speculeren hoe de individuele genetische varianten het risico op preeclampsie precies beïnvloeden. Het is in ieder geval wel duidelijk dat het hebben van een bepaalde genetische variant niet voldoende is om tot preeclampsie te leiden. Er zijn namelijk vrouwen die wel een bepaalde genetische variant hebben maar nooit preeclampsie krijgen, terwijl andere vrouwen zonder ‘risico’ variant wel preeclampsie ontwikkelen. Kortom, waarschijnlijk is een combinatie van verschillende risicofactoren nodig om de ziekte te ontwikkelen.

HOOFDSTUK 3 gaat over een andere risicofactor voor het ontwikkelen van preeclampsie, namelijk het hebben van een auto-immuunziekte, zoals het antifosfolipiden syndroom en/of systemische lupus erythematoses. Muismodellen hebben laten zien dat het complement systeem—een onderdeel van het immuunsysteem—een belangrijke rol speelt bij het ontstaan van zwangerschapscomplicaties in het kader van auto-immuunziekten. Over de mechanismen in de humane zwangerschap is echter minder bekend. Dit hoofdstuk beschrijft de experimenten die zijn uitgevoerd om uit te zoeken welke route van het complement systeem geactiveerd is in placenta's van vrouwen met bovengenoemde auto-immuunziektes. Hiervoor werden placenta's van vrouwen met auto-immuunziekten vergeleken met placenta's van gezonde controles. Het bleek dat de aanwezigheid van C4d—een stabiele marker voor complement activatie—zelden aanwezig was in placenta's van gezonde controles. Ook liet deze studie zien dat de aanwezigheid van C4d sterk geassocieerd is met intra-uteriene vruchtdood in vrouwen met auto-immuunziekten. Hieruit kan geconcludeerd worden dat klassieke route activatie in de placenta waarschijnlijk een rol speelt bij het ontstaan van zwangerschapscomplicaties bij vrouwen met auto-immuunziekten. In deze studie kwam preeclampsie bovendien relatief vaak voor onder vrouwen met de auto-immuunziekten antifosfolipiden syndroom en systemische lupus erythematoses.

Het was al langer bekend dat preeclampsie sterk geassocieerd is met deze auto-immuunziekten. Tegelijkertijd weten we inmiddels dat het complement systeem een belangrijke rol speelt bij het ontstaan van zwangerschapscomplicaties in vrouwen met auto-immuunziekten. Deze twee observaties samen hebben geleid tot de hypothese dat complement activatie in de placenta ook een rol zou kunnen spelen bij het ontstaan van preeclampsie.

De experimenten die werden uitgevoerd om deze hypothese te toetsen, zijn beschreven in **HOOFDSTUK 4**. De belangrijkste boodschap van dit hoofdstuk is dat er een sterke associatie bestaat tussen preeclampsie en de aanwezigheid van C4d—een belangrijke marker voor complement activatie. Een tweede boodschap van dit hoofdstuk is dat niet alleen activatie van het complement systeem is toegenomen bij preeclampsie maar dat ook de regulatie van het complement systeem veranderd is. Preeclampsie bleek namelijk geassocieerd te zijn met een sterke upregulatie van twee belangrijke complement remmers in de placenta.

De experimenten beschreven in hoofdstuk 4 werden uitgevoerd met relatief kleine patiënt- en controle groepen. Hierdoor was het moeilijk om relaties tussen complement activatie en de ernst van preeclampsie vast te stellen. De relatie tussen complement activatie en de ernst van preeclampsie werd verder onderzocht in de studie beschreven in **HOOFDSTUK 5**. Voor deze studie werden meer dan honderd placenta's van vrouwen met preeclampsie verzameld. Vervolgens werd onderzocht of de aanwezigheid van C4d in deze placenta's gerelateerd is aan de klinische manifestaties van preeclampsie. De belangrijkste boodschap van dit hoofdstuk is dat onder vrouwen met preeclampsie de aanwezigheid van C4d in de placenta geassocieerd is met de mate van placenta dysfunctie. Vergeleken met preeclamptische vrouwen zonder C4d, hebben vrouwen met preeclampsie én C4d een kortere zwangerschapsduur, een hogere incidentie van intra-uteriene vruchtdood, significant kleinere placenta's en meer tekenen van een slechte doorbloeding van de placenta. Hieruit kan geconcludeerd worden dat complement activatie in de placenta gerelateerd is aan de mate van placenta dysfunctie.

Uiteindelijk is preeclampsie een ziekte van de placenta, waarbij alle risicofactoren er waarschijnlijk gezamenlijk voor zorgen dat de placenta slecht wordt aangelegd. Het resultaat is dat de placenta anti-angiogenetische factoren (zoals sFlt-1) gaat produceren. De experimenten beschreven in **HOOFDSTUK 6** gaan in op de angiogenetische aspecten van preeclampsie. Het idee is dat sFlt-1 vanuit de placenta wordt uitgescheiden in de bloedsomloop van de moeder, alwaar het voor schade aan de bloedvaten zorgt. Het is nog niet helemaal duidelijk hoe sFlt-1 in het bloed van de moeder terecht komt. Een recente hypothese stelt dat klompjes cellen van de placenta—de zogenaamde syncytiale knoppen—worden losgelaten in het bloed van de moeder. Op deze knoppen bevinden zich de anti-angiogenetische factoren. Hoofdstuk 6 laat zien dat de knoppen binnen de placenta de belangrijkste productie bron zijn van sFlt-1. Deze knoppen worden inderdaad in de bloedsomloop van de moeder losgelaten, aangezien ze terug te vinden zijn in de longen van vrouwen die zijn overleden aan preeclampsie. Het zou goed kunnen dat het circuleren van deze stukjes placenta ervoor zorgt dat een vrouw met preeclampsie ook ná de bevalling nog complicaties van preeclampsie kan ontwikkelen.

De bevindingen beschreven in dit proefschrift laten zien dat zowel genetische, immunologische als angiogenetische aspecten een rol spelen bij preeclampsie. Verder onderzoek zal nodig zijn om de relaties tussen deze verschillende aspecten te ontrafelen en om uit te zoeken wat oorzaak en wat gevolg is in het ontstaan van preeclampsie.

CURRICULUM VITAE

Aletta Buurma werd op 26 november 1988 geboren te Lelystad. In 2001 werd zij toegelaten tot het tweetalig vwo aan het Alfrink College te Zoetermeer. Vier jaar later kreeg zij de kans om deel te nemen aan het Pre-University College van de Universiteit Leiden. In 2007 behaalde zij haar eindexamen vwo, het International Baccalaureate English A2 en het Pre-University College diploma. Datzelfde jaar begon zij aan de opleiding Geneeskunde aan het Leids Universitair Medisch Centrum (LUMC).

Tijdens haar studie Geneeskunde nam zij deel aan een intracurriculaire uitwisseling met het Karolinska Institutet te Stockholm, Zweden. Hierna werd zij toegelaten tot het Excellente Studenten Traject en begon zij haar onderzoek op de afdeling Pathologie van het LUMC. Onder leiding van dr. Hans Baelde, dr. Kitty Bloemenkamp en prof. dr. Jan Anthonie Bruijn werd de basis gelegd voor het huidige proefschrift. Eind 2010 ontving zij een beurs in het kader van het MD/PhD-traject van het LUMC. Met deze beurs kon zij, na het voltooien van de pre-klinische fase in 2011, gedurende twee jaar voltijd onderzoek verrichten.

In februari 2013 begon zij aan haar co-schappen en in december 2014 hoopt zij het artsexamen te behalen.

Aletta Buurma was born on the 26th of November 1988 in Lelystad, the Netherlands. In 2001 she was admitted to the Alfrink College secondary school in Zoetermeer. Four years later, she was given the opportunity to participate in Pre-University College at the University of Leiden. In 2007 she graduated from secondary school and also received the International Baccalaureate English A2 and the Pre-University College certificate.

That same year, she started medical school at the Leiden University Medical Center (LUMC). During her medical study, she participated in an intracurricular exchange with the Karolinska Institutet, Stockholm, Sweden. After this, she was awarded a research scholarship within the Excellent Student Program of the LUMC and she started her research project at the department of Pathology, LUMC. Under the guidance of dr. Hans Baelde, dr. Kitty Bloemenkamp and prof. dr. Jan Anthonie Bruijn the foundation of the current thesis was built. In 2010, she received a scholarship within the MD/ PhD Program of the LUMC. This scholarship gave her the opportunity to work fulltime as a PhD student for two years.

In February 2013 she started her clinical rotations and she hopes to receive her Medical Degree in December 2014.

BIBLIOGRAPHY

Buurma A*, Penning ME*, Prins F, Schutte JM, Bruijn JA, Wilhelmus S, Rajakumar A, Bloemenkamp KW, Karumachi SA, Baelde HJ. Preeclampsia is associated with the presence of transcriptionally active placental fragments in the maternal lung. *Hypertension*, in press.

Buurma A, Turner RJ, Driessen JH, Mooyaart AL, Schoones JW, Bruijn JA, Bloemenkamp KW, Dekkers OM, Baelde HJ. Genetic variants in pre-eclampsia: a meta-analysis. *Hum Reprod Update* 2013; 19(3):289-303

Buurma A, Cohen D, Veraar K, Schonkeren D, Claas FH, Bruijn JA, Bloemenkamp KW, Baelde HJ. Preeclampsia is characterized by placental complement dysregulation. *Hypertension* 2012; 60(5):1332-1337.

Cohen D, **Buurma A**, Goemaere NN, Girardi G, le Cessie S, Scherjon S, Bloemenkamp KW, de Heer E, Bruijn JA, Bajema IM. Classical complement activation as a footprint for murine and human antiphospholipid antibody-induced fetal loss. *J Pathol* 2011; 225(4):502-511.

Buurma A, Cohen D. Ovariectomy of ovaria in situ laten bij hysterectomie? *Ned Tijdschr Geneesk* 2011; 155:A3673

*Both authors contributed equally.



Stellingen

BEHOREND BIJ HET PROEFSCHRIFT: **ON THE PATHOLOGY OF PREECLAMPSIA. GENETIC VARIANTS, COMPLEMENT DYSREGULATION, AND ANGIOGENESIS.**

1. Genetic variants in the coagulation and fibrinolysis system as well as the renin-angiotensin system are associated with preeclampsia. (*this thesis*)
2. Placental C4d is strongly related to fetal loss in the setting of the autoimmune diseases systemic lupus erythematosus and antiphospholipid syndrome. (*this thesis*)
3. Preeclampsia is associated with placental complement dysregulation. (*this thesis*)
4. Presence of C4d in the preeclamptic placenta is related to placental dysfunction and injury. (*this thesis*)
5. sFlt-1 loaded placental material is retained in maternal lungs, and particularly in those of preeclamptic women. (*this thesis*)
6. The placenta—and particularly the diseased placenta—sends out a variety of messages to the mother.
7. Like no snowflake in an avalanche ever feels responsible, no single factor can be held accountable for the development of preeclampsia.
8. Autoimmune mechanisms may participate in the pathophysiology of preeclampsia.

9. A researcher must train herself to notice what she sees.

10. Intelligence without ambition is a bird without wings.
(*Salvador Dali, 1904-1989*)

11. If it is important to you, you will find a way. If not, you will find an excuse.

12. The grass is greener where you water it.

13. Your work is going to fill a large part of your life, and the only way to be truly satisfied is to love what you do. If you haven't found it yet, keep looking. Don't settle. As with all matters of the heart, you'll know when you find it.
(*Steve Jobs, Stanford commencement address, 2005*)