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Endothelial pathology in preeclampsia

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ENDOTHELIAL PATHOLOGY IN PREECLAMPSIA

Rosanne J. Turner

Endothelial pathology in preeclampsia

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Colophon

The studies described in this thesis were performed at the Department of Pathology (Head: Prof. Dr. V.T.H.B.M. Smit) of the Leiden University Medical Center, the Netherlands

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Endothelial pathology in preeclampsia

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Chapter 1

General introduction and outline of thesis

GENERAL INTRODUCTION AND OUTLINE OF THESIS

Preeclampsia is a frequent complication of pregnancy that involves one of the largest organs in the human body: the vascular system. Likewise, the hallmarks of this syndrome are hypertension and kidney dysfunction.[1] This thesis focuses on endothelial pathology in preeclampsia, and, specifically, on elucidating the interplay between angiogenesis, coagulation and inflammation in the development of the syndrome.

Preeclampsia used to be defined as the development of hypertension, systolic blood pressure >140 mmHg or diastolic blood pressure >90 mmHg, after 20 weeks gestation, measured at least twice on separate occasions, and onset of proteinuria.[1, 2] Preeclampsia is generally known as a relatively mild and frequent pregnancy complication: the incidence of preeclampsia varies from 1 to 5%.[3, 4] However, the course of the disease can worsen abruptly and hypertensive disorders of pregnancy account for nearly 18% of all maternal deaths worldwide;[5] complications such as renal insufficiency, liver dysfunction, neurological complications (e.g. eclampsia and stroke), thrombocytopenia, haemolysis and foetal growth impairment can develop and may require intervention.[1] To account for the wide spectrum of complications that can develop with preeclampsia, in 2014, the definition of preeclampsia was updated to hypertension developing after 20 weeks gestation and the coexistence of at least one of the following complications: proteinuria, renal insufficiency, liver involvement, neurological- or haematological complications, or foetal growth restriction.[1] Since the cohorts collected for the work in this thesis were collected before 2014, they fulfil the criteria of the former definition of preeclampsia, which is mentioned and cited throughout the chapters in this thesis.

The current treatment of preeclampsia is purely symptomatic. Women with mild preeclampsia are monitored closely. MgSO4 has to be commenced as prevention of eclampsia and blood pressure has to be lowered by administering antihypertensive therapy; calcium channel antagonists and beta blockers are used to lower blood pressure when this exceeds 160 mmHg systolic blood pressure or 110 mmHg diastolic blood pressure to prevent cerebrovascular complications.[6] Drugs targeting the renin-angiotensin-aldosterone system (RAAS) might seem a logical therapeutic option because of

their positive effect on kidney function, but cannot be used during pregnancy because of their potential adverse effects when used in second or third trimesters of pregnancy; foetal development is impaired through placental hypoperfusion and through direct effects of angiotensin-converting enzyme blockers or angiotensin II receptor blockers on foetal kidney development.[7]

Currently, the only cure for preeclampsia is delivery of the placenta, and, inevitably, the foetus. To postpone delivery in women with preeclampsia at <37 weeks gestation, patients are closely monitored and high blood pressure is treated with antihypertensive agents. However, delivery is necessary when maternal or foetal complications develop, e.g. inability to control maternal blood pressure, progressive symptoms of HELLP syndrome (hemolysis, elevated liver enzymes and low platelet count syndrome) or eclampsia, placental abruption or signs of worsening foetal condition.[1]

Likewise, the incidence of preterm birth, spontaneous or iatrogenic, is higher in pregnancies complicated by preeclampsia as compared to uncomplicated pregnancies.[8] Severe preeclampsia has been reported to increase the risk of birth before 33 weeks of gestation 80 times.[9] Preterm birth is associated with several adverse health outcomes, e.g. neonatal mortality, pulmonary and cardiovascular complications, such as diabetes and obesity, and neurodevelopmental disturbances.[10, 11] In addition, female offspring born to a pregnancy complicated by hypertension has a higher risk of placental insufficiency during their own pregnancies as well, thereby passing the risk for hypertension from generation to generation.[12] The seriousness of the complications of the syndrome and its treatment (preterm birth) call for better treatment options that target the syndrome upstream in its pathogenesis. However, to do so, a better understanding of the pathways leading up to the syndrome has to be established.

The first part of this introductory chapter (*part 1 - the two-stage aetiology of preeclampsia*) describes the changes in the placenta and the maternal endothelium during the development of the syndrome. This part focuses in particular on the interplay between these two organs. The second part (*part 2 - risk factors for developing preeclampsia*) concentrates on known risk factors for developing preeclampsia: clues for the pathogenesis of the

syndrome may lie within these risk factors. The risk factors within the genetic and lifestyle compartments mainly involve endothelial dysfunction, and especially coagulation and inflammation. Therefore, part 3 of this introduction (*part 3 - vascular pathology and hypercoagulation*) describes the role of the coagulation system in preeclampsia and part 4 (part 4 - *immunologic imbalance in preeclampsia*) focuses on abnormalities in the functioning of the immune system in preeclampsia. Subsequently, part 5 (*part 5: thrombomodulin; a mediator of inflammation and coagulation*) will describe a pathway that regulates both inflammation and coagulation, and that could potentially be a new player in preeclampsia. Part 6 (*part 6 - kidney involvement in preeclampsia*) will elaborate on the organ mainly affected by preeclampsia, the kidney, and in particular on the possible interplay between placental factors and kidney dysfunction. The last chapter (*part 7 - this thesis*) will describe the research questions that arise from the previous chapters and that comprise the work performed in this thesis, followed by an outline of thesis chapters.

PART 1 - THE TWO-STAGE AETIOLOGY OF PREECLAMPSIA

Impaired placental function in preeclampsia

The placenta is an organ exclusively formed for pregnancy. It forms the sole surface where contact between foetus and mother is established, and where exchange of oxygen and nutrients can take place. The placenta consists of a maternal and a foetal part. The maternal part is composed of the decidua: this is former endometrium, the lining of the uterus that has grown and has become vascularized to facilitate implantation of the blastocyst. The foetal part consists of trophoblast cells derived from the outer layer of the blastocyst: they invade the decidua and build the branching structure of the villous tree. Villi are thin, protruding portions of the foetal part of the placenta. They float in the maternal decidual blood, where their specialized outer cell lining, the syncytiotrophoblast, composed of a syncytium of trophoblast cells, facilitates the exchange of oxygen and nutrients.[13]

To ensure sufficient blood supply to this fast-growing organ, the arteries in the decidua and myometrium, the “spiral arteries” undergo substantial changes during early pregnancy. Foetal trophoblast cells invade the spiral arteries and replace the internal elastic lamina and underlying smooth muscle

layer by loose, fibrinoid matrix.[14] This results in wider spiral arteries with a lower resistance, leading to increased placental blood flow with a low arterial pressure. In preeclampsia, this process appears to fail: the lumen of the decidual arteries remains narrow and smooth muscle cells remains present.[15] This supposedly leads to underperfusion of the placenta from week 12 of pregnancy.[16, 17]

This underperfusion leads to hypoxia and ischemia reperfusion injury in the developing placenta, resulting in impaired growth of both the foetus and the placenta. For the foetus, there is an increased risk of growth restriction or even stillbirth. In the placenta, changes typical for oxidative stress are seen. The villi show immature ageing, often with maldeveloped, small vessels.[13] The most striking observation is the increase of syncytial knots, aggregations of syncytial nuclei, that can detach and get launched into the maternal circulation.[18] Probably as a result of the oxidative stress, the protein synthesis in the trophoblast cells gets disorganized, and the placenta produces excessive levels of anti-angiogenic factors soluble FMS-like tyrosine kinase 1 (sFlt-1) and soluble endoglin (sEng).[19] These factors bind to vascular endothelial growth factor (VEGF) and tumour growth factor beta (TGF-B), respectively, and prevent them from binding to their corresponding receptors on endothelial cells. These cytokines are essential for normal vascular function and a decrease in their availability plays a key role in the development of endothelial dysfunction.[20, 21]

Systemic endothelial dysfunction in preeclampsia

The placenta may be the source of preeclampsia, as its removal is the cure of the syndrome, but the cells directly responsible for its manifestation in the pregnant mother are the endothelial cells. Endothelial cells make up the lining of all blood vessels in the body; they form a selective barrier between the circulating blood and the underlying tissue. They can also regulate blood pressure by producing vasoactive substances and communicate with the smooth muscle cells in the underlying vessel wall. Further, when threatening situations for the circulation or tissue arise, endothelial cells can become *activated*. For instance, when the circulatory loop gets interrupted by damage to a vessel, the endothelial cells initiate the formation of a blood clot to prevent blood leaking out of the vessel.[20] Endothelial cells also assist in the

fight against pathogens: when activated, they attract cells or components of the immune system by expressing chemoattractant molecules.[20]

Under unthreatening, basic circumstances, these functions of the endothelium are delicately balanced. However, endothelial cell signalling is disturbed in preeclampsia and the endothelial cells appear to become excessively activated throughout all vessels in the mother's body: this is called systemic endothelial dysfunction.[19] The endothelial activation results in increased production of vasoactive substances, such as endothelin-1, and the smooth muscle cells in the vessel walls contract, giving rise to hypertension.[22] This vasoconstriction can even lead to tissue necrosis in liver, heart and brain in extreme cases.[23] Further, the endothelial cells are in a hyperinflammatory state, as confirmed by increased levels of cytokines in maternal serum.[24, 25] Coagulation is excessively activated, leading to overuse of coagulation factors and subsequent low levels of platelets as seen in the HELLP syndrome.[26] These mechanisms are explored in-depth in parts 3 and 4 of this introductory chapter.

Contributing to this endothelial dysfunction is the placenta, with its production of excessive levels of anti-angiogenic factors sFlt-1 and sEng. The resulting decreased availability of VEGF and TGF-B is detrimental to endothelial cell signalling. VEGF, specifically, is essential for endothelial cell survival, proliferation, migration and adequate regulation of permeability.[27] TGF-B is involved in intracellular survival pathways as well and regulates vascular tone and inflammatory pathways.[28] Disruption of the balance between pro- and anti-angiogenic factors both in animal models and in patients receiving anti-angiogenic treatment results in the development of signs resembling preeclampsia, e.g. hypertension and proteinuria.[21, 29, 30] Restoring the angiogenic imbalance through treatment with TGF-B or VEGF might seem a logical option, but levels of angiogenic factors have to be maintained in a small window: high levels of angiogenic factors are linked with inflammation and kidney disease.[31, 32]

PART 2 - RISK FACTORS FOR DEVELOPING PREECLAMPSIA

Given the above, the aetiology of preeclampsia comprises deviations of normal adaptations to pregnancy in both maternal (e.g. decidual arteries and endothelium) and foetal (placenta) organs. However, the precise pathogenesis of the syndrome has not been elucidated yet. Clues for the pathophysiology of a disease often lie within its risk factors as they can reveal the physical pathways involved. Therefore, this chapter will give an overview of the risk factors discovered for preeclampsia so far.

Genetic predisposition

Preeclampsia has a clear genetic component. Having a family history of preeclampsia gives a 3 fold increased risk of developing the syndrome.[33] Consequently, many studies investigating possible genes associated with preeclampsia have been performed. The first large set of mutations to be associated with preeclampsia lies within genes from the coagulation and fibrinolytic systems; women with inherited thrombophilia have a higher risk of developing the syndrome.[34] For example, a mutation in the prothrombin gene, enhancing its function, has been reported frequently.[35-37] Also, mutations in the gene encoding for factor V, making it less susceptible to inactivation by protein C, and leading to excessive activation of the coagulation cascade, are associated with the syndrome.[35, 37] Mutations within the immune system form the second group; both mutations in the innate immune system, such as in the complement system (e.g. complement factor H and factor C3),[38, 39] and mutations in signalling of the adaptive immune system, such as in the interleukin family, have been reported to be associated with preeclampsia.[40, 41] Further, mutations in the STOX1-gene, involved in trophoblast function and implantation of the placenta, showed a strong correlation with severe preeclampsia in a Dutch patient cohort.[42] In other populations these results could not be replicated.[43]

Despite the clear genetic component, no gene has been proven to be the sole cause of preeclampsia; the syndrome is a multifactorial disease. Further, genome-wide association studies on preeclampsia show inconsistent results.[44] This directs the search for risk factors of preeclampsia further to non-genetic maternal and foetal characteristics associated with preeclampsia.

Maternal and foetal characteristics

When investigating maternal predisposing factors, vascular pathology appears to be the most prominent again. In particular, pre-existing medical conditions affecting the cardiovascular system increase the risk of developing preeclampsia. Pre-existing diabetes quadruples the risk, and hypertension increases the risk of developing preeclampsia up to 7 times.[33] Interestingly, women who suffered from preeclampsia are also at greater risk for developing cardiovascular disease later in life. There appears to be an underlying cause, perhaps genetic, enhancing the risk of both preeclampsia and cardiovascular disease in this women, with preeclampsia acting as a second hit, accelerating vascular damage.[45] Intriguingly, the incidence of preeclampsia has increased over the last few decades, indicating that globally the Western lifestyle of the modern world has its impact on the syndrome.[46] Obesity indeed increases the risk of developing preeclampsia up to five times.[33] Weight loss in obese women restores this risk to the general population's risk.[47] Obesity is associated with endothelial dysfunction, e.g. excessive inflammation, resistance to insulin signalling, apoptosis, and even with an angiogenic imbalance.[48]

On the other end of the spectrum, the immunologic risk factors appear. Nulliparity triples the risk for preeclampsia; as well as new paternity and limited exposure to a partner's semen prior to conception.[10, 33, 49] These associations suggest an immunological mechanism, where later pregnancies are protected against a reaction to paternal antigens through immunomodulatory mechanisms.[48] The immunological basis of preeclampsia is strengthened by the association between autoimmune diseases, such as systemic lupus erythematosus and antiphospholipid antibody syndrome, and preeclampsia.[50, 51] Further, women who became pregnant after receiving oocyte donation, and who subsequently carry a completely allogeneic foetus, are also at increased risk of developing preeclampsia.[52] The immunologic risk factors and mechanisms are elaborated in Part 4 of this introduction.

In conclusion, both genetic and acquired risk factors for preeclampsia fall apart in two categories: vascular and immunologic risk factors. The following two chapters will discuss the role of each of these systems in preeclampsia in depth.

PART 3 - VASCULAR PATHOLOGY AND HYPERCOAGULATION IN PRE-ECLAMPSIA

The role of the haemostatic system in preeclampsia started to raise interest in the first half of the 20th century. Physicians then first started to notice large thrombi in brain and liver tissue from women who died of 'toxaemia of pregnancy', i.e. preeclampsia.[53] Furthermore, pathological changes in organs from women who died after preeclampsia resemble damage due to reduced perfusion, showing infarction, necrosis and deposition of microthrombi.[54]
But how do these thrombi develop? The following chapter describes the pathophysiology of thrombosis, and elaborates on the specific pathways of haemostasis that play a role in preeclampsia.
The pathophysiology of thrombosis rests on three pillars: endothelial injury, abnormal blood flow and hypercoagulability. This triad is known as "Virchow's triad", named after one of the founders of thrombosis research, Rudolph Virchow (1821-1902). It depicts the interplay between the three components that increase the risk of the formation of a thrombus, a plug of activated blood platelets held together by a network of fibrin that (partly) obstructs a blood vessel.

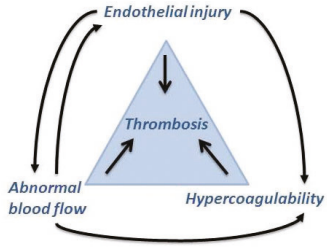

Figure 1, Virchow's triad
Virchow's triad depicts the interplay between the three factors leading to thrombosis.

The first pillar, endothelial injury, is considered to be a dominant part of the triad, since endothelial injury can initiate thrombosis on its own. At baseline conditions, the endothelium exhibits antithrombotic features. First, the endothelium has antiplatelet functions, which means that the endothelium prevents platelets from being activated by extracellular matrix, and inhibits

platelet adhesion through producing prostacyclin and nitric oxide. Second, several membrane-associated proteins on the endothelium possess anticoagulant properties, such as the protein C activators thrombomodulin and the endothelial protein C receptor. Lastly, the endothelium also produces tissue plasminogen activator, which clears fibrin deposits and assists in fibrinolysis (degradation of a thrombus). However, when endothelial cells are activated, i.e. under inflammatory conditions, they exhibit opposite, prothrombotic properties. Von Willebrand factor expressed by endothelial cells helps platelets to adhere to the extracellular matrix underlying the endothelium, activating the platelets. Activated endothelial cells also express tissue factor, the activator of the extrinsic coagulation cascade (see below), which results in the formation of a fibrin network. Lastly, activated endothelial cells produce plasminogen activator inhibitor, which suppresses fibrinolysis.[20]

The second pillar consists of abnormal blood flow. Normal blood flow is laminar: the blood flows in layers moving parallel to the blood vessel, with the middle layer, which contains the platelets, having the highest velocity and the outer layers (or "clear zone"), consisting of plasma alone, having a velocity reaching zero. Alterations of blood flow, caused for example by hypertension or stenosis, lead to disturbance of this laminar flow, causing turbulence and stasis. Turbulence facilitates the movement of platelets from the middle layer to the outer layer, the clear zone. This brings the platelets close to the endothelium and subsequently facilitates platelet activation. Turbulent flow can also damage the endothelium, leading directly to endothelial activation, exposure of underlying extracellular matrix and platelet activation. On the other hand, the corresponding stasis of blood (e.g. behind a stenosis) leads to accumulation of clotting factors and subsequent excessive activation of the coagulation cascade. In addition to the latter, endothelial activation and subsequent contraction of the vessel wall can further contribute to the development of abnormal blood flow by decreasing the vessel lumen. In summary, we can conclude that the first two pillars of the triad, endothelial injury and abnormal blood flow, are tightly intertwined.[20]
The third pillar, hypercoagulability, is defined as an alteration of the coagulation pathways that predisposes to thrombosis.[20] The coagulation pathways contribute to the formation of a thrombus by being ultimately

responsible from the conversion from fibrinogen to fibrin. The two pathways consist of several clotting factors, which are produced in the liver, and activate each other in the form of a cascade, as illustrated in Figure 2.

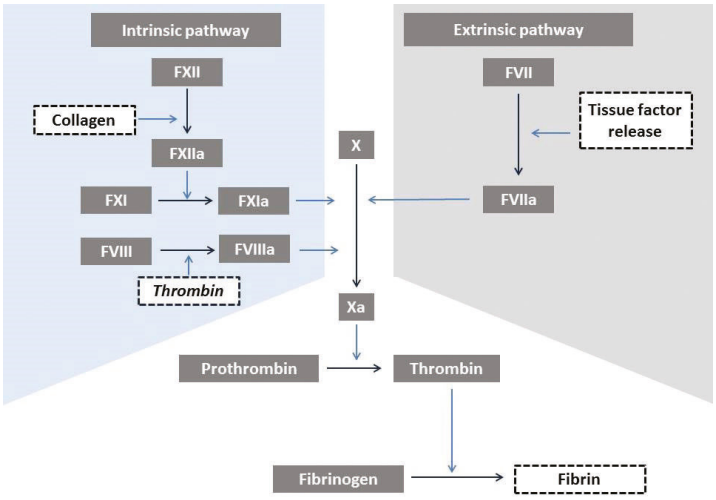

Figure 2, two pathways leading to coagulation

This figure illustrates the two pathways initiating coagulation and their final common pathway. Coagulation can be activated through the intrinsic pathway, after collagen exposure, or through the extrinsic pathway, after release of tissue factor from the subendothelial space. Coagulation cascade activation ultimately results in the formation of fibrin strands which form the framework for the blood clot.[20]

The extrinsic pathway of coagulation is the most physiologically relevant pathway for coagulation occurring after vascular damage. It is initiated by tissue factor, a protein released from the subendothelial space after endothelial damage. Tissue factor release results in the activation of the extrinsic coagulation pathway; through FVII and FX activation, prothrombin is converted to thrombin and fibrinogen is subsequently converted to fibrin monomers. Tissue factor is abundantly expressed on the placenta, in trophoblastic tissue and in amniotic fluid.[55] The second coagulation pathway, the intrinsic pathway, is activated when FXII (Hageman Factor) is exposed to thrombogenic surfaces, such as collagen. It appears only logical that the

cascade of activation of clotting factors has to be retained to the site of vascular injury. Here, the endothelium plays a leading role by expressing anticoagulant and fibrinolytic factors, as described above.

Systemic coagulation in preeclampsia

In preeclampsia, each of the three pillars of Virchow's triad is disrupted. As described in the introductory paragraph, endothelial dysfunction is characteristic for preeclampsia. This contributes to abnormal blood flow, i.e. hypertension. Further, endothelial activation contributes to hypercoagulability. Pregnancy itself is a hypercoagulable state.[56] Levels of all clotting factors increase, and there is a reduction in concentrations of the anticoagulants protein C and protein S.[56] These changes are presumed to be a preparation for the haemostatic challenge of childbirth.[57] However, in preeclampsia the hypercoagulable state is exaggerated, characterized by increased levels of key coagulation markers tissue factor, factor VIII consumption, fibrinogen and von Willebrand factor compared to normal pregnancy.[57] The anticoagulation system is also disrupted, as shown by reduced antithrombin levels and a decreased ratio of tissue factor pathway inhibitor and tissue factor.[57] Ultimately, preeclampsia can even be complicated by disseminated intravascular coagulation (DIC).[58] DIC results from diffuse activation of intravascular coagulation, leading to excessive consumption of coagulation factors and subsequent haemorrhage, e.g. in the brain.[1]

Hypercoagulation in the placenta

Both the observance of the thrombi-prone state in women with preeclampsia and the link with inherited thrombophilia in these patients have led to hypotheses about the role of thrombi and coagulation in the development of preeclampsia, i.e. in the development of placental dysfunction; decidual thrombi have been proposed to be within the aetiology of preeclampsia as early as 1947.[59] The flow in the placental bed reaches almost zero, which, following Virchow's triad, should increase the risk of haemostasis. But still, the "endothelial" like syncytiotrophoblast cells lining the villi succeed to prevent excessive coagulation in normal pregnancies. In preeclampsia there is increased deposition of perivillous fibrin, indicative of failure to prevent placental coagulation and expression of the thrombin receptor PAR-1 is intensified.[60, 61] The amount of placental infarction is associated with the

severity of preeclampsia.[62] In the decidual vessels, thrombosis is associated with perinatal mortality in preeclampsia.[63] So, taken all the above examples of excessive coagulation in the maternal vessels and the placenta into account, hypercoagulation is a key feature of the pathogenesis of preeclampsia.

Treatment & coagulation

The long-established association between coagulation and preeclampsia has led to many clinical trials on the use of anticoagulants for the treatment and prevention of the syndrome, but no ultimate solution has been found so far. Drugs from the heparin family have been studied extensively; heparin acts through enhancing the effectiveness of the anticoagulant antithrombin, and also through inhibition of the complement system (this is described in-detail in part 3).[64] Modest effects from using low-molecular weight heparin for the secondary prevention of preeclampsia have been reported; the Dutch FRUIT study revealed a risk reduction of 8.7% for the onset of hypertensive disorders before 34 weeks of pregnancy.[65, 66] Another well-known drug, aspirin, also has shown positive but modest effects (RR 0.9, 95% CI 0.84 – 0.97) on preeclampsia occurrence and improves pregnancy outcome.[67, 68] The positive effects of aspirin might lie within its double mode of action: it blocks platelet aggregation, and also modulates inflammatory responses by decreasing prostaglandin production, reflecting the multifactorial aetiology of preeclampsia. Trials on the new anticoagulants, e.g. rivaroxaban or dabigatran cannot be initiated because of their potential teratogenic effects; animal studies have revealed early pregnancy loss and foetal harm.[69]

PART 4 - IMMUNOLOGIC IMBALANCE IN PREECLAMPSIA

Pregnancy is a semi-allogeneic situation where the maternal immune system encounters paternal and foetal antigens. To achieve successful embryo-implantation and pregnancy, a tolerant immune environment has to be established, with changes in both the innate and adaptive immune systems. Further, both innate and adaptive immune cells are involved in early stages of placental development; they assist in controlled removal of native spiral artery cells, allowing the trophoblast cells to invade.[70] In preeclampsia, changes in this tolerant, constructive state of the immune system have been described. This chapter will describe each component of the immune system involved in the pathogenesis of preeclampsia, from innate to adaptive immunity.

Innate immunity: the complement system

The complement system is the initiator of activity of the innate immune system. Its pathways are activated by immune complexes, microbial carbohydrates and pathogen surfaces.[20] Completion of each the three pathways of the complement cascade results in the formation of the membrane attack complex, which results in cell lysis, cell destruction by phagocytes and the production of the anaphylatoxins C3a and C5a, activators of chemotaxis, cytokine production and vascular permeability.[71] The complement system can even be activated spontaneously via the alternative pathway. In normal pregnancy, the expression of complement-regulatory proteins by the syncytiotrophoblast is sufficient to prevent excess complement activation.[72, 73] However, in preeclampsia, there is an increase in complement pathway activation in the placenta, as detected by increased abundance of the marker of complement activation C4d, and this is associated with lower gestational age at birth.[74] Although immunological markers in maternal serum do not reflect the immune response at the fetomaternal interface completely,[75] markers of excessive complement activation in preeclampsia are found in maternal serum as well.[75, 76] Further, preeclampsia is associated with complement deposition in the kidney.[77] Further proof for complement system activation as a cause for preeclampsia comes from a study by Qing et al; placenta-specific inhibition of the final complement enactor C3 in a mouse model prevented placental dysfunction but also systemic symptoms of preeclampsia, such as proteinuria and renal pathology.[78]

Macrophages and dendritic cells as immunomodulators

Decidual macrophages play an immunomodulatory role in placenta by maintaining the balance between pro-inflammatory Th1 cells and anti-inflammatory Th2 cells and by producing anti-inflammatory cytokines IL4 and IL10.[73] They also facilitate implantation of the embryo and placenta tissue remodelling by clearing apoptotic cells and promoting trophoblast cell survival.[79] During normal pregnancies, decidual macrophages polarize towards the M2 macrophage type, which repairs tissue and inhibits inflammation.[73, 80] A shift towards dominance of the pro-inflammatory M1 type has been proposed to play a role in pathological pregnancies such as preeclampsia.[81] For example, a regulator of M2 polarization, sHLAG5, is decreased tremendously in preeclampsia.[82] Additionally, these immature dendritic cells induce immunological tolerance and activate T regulatory cells.[73] Disturbances in the maturation of dendritic cells have been described in the HELLP syndrome.[83] T regulatory cells decrease the activity of CD4 T-cells and prevent an immune reaction to HLA DR antigens that are released from trophoblast cell debris. Decreased presence of T regulatory cells in blood and in the decidua are associated with recurrent miscarriages.[73]

Natural killer cells assist in trophoblast invasion

During preparation for embryo implantation, numbers of uterine natural killer cells rapidly increase. These cells are of the "CD56 bright" phenotype, i.e. they produce cytokines involved in trophoblast invasion and angiogenesis instead of being cytotoxic.[73] HLA-C on the trophoblast cells plays a major role in the recognition of trophoblast cells by uterine NK cells during trophoblast invasion. Failure of this recognition process is associated with impaired placentation and certain combinations of variants of uterine NK cell receptors and HLA-C are associated with preeclampsia.[73] Uterine NK cells are associated with immature dendritic cells remaining abundant in the decidua throughout pregnancy.

The adaptive immune system

Lastly, the humoral adaptive immune system plays a role in the development of preeclampsia as well. In preeclampsia, there is a shift from maternal Th2 to Th1 cell activity.[73] The cytokines produced by Th1 cells contribute to B-cell autoantibody production.[70] Preeclampsia is associated with abundance of a

subset of B-cells prone to synthesizing autoantibodies throughout pregnancy.[73] The syndrome has been linked to angiotensin II receptor antibody production, which results in excessive stimulation of this receptor in the placenta and subsequent production of anti-angiogenic factors.[73] Interestingly, the preparation of the maternal immune system for pregnancy even starts before implantation of the embryo, as a reaction by antigen presenting cells to seminal fluid.[84] A schematic overview of the role of the different components of the innate and adaptive immune systems in pregnancy and preeclampsia, as described above, is given below.

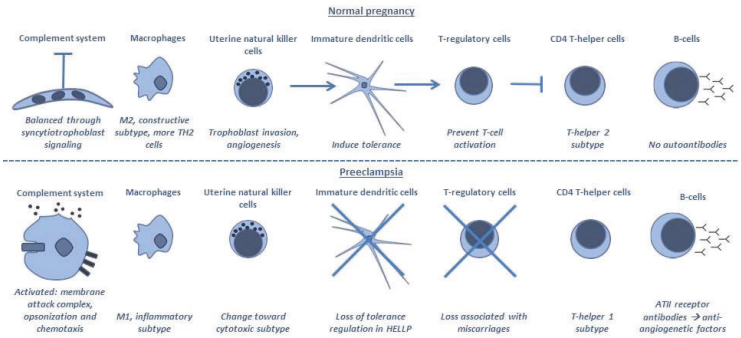

Figure 3, the inflammatory response in normal pregnancy compared to preeclampsia

Components from the innate and adaptive immune systems with their role in normal pregnancy and their changed activity in preeclampsia are depicted.

In summary, the immune system is involved in the early changes in the placenta in preeclampsia, illustrated by changes in placental immune cells and subsequent disturbances in placental growth, and also appears to be disturbed during later stages of preeclampsia, where systemic involvement develops, for example with increased systemic complement activation or autoantibodies. Despite the immune system being an obvious key player in preeclampsia, no effective treatment targeting the immune system has been discovered yet, in spite of abounding clinical trials targeting either these late or early effects.[85] These have been performed for dexamethasone, anti-oxidants and immunomodulating supplements such as vitamin D (which showed a small effect). A case report on inhibiting the complement system

in HELLP syndrome showed promising results in one case, but larger clinical trials have not been performed yet.[86]

PART 5 - THROMBOMODULIN: A MEDIATOR OF COAGULATION AND INFLAMMATION

Taking all of the above into consideration, both dysregulation of coagulation and the immune system appear to be major pathways leading up to preeclampsia. However, studies targeting the coagulation or immune systems independently do not seem to result in resolution of the syndrome yet. Other pathways, creating links between the angiogenic imbalance of preeclampsia, coagulation and inflammation might bring a new twist, and hopefully progression to preeclampsia research. One of these candidate pathways is the thrombomodulin signalling pathway; the following chapter will give an overview of this promising player in preeclampsia.

Thrombomodulin physiology

Thrombomodulin is a transmembrane glycoprotein expressed by the endothelium throughout the body and on the syncytiotrophoblast lining the placental villi.[87] Thrombomodulin signalling comprises three distinct pathways, as illustrated in Figure 4: modulation of coagulation, inflammation, and cell survival. In detail, thrombomodulin can form a complex with thrombin, thereby inactivating the latter, and thus inhibiting the coagulation cascade. Further, the thrombomodulin-thrombin complex facilitates activation of protein C by the endothelial protein C receptor, thereby inhibiting the coagulation cascade even further.[88] Thrombomodulin, when bound to thrombin, can also activate the intracellular PAR-1 receptor. The PAR-1 receptor is a versatile receptor that can stimulate opposite pathways, when activated by specific molecules. When activated by thrombomodulin, PAR-1 inhibits inflammatory and anti-angiogenic signalling by endothelial cells. Further, PAR-1 activation stimulates cell survival pathways. Thrombomodulin on its own can also inhibit inflammation through activation of TAFI, which inhibits the final products of the complement system.[88]

Thrombomodulin is essential for the development and maintenance of a healthy circulation; in animal models, thrombomodulin knockout results in embryonic lethality and massive thrombosis.[89, 90] Mutations in the

thrombomodulin gene are associated with the haemolytic uremic syndrome in human patients, a disease characterized by endothelial dysfunction and excessive complement activation.[91] Further, low levels of thrombomodulin are associated with increased apoptosis and albuminuria in diabetic nephropathy, and restoring thrombomodulin signalling returned apoptosis and albuminuria to levels similar as in control mice.[92]

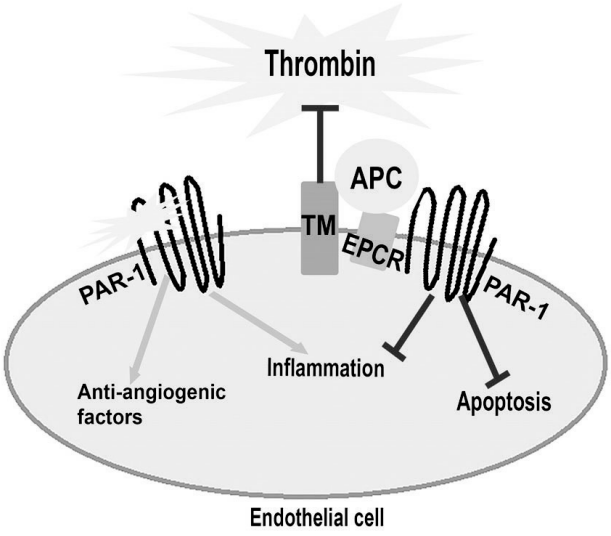

Figure 4, effects of thrombin and thrombomodulin signalling in endothelial cells
When thrombin (star figure) initiates signalling through PAR-1 in endothelial cells, intracellular pathways resulting in the release of anti-angiogenic factors and activation of inflammation are activated. In contrast, when thrombin binds to thrombomodulin, together with APC and EPCR, PAR-1 activation results in inhibition of inflammation and apoptosis pathways.
TM, thrombomodulin; APC, activated protein C; EPCR, endothelial protein C receptor

Thrombomodulin in disease

Thrombomodulin can be cleaved under inflammatory circumstances by metalloprotease-like proteins; the waste product can then be detected in serum as soluble thrombomodulin.[88, 93] Thrombomodulin cleaving does not appear to result in a functional soluble product.[88] However, soluble

thrombomodulin has been proven to be a useful biomarker of endothelial dysfunction, and is elevated in a variety of inflammatory conditions.[94-97] In preeclampsia, levels of soluble thrombomodulin increase as well.[98-100] Up to date, it is not known if the cleavage of thrombomodulin from the endothelium and syncytiotrophoblast indeed leads to loss of the thrombomodulin protein from the vessel surface, or if the cleavage is compensated with increased production of the protein. Nevertheless, thrombomodulin is an upcoming target for treatment in diseases characterized by dysregulation of inflammation and coagulation. Thrombomodulin has been proven to be effective, for example, in the treatment of sepsis and thrombotic thrombocytopenic purpura.[101, 102]

PART 6 - KIDNEY INVOLVEMENT IN PREECLAMPSIA

Kidney physiology

The kidneys are responsible for the homeostasis of a variety of vital functions. In short, the kidneys regulate excretion of metabolites, resorption of vital nutrients, acid-base homeostasis, blood osmolality, blood pressure and also excrete a variety of hormones involved in blood and bone maintenance.[20] Excretion of waste products is facilitated by the special structure of the filtration apparatus of the kidney: the glomerulus. A glomerulus consists of a clew of capillaries, supplied by an afferent and efferent arteriole. The hydrostatic pressure in the glomerulus is about 40 mmHg, resulting in a net filtration pressure of 20mmHg, the biggest net extravasation pressure in the human body.[103] On top of that, the capillaries are lined with specialized fenestrated endothelium, allowing small molecules and ions to pass through. The molecules that can pass then have to cross the negatively charged glomerular basement membrane that lies around the endothelium, thereby blocking the crossing of large proteins. Lastly, the filtrate has to pass the specialized epithelium of the glomerular filtration barrier; the podocytes. These epithelial-like cells are attached to the glomerular basement membrane with thousands of 'foot processes': long cell projections with slit-diaphragms in between that facilitate further selective crossing of molecules. The podocytes are presumed to be the most crucial component of the glomerular filtration barrier with respect to the retaining of proteins; damage to the podocytes is associated with the presence proteinuria.[103]

Signs of kidney disease in preeclampsia

The kidneys are major players in the manifestation of preeclampsia; failure of the kidneys to maintain their normal function are among the first symptoms women with preeclampsia present themselves with.[1] The disturbances in kidney function in preeclampsia first present as proteinuria.[1] This indicates failure of the kidneys to retain vital nutrients in the blood: the glomerular filtration barrier has lost its selectivity and proteins can go across and are excreted in the urine. This can result in nephrotic syndrome, where the low osmolality of the blood allows plasma to extravasate, resulting in swelling of feet and ankles.[104] When severe preeclampsia develops, kidney failure can deteriorate as well. The kidneys fail to excrete metabolites and levels of uric acid and other waste products in the blood increase. [105] Proteinuria and kidney failure in preeclampsia are generally reversible,[106] but a small number of women remain at an increased risk of developing kidney disease in later life.[107]

The histopathology of preeclamptic nephropathy

The background for the above-mentioned symptoms of preeclamptic nephropathy can be found with histopathological evaluation of the kidney from a patient with preeclampsia. The podocytes show pathologic changes on electron microscopy: their foot processes retract and lose their filtration function.[108] Further, podocyte turnover is increased and detached podocytes can be retrieved from the urine from women with preeclampsia, this is called podocyturia.[109] The endothelial cells show detrimental changes on light microscopy, called glomerular endotheliosis: they become swollen and can take up the whole capillary lumen, making the glomeruli appear 'bloodless'.[110]

Angiogenic imbalance and the kidney

The above gives rise to one big question: why is especially the kidney the number one target organ in preeclampsia? What pathogenesis could underlie these symptoms? All other organs of the body have blood vessels with dysfunctional endothelium as well, but they do not show dysfunction until much later in the course of the disease. The answer possibly lies within the particular dependence on VEGF of all cells in the glomerular filtration barrier. Both podocytes and glomerular endothelial cells need tightly regulated levels of available VEGF to maintain their function. Podocytes are the main source

of VEGF in the glomerulus; podocyte-specific knockout of VEGF expression results in endothelial and podocytal pathology.[111, 112] Animal models with overexpression of sFlt-1 and soluble endoglin develop renal changes somewhat similar to those in preeclampsia, for example with glomerular endothelial cell swelling and podocyte changes.[113] However, the angiogenic imbalance is not the sole explanation for the changes observed in preeclamptic nephropathy: excessive coagulation, endothelial damage or mechanical damage to the glomerular filtration barrier might be co-players in the aetiology.

PART 7 - THIS THESIS

Despite decades of research, it is still unclear what underlies the pathophysiologic mechanisms of and susceptibility for preeclampsia. Suitable management strategies have not been developed yet, probably because of the latter. Further research on the pathophysiology of preeclampsia might reveal new pathways to target.
This thesis touches upon the three main pathways leading to endothelial dysfunction in preeclampsia: angiogenesis, coagulation and inflammation. These pathways will be investigated on several levels: in the genome, in the placenta, and in the organs from women with preeclampsia.

In particular:

- The contribution of genetic variants in the development of preeclampsia
- The role of thrombomodulin and its modulating effects on inflammation and coagulation in preeclampsia
- The role of VEGF in kidney disease

Aims of this thesis

- To determine which genetic variants are reproducibly and significantly associated with preeclampsia
- To investigate the role of thrombomodulin in the development of placental dysfunction and the angiogenic imbalance in preeclampsia
- To explore the endothelial protective properties of thrombomodulin in the kidney during preeclamptic nephropathy
- To explore the interplay between vascular endothelial growth factor, the endothelium and podocytes in kidney pathology in preeclampsia

- To investigate whether alternative splicing of vascular endothelial growth factor plays a role in the development of renal damage

Thesis outline

Overall, the work described in this thesis focuses on the pathways leading to endothelial dysfunction in preeclampsia. Several genes involved in the regulation of coagulation and inflammation on the endothelium have been described in preeclampsia, but genome-wide association and other genetic studies yielded inconsistent results. Therefore, **chapter 2** of this thesis describes a meta-analysis performed to investigate which genetic variants are reproducibly and significantly associated with preeclampsia. Since a comprehensive amount of literature, as described in this introduction, as well as **chapter 2** of this thesis point towards coagulation and inflammation as major players in preeclampsia, chapter 3 and 4 focus on the role of a mediator of coagulation and inflammation, thrombomodulin, in this syndrome. **Chapter 3** describes the work performed to investigate the role of thrombomodulin in the placenta, and the possible connection between thrombomodulin and the angiogenic imbalance of preeclampsia. Since it is unknown if thrombomodulin cleaving under inflammatory circumstances leads to thrombomodulin loss on the endothelium, the role of thrombomodulin in the kidney in preeclampsia is explored in **chapter 4**. Besides coagulation and inflammation, the angiogenic imbalance appears to be the major role player in preeclampsia, especially in the kidney. In **chapter 5**, the cross-talk between the endothelium and podocytes through VEGF in the kidney in preeclampsia is explored. To further explore this cross-talk, we investigated how VEGF mRNA is spliced in the human and rodent kidney, and if this splicing is dysregulated during glomerulopathy. These experiments are described in **chapter 6**. In **chapter 7**, the general discussion, the discoveries from this thesis are summarized and placed in a general perspective. The general discussion is followed by a summary in Dutch.

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Chapter 2

Genetic Variants in Preeclampsia: a Meta-Analysis

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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 pre-eclampsia. We identified 22 replicated genetic variants, of which 7 remained significantly associated with pre-eclampsia 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 pre-eclampsia. Importantly, many of these variants are also risk factors for developing cardiovascular disease, revealing that pre-eclampsia and cardiovascular disease have shared genetic risk factors. The contribution of the identified genetic variants in the pathogenesis of pre-eclampsia 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 (Steegers *et al.*, 2010). Furthermore, women with preeclampsia have an increased risk of developing cardiovascular disease later in life (Bellamy *et al.*, 2007). 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 (Arngrimsson *et al.*, 1990, Chesley *et al.*, 1986, Cincotta *et al.*, 1998, Esplin *et al.*, 2001, Nilsson *et al.*, 2004, Sutherland *et al.*, 1981). Furthermore, the prevalence of preeclampsia differs between various ethnic groups (Steegers *et al.*, 2010). However, the underlying genetics are complex, and it is currently unclear which 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 (Chappell and Morgan, 2006). 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 (Ioannidis *et al.*, 2001). 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 (Brown et al., 2001). 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 (AB and RT)

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 (Higgins *et al.*, 2003). Bias due to small study size was tested using a stratified analysis for study size as described previously (Serrano *et al.*, 2006). 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 pre-eclampsia (Fig. 1). We identified 22 polymorphisms in 15 genes that were reproducibly associated with pre-eclampsia. 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 pre-eclampsia (Fig. 2a). The remaining 15 reproduced variants were not associated with preeclampsia following meta-analysis (Fig. 2b). The odds ratios of the significant associations with pre-eclampsia 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 Supplementary data, Table II show that some studies included only women with severe pre-eclampsia.

Genetic variants involved in coagulation and fibrinolysis

Five genetic variants in four genes that are related to coagulation and fibrinolysis were associated reproducibly with pre-eclampsia. Of these five variants, four were still associated with pre-eclampsia after the meta-

analysis. Two variants in coagulation factor V (FV), rs6025 and rs6020, remained associated with pre-eclampsia in the meta-analysis. The variant rs6025, which is also known as Factor V Leiden, was a frequently studied polymorphism in pre-eclampsia, 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 variant rs6020 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 variant rs1799963 of the coagulation factor II (F2) gene (also known as prothrombin) was investigated in 30 studies and was associated with pre-eclampsia 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 variant rs1799889 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 the 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 ≥200 cases.

Genetic variants involved in the renin-angiotensin system

The angiotensin I converting enzyme (ACE) rs4646994 variant has been studied frequently in pre-eclampsia, 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 ≥200 cases. Both rs699 and rs4762 variants in angiotensinogen (AGT) were studied in 21 and 5 studies, respectively. The variant rs699 was not associated with pre-eclampsia after meta-analysis, with a pooled odds ratio of 1.23 (95% CI 0.98–1.54). The variant rs4762 was also not associated with pre-eclampsia, 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 pre-eclampsia after meta-analysis.

Genetic variants involved in oxidative stress

Three variants in the nitric oxide synthase 3 (NOS3) gene were reproducibly associated with pre-eclampsia, but none was still associated with pre-eclampsia 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 pre-eclampsia 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 pre-eclampsia 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 pre-eclampsia but were not associated with pre-eclampsia 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 variants rs1800590 and rs268 in the lipoprotein lipase (LPL) gene were reproduced in pre-eclampsia, but only rs268 remained associated with pre-eclampsia 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 pre-eclampsia following the meta-analysis. The rs3025039 variant in the vascular endothelial growth factor (VEGF) gene was reproduced in pre-eclampsia, 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 pre-eclampsia. Meta-analysis for several individual genetic variants in the setting of pre-eclampsia has been performed previously. However, the present study provides the first complete and comprehensive overview of all genetic variants that are reproducibly associated with pre-eclampsia. These data may shed light on the pathogenesis of pre-eclampsia 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 pre-eclampsia. The results of this meta-analysis suggest that the following systems may play a role in the pathogenesis of pre-eclampsia: 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 pre-eclampsia.

One genetic variant involved in the renin–angiotensin system remained associated with pre-eclampsia 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 (Serrano et al., 2006). The ACE rs4646994 variant is known to be associated with increased activity of the angiotensinconverting enzyme (Sayed-Tabatabaei et al., 2006), which could increase the conversion of angiotensin I into angiotensin II, thus affecting the regulation of blood pressure and blood volume.

Pre-eclampsia is associated with an exaggerated maternal inflammatory response. Therefore, various candidate genes involved in inflammation have been studied in the setting of pre-eclampsia; only one genetic variant in CTLA-4 remained associated with pre-eclampsia after our meta-analysis. No previous meta-analysis of this variant in the setting of pre-eclampsia 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 (Teft et al., 2006; Sun et al., 2008). Carrying the G allele of CTLA-4 could contribute to the maternal

inflammatory response, thereby increasing the risk of developing pre-eclampsia.

With respect to genes involved in lipid metabolism, one variant in LPL remained associated with pre-eclampsia following the meta-analysis. No previous meta-analysis of this variant in the setting of pre-eclampsia has been published to date. The G allele of LPL rs268 is associated with reduced LPL activity and dyslipidemia (Fisher et al., 1997). Because dyslipidemia can contribute to endothelial cell dysfunction, carriers of the G allele may have an increased risk for developing pre-eclampsia (Mayret-Mesquiti et al., 2007).

After meta-analysis, several factors involved in coagulation and fibrinolysis remained associated with pre-eclampsia, which is largely in line with previous meta-analyses (Lin and August, 2005; Dudding et al., 2008). Normal pregnancy is associated with the development of a hypercoagulable, hypofibrinolytic state, which is exaggerated in pre-eclampsia. Thrombophilias can increase the risk of developing pre-eclampsia via placental thrombosis and effects on both trophoblast growth and differentiation (Isermann et al., 2003). The A allele of F2 rs1799963 is associated with both higher prothrombin levels and an increased risk of thrombosis (Kyrle et al., 1998; Ceelie et al., 2004). Two variants in FV are associated with pre-eclampsia. 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 C (Le et al., 2000) 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 (Ye et al., 1995). By increasing the inhibition of fibrinolysis, this genetic variant may contribute to the exaggerated hypercoagulable state that characterizes women with pre-eclampsia.

In accordance to previous meta-analyses, many genetic variants did not remain associated with pre-eclampsia following meta-analysis (Medica et al., 2007; Bombell and McGuire, 2008; Molvarec et al., 2008a, b, c; Xie et al., 2011). Perhaps this is due to the clinical variety of the cases that were included in the studies. Some studies, for instance, included only women with severe

pre-eclampsia. It is, however, also likely that there is a true lack of association between pre-eclampsia 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 (Ioannidis and Trikalinos, 2005; Healy et al., 2006).

It is important to note that epidemiological studies have revealed a relationship between pre-eclampsia and cardiovascular morbidity and mortality later in life (Jonsdottir et al., 1995; Hannaford et al., 1997; Irgens et al., 2001; Smith et al., 2001; Rodie et al., 2004). Women who have had pre-eclampsia are more likely to develop cardiovascular disease, and pre-eclampsia and cardiovascular disease share various risk factors, including obesity, hypertension and diabetes (Steegers et al., 2010). 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 variants are all associated with coronary disease (Ye et al., 2006). 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 (Sagoo et al., 2008). Thus, pre-eclampsia 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 pre-eclampsia. It would be interesting to investigate whether a combination of environmental and genetic risk factors can predict what women with pre-eclampsia 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 pre-eclampsia and for which independent replication was available. This approach has been described previously (Mooyaartetal.,2011)and aims to reduce the likelihood of reporting false-positive associations. However, by selecting only the genetic variants that are reproducibly associated with pre-eclampsia, 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 pre-eclampsia (Serrano et al., 2006), evidence for small-study bias has previously been reported with respect to both neurological and cardiovascular diseases (Keavney et al., 2000; Healy et al., 2006). 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 insights (McCarthy *et al.*, 2008) 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. 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.

SUPPLEMENTARY DATA

Supplementary data are available *at http://humupd.oxfordjournals.org/.*

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TABLES

Table I. Random-effects meta-analysis of reproduced variants for preeclampsia

Variant by gene	Minor allele	Studies (n)	Cases (n)	Control subjects (n)	OR (95% CI)	I^2 (%)	p value for funnel plot asymmetry*	Location	Function/ consequence
ACE									
rs4646994	Deletion	20	2855	4582	1.20 (1.08-1.34)	47	0.044	In an intron	Higher serum ACE levels (Sayed-Tabatabaei et al., 2006)
AGT									
rs699	C	21	2104	4530	1.23 (0.98-1.54)	81	0.116	In the gene	Higher plasma angiotensinogen levels (Jeunemaitre et al.,1992)
rs4762	T	5	497	1395	1.25 (0.67-2.30)	80	0.327	In the gene	Conflicting data(Balam-Ortiz et al.,2011 ; Jeunemaitre et al., 1992)
APOE									
rs429358 rs7412	E2	7	554	712	0.86 (0.66-1.13)	2	0.881	In the gene	Hyperlipoproteinemia (Utermann,1987)
AT1R									
rs5186	C	9	886	1230	1.13 (0.95-1.33)	0	0.022	In the 3' untranslated region	Increased response to angiotensin II (van Geel et al., 2000)

Variant by gene	Minor allele	Studies (n)	Cases (n)	Control subjects (n)	OR (95% CI)	I^2 (%)	p value for funnel plot asymmetry*	Location	Function/ consequence
CTLA4									
rs231775	G	4	353	536	1.24 (1.01-1.52)	3	1	In the gene	Higher T-cell activation and proliferation rates (Sun et al., 2008)
F2									
rs1799963	A	30	3329	4878	1.95 (1.43-2.66)	8	0.133	In the 3' untranslated region	Elevated prothrombin levels (Poort et al.,1996)
FV									
rs6020	A	2	266	336	1.94 (1.05-3.60)	60	0.317	In the gene	Poor response to activated protein C (Le et al, 2000)
rs6025	A	40	4373	6446	1.95 (1.56-2.45)	34	0.456	In the gene	Poor response to activated protein C (Bertina et al.,1994)

Variant by gene	Minor allele	Studies (n)	Cases (n)	Control subjects (n)	OR (95% CI)	I^2 (%)	p value for funnel plot asymmetry*	Location	Function/ consequence
IL-10									
rs1800896	G	8	1075	1360	0.91 (0.74-1.12)	64	0.621	In the promotor region	Lower serum IL-10 levels (Wang et al, 2011)
LPL									
rs1800590	G	3	395	579	2.27 (0.62-8.24)	72	0.602	In the 5' untranslated region	No changes in lipid profiles (Sagoo et al, 2008)
rs268	G	4	530	933	2.42 (1.25-4.68)	21	1	In the gene	Adverse lipid profiles (Sagoo et al, 2008)
MTHFR									
rs1801133	T	45	5418	7271	1.06 (0.97-1.16)	45	0.531	In the gene	Elevated plasma homocysteine levels (Frosst et al, 1995)

Variant by gene	Minor allele	Studies (n)	Cases (n)	Control subjects (n)	OR (95% CI)	I^2 (%)	p value for funnel plot asymmetry*	Location	Function/ consequence
NOS3									
27bp repeat	4a	14	1593	2239	1.14 (0.90-1.43)	63	0.071	In an intron	Altered nitrite and nitrate levels (Wang and Wang, 2000)
rs2070744	C	11	1571	2202	1.08 (0.95-1.23)	28	0.484	In the promotor region	Reduced eNOS gene promotor activity (Nakayama et al., 1999)
rs1799983	T	24	2825	4048	1.19 (1.00-1.42)	68	0.960	In the gene	Reduced nitrate, nitrite and nitric oxide production (Sofowora et al, 2001; Veldman et al, 2002)
SERPINE1									
rs1799889	4G	11	1283	1661	1.17 (1.03-1.33)	10	0.102	In the promotor region	Higher PAI-1 levels (Lin et al, 2009; Rallidis et al, 2010; Ye et al, 1995)

Variant by gene	Minor allele	Studies (n)	Cases (n)	Control subjects (n)	OR (95% CI)	I^2 (%)	p value for funnel plot asymmetry*	Location	Function/ consequence
TLR4									
rs4986790	G	4	723	614	1.07 (0.48-2.40)	78	0.497	In the gene	Dampened inflammatory response (Kiechi et al, 2002)
rs4986791	T	3	614	461	1.20 (0.45-3.20)	79	0.602	In the gene	Dampened inflammatory response (Kiechi et al, 2002)
TNF-alpha									
rs1800629	A	12	1592	1837	1.17 (0.91-1.49)	55	0.237	In the gene	Higher TNF-alpha gene expression (Kroeger et al, 1997)
rs1799724	T	3	390	385	0.66 (0.33-1.31)	85	0.602	Near the gene	Higher TNF-alpha serum levels (Puthotu et al, 2009)
VEGF									
rs3025039	T	3	377	514	1.36 (0.64-2.91)	88	0.602	In the gene	Lower VEGF levels (Al-Habboubi et al, 2011; Ruggiero et al, 2011)

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

Table II. References of the included articles, per genetic variant

Variant by gene	
ACE	
rs4646994	Aggarwal et al., (2010) Aggarwal et al., (2011) Benedetto et al., (2007) Bouba et al., (2003) Choi et al., (2004) Galao et al., (2004) Gurdol et al., (2004) Heiskanen et al., (2001) Kaur et al., (2005) Kobashi et al., (2005) Li et al., (2007) Mando et al., (2009) Miskovic et al., (2008) Morgan et al., (1999) Procopciuc et al., (2009) Roberts et al., (2004) Salimi et al., (2011) Serrano et al., (2006)2006 Serrano et al., (2006), Uma et al., (2010) Wang et al., (2006)
AGT	
rs699	Aggarwal et al., (2010) Aggarwal et al., (2011) Bashford et al., (2001) Benedetto et al., (2007) Bouba et al., (2003) Choi et al., (2004) Dissanayake et al., (2009) Guo et al., (1997)** Jenkins et al., (2008) Knyrim et al., (2008) Kobashi et al., (1999) Morgan et al., (1999) Procopciuc et al., (2009) Roberts et al., (2004) Tempfer et al., (2004) Wang et al., (2006) Ward et al., (1993) Yoshida et al., (2008) Zhang et al., (2003)
rs4762	Choi et al., (2004) Dissanayake et al., (2009) Knyrim et al., (2008) Procopciuc et al., (2009) Wang et al., (2006)
APOE	
rs429358, rs7412	Belo et al., (2004) Bernard et al., (2007) Chikosi et al., (2000) Francoual et al., (2002) Nagy et al., (1998) Procopciuc et al., (2011) Stiefel et al., (2009)
AT1R	
rs5186	Akbar et al., (2009) Benedetto et al., (2007) Bouba et al., (2003) Li et al., (2007) Morgan et al., (1998) Plummer et al., (2004) Procopciuc et al., (2009) Salimi et al., (2011) Seremak-Mrozikiewicz et al., (2005)
CTLA4	
rs231775	Best et al., (2011) Jaaskelainen et al., (2008) Pendeloski et al., (2011) Samsami et al., (2005)

Variant by gene	
F2	
rs1799963	Agorastos et al., (2002) Alfirevic et al., (2001) Benedetto et al., (2002) Best et al., (2009) Dalmaz et al., (2006) de Groot et al., (1999) Demir et al., (2006) Dogan et al., (2011) Dusse et al., (2007) Fabbro et al., (2003) Gerhardt et al., (2005) Grandone et al., (1999) Higgins et al., (2000) Hiltunen et al., (2009) Jarvenpaa et al., (2006) Kankova et al., (2000) Karakantza et al., (2008) Kupferminc et al., (1999) Kupferminc et al., (2000) † Kupferminc et al., (2000) Larciprete et al., (2007) Livingston et al., (2001) Malek-Khosravi et al., (2011) Mello et al., (2005) Mendilcioglu et al., (2011) Morrison et al., (2002) O'Shaughnessy et al., (2001)2001 Seremak-Mrozikiewicz et al., (2010) Tempfer et al., (2004) Yalinkaya et al., (2006)
FV	
rs6020	Faisel et al., (2004) Watanabe et al., (2001)
rs6025	Aggarwal et al., (2011) Agorastos et al., (2002) Alfirevic et al., (2001) Benedetto et al., (2002) Best et al., (2009) Currie et al., (2002) Dalmaz et al., (2006) Davalos et al., (2005) de Groot et al., (1999) Demir et al., (2006) Dizon-Townson et al., (1996) Dogan et al., (2011) Fabbro et al., (2003) Faisel et al., (2004) Gerhardt et al., (2005) Grandone et al., (1999) Hiltunen et al., (2009) Jarvenpaa et al., (2006) Kankova et al., (2000) Karakantza et al., (2008) Karimi et al., (2012 Kim et al., (2001) Larciprete et al., (2007) Lindoff et al., (1997) Livingston et al., (2001) Malek-Khosravi et al., (2011) Mello et al., (1999) Mello et al., (2005) Mendilcioglu et al., (2011) Morrison et al., (2002) Murphy et al., (2000) Nagy et al., (1998) Omar et al., (2008) O'Shaughnessy et al., (1999) Prasmusinto et al., (2004) Rigo et al., (2000) Seremak-Mrozikiewicz et al., (2010) Tempfer et al., (2004) von Tempelhoff et al., (2000) Yalinkaya et al., (2006)
IL-10	
rs1800896	Daher et al., (2006) de Groot et al., (2004) de Lima et al., (2009) Haggerty et al., (2005) Kamali-Sarvestani et al., (2006) Mirahmadian et al., (2008) Stonek et al., (2008) Vural et al., (2010)

Variant by gene	
LPL	
rs1800590	Hubel et al., (1999) Kim et al., (2001) Pappa et al., (2011)
rs268	Bernard et al., (2007) Hubel et al., (1999) Kim et al., (2001) Zhang et al., (2006)
MTHFR	
rs1801133	Aggarwal et al. (2011), Also-Rallo et al. (2005), Canto et al. (2008), Chikosi et al. (1999), Dalmaz et al. (2006), Davalos et al. (2005), De Maat et al. (2004), Demir et al. (2006), Dogan et al. (2011), Dusse et al. (2007), Fabbro et al. (2003), Gerhardt et al. (2005), Grandone et al. (1999), Hill et al. (2011), Hiltunen et al. (2009), Jaaskelainen et al. (2006), Jarvenpaa et al. (2006), Kaiseret al. (2001), Kankova et al. (2000), Kim et al. (2001), Klai et al. (2011), Kobashi et al. (2000), Laivuori et al. (2000), Larciprete et al. (2007), Livingston et al.(2001), Mendilcioglu et al.(2011), Morrison et al. (2002), Murphy et al. (2000), Nagy et al. (2007) ,O'Shaughnessy et al. (1999), Pegoraro et al. (2004), Perez-Mutul et al. (2004), Powers et al. (1999), Prasmusinto et al. (2002)***, Procopciuc et al. (2010), Rajkovic et al. (2000), Rigo et al. (2000), Sohda et al. (1997), Stiefel et al. (2009), Watanabe et al. (2001), Williams et al.(2004),Yilmaz et al. (2004), Yoshida et al. (2008).
NOS3	
27bp repeat	Aggarwal et al., (2010) Bashford et al., (2001) Benedetto et al., (2007) Chen et al., (2007) Diaz-Olguin et al., (2011) Fatini et al., (2006) Grandone et al., (2003) Ozturk et al., (2011) Salimi et al., (2011) Sandrim et al., (2008) Sandrim et al., (2010) Serrano et al., (2004) Tempfer et al., (2001) Zdoukopoulos et al., (2011)
rs2070744	Aggarwal et al., (2010) Diaz-Olguin et al., (2011) Fatini et al., (2006) Kim et al., (2008) Salimi et al., (2011) Sandrim et al., (2008) Sandrim et al., (2010) Seremak-Mrozikiewicz et al., (2011) Serrano et al., (2004) Tempfer et al., (2004) Zdoukopoulos et al., (2011)

Variant by gene	
rs1799983	Aggarwal et al., (2010) Best et al., (2010) Chen et al., (2007) Diaz-Olguin et al., (2011) Fatini et al., (2006) Hakli et al., (2003) Kim et al., (2006) Kim et al., (2008) Kobashi et al., (2001) Landau et al., (2004) Nishizawa et al., (2009) Ozturk et al., (2011) Pappa et al., (2011) Sandrim et al., (2008) Sandrim et al., (2010) Serrano et al., (2004) Sharma et al., (2011) Singh et al., (2010) Tempfer et al., (2004) Turan et al., (2010) Yaghmaei et al., (2011) Yoshimura et al., (2000) Yoshimura et al., (2003) Zdoukopoulos et al., (2011)
SERPINE1	
rs1799889	Dalmaz et al., (2006) De Maat et al., (2004) Fabbro et al., (2003) Gerhardt et al., (2005) Hakli et al., (2003) Kankova et al., (2000) Larciprete et al., (2007) Morrison et al., (2002) Pegoraro et al., (2003) Tempfer et al., (2004) Yamada et al., (2000)
TLR4	
rs4986790	Franchim et al., (2011) Molvarec et al., (2008) van Rijn et al., (2008) Xie et al., (2010)
rs4986791	Molvarec et al., (2008) van Rijn et al., (2008) Xie et al., (2010)
TNF-alpha	
rs1800629	Canto-Cetina et al., (2007) Chen et al., (1996) Daher et al., (2006) de Lima et al., (2009) Dizon-Townson et al., (1998) Freeman et al., (2004) Haggerty et al., (2005) Kaiser et al., (2004) Levesque et al., (2004) Mirahmadian et al., (2008) Molvarec et al., (2008) Pazarbasi et al., (2007) Saarela et al., (2005) Stonek et al., (2008) Vural et al., (2010)
rs1799724	Canto-Cetina et al., (2007) Heiskanen et al., (2002) Pazarbasi et al., (2007)
VEGF	
rs3025039	Kim et al., (2008) Papazoglou et al., (2004) Shim et al., (2007)

** 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

FIGURES & LEGENDS

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

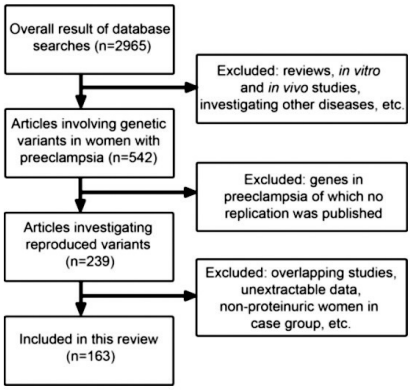
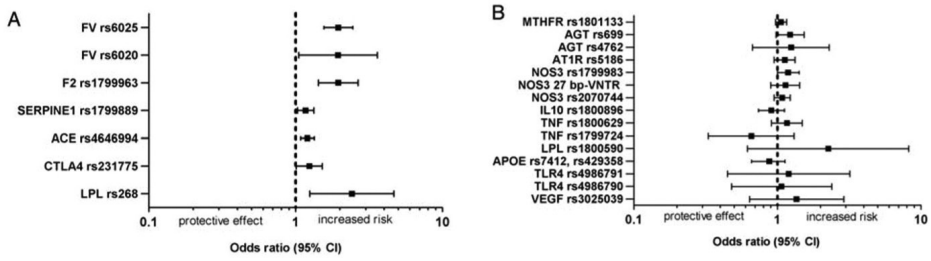


Figure 2. Odds ratios (with 95% confidence intervals) for the genetic variants that were reproducibly associated with preeclampsia.



A. All genetic variants that were reproduced in an independent study and significantly associated with preeclampsia following the meta-analysis.
B. All genetic variants that were reproduced in an independent study, but were not significantly associated with preeclampsia following the meta-analysis.

Chapter 3

Loss of Thrombomodulin in Placental Dysfunction in Preeclampsia

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ABSTRACT

Objective

Preeclampsia is a pregnancy-specific syndrome characterized by placental dysfunction and an angiogenic imbalance. Systemically, levels of thrombomodulin, an endothelium- and syncytiotrophoblast-bound protein that regulates coagulation, inflammation, apoptosis, and tissue remodeling, are increased. We aimed to investigate placental thrombomodulin dysregulation and consequent downstream effects in the pathogenesis of preeclampsia.

Approach and Results

Placentas from 28 preeclampsia pregnancies, 30 uncomplicated pregnancies and 21 pregnancies complicated by growth restriction as extra controls were included. Immunohistochemical staining of thrombomodulin, caspase-3, and fibrin was performed. Placental mRNA expression of thrombomodulin, inflammatory markers, matrix metalloproteinases 2 and 9, and soluble Flt-1 were measured with qPCR. Thrombomodulin mRNA expression was determined in vascular endothelial growth factor-transfected trophoblast cell lines.

Thrombomodulin protein and mRNA expression were decreased in preeclampsia as compared to both control groups ($P=0.001$). Thrombomodulin mRNA expression correlated with maternal body mass index ($P< 0.01$), and diastolic blood pressure ($P<0.05$) in preeclampsia. An increase in placental apoptotic cells was associated with preeclampsia ($P<0.001$). Thrombomodulin expression correlated positively with matrix metalloproteinase expression ($P<0.01$) in preeclampsia, but not with fibrin deposits or inflammatory markers Placental soluble Flt-1 expression correlated with decreased thrombomodulin expression. Vascular endothelial growth factor induced upregulation of thrombomodulin expression in trophoblast cells.

Conclusions

Decreased thrombomodulin expression in preeclampsia may play a role in placental dysfunction in preeclampsia and is possibly caused by an angiogenic imbalance. Hypertension and obesity are associated with thrombomodulin downregulation. These results set the stage for further basic and clinical

research on thrombomodulin in the pathogenesis of preeclampsia and other syndromes characterized by endothelial dysfunction.

NONSTANDARD ABBREVIATIONS

sFlt-1, soluble Flt-1
APC, activated protein C
IUGR, intra-uterine growth restriction
VEGF, vascular endothelial growth factor
MMP, matrix metalloproteinase

INTRODUCTION

Preeclampsia complicates 2–8% of all pregnancies and is a leading cause of maternal and fetal morbidity and mortality.[1, 2] The exact etiology of the syndrome is unknown, but impaired placentation plays a key role in its pathogenesis.[3] In preeclampsia, placental production of anti-angiogenic factors such as soluble Flt-1 (sFlt-1) and soluble endoglin is increased, which results in deprivation of essential survival factors in the endothelium and syncytiotrophoblast.[3] This contributes to the development of endothelial dysfunction, causing increased systemic vascular resistance, high blood pressure, and hypercoagulability. [3] Further, a hyper-inflammatory state develops, characterized for example by complement deposits in the kidneys and in the placenta. [4, 5]

Thrombomodulin is essential for the maintenance of endothelium; it is a transmembrane glycoprotein found on the endothelium of arteries, venules, and capillaries, and on the syncytiotrophoblast in the placenta.[6] Inflammation results in the release of thrombomodulin from endothelial cells into the circulation, leading to decreased expression of thrombomodulin on the endothelial surface.[7] Whether this cleaved form of thrombomodulin is functional remains unclear, but soluble thrombomodulin concentrations can be used to monitor inflammatory conditions.[8] In women with preeclampsia, maternal serum levels of cleaved soluble thrombomodulin in serum are elevated.[9–11] The placenta is a possible source of this soluble thrombomodulin, which could result in decreased available thrombomodulin on the syncytiotrophoblast.[12] Vascular endothelial growth factor stimulates thrombomodulin expression,[8] so the angiogenic imbalance in the placenta

in preeclampsia could lead to a decrease in placental thrombomodulin expression.

One of the pathways through which thrombomodulin exerts its protective effects on endothelium is by activating protein C. In mice, loss of thrombomodulin from the endothelium results in disruption of the activated protein C (APC) pathway, which leads to lethal massive thrombosis.[13] APC has an anticoagulant function by cleaving activated cofactors FV and FVIII. In addition to its anticoagulant effects, APC has an important cytoprotective function for endothelium through the endothelial protein C receptor: namely, direct anti-inflammatory and anti-apoptotic effects on endothelial cells.[14] In an experimental mouse model, this anti-apoptotic effect protected against diabetic nephropathy by inhibiting endothelial apoptosis.[15] Furthermore, APC plays a role in tissue remodeling by activating matrix metalloproteinase (MMP) 2 and 9, which are involved in placental development and are dysregulated in preeclampsia.[16-18]

We set out to investigate whether placental thrombomodulin dysregulation plays a role in the pathogenesis of preeclampsia. Therefore, we measured thrombomodulin expression in placentas from women with preeclampsia and compared this with thrombomodulin expression in placentas from control subjects with uncomplicated pregnancies, and in placentas from control subjects with pregnancies complicated by intra-uterine growth restriction (IUGR) to confirm that our findings were preeclampsia-specific rather than a consequence of lower gestational age or birth weight in our case group. Further, we investigated whether thrombomodulin expression in the placenta is correlated with its downstream effects; fibrin depositions, cell survival, inflammation, and MMP expression in the placenta, and if the angiogenic imbalance of preeclampsia is associated with a decrease in thrombomodulin expression.

MATERIALS AND METHODS

Samples

We studied 79 placentas obtained from women who delivered at the Leiden University Medical Center Department of Obstetrics between 2002 and 2011. After delivery all placentas were macroscopically examined and samples

from pre-defined locations on the placenta and fetal membranes were taken according to a standard protocol. For this study, tissue samples from lateral sites of the basal plate of the placenta were selected to study mature and terminal villi. The placentas were divided between a case group of 28 placentas obtained from women with mild or severe preeclampsia and a healthy control group of 30 placentas obtained from women with uncomplicated pregnancies that resulted in term livebirths. Preeclampsia were diagnosed according to ISSHP guidelines, with severe preeclampsia being defined as the presence of diastolic blood pressure >110 mmHg, proteinuria > 3 g / 24H, or HELLP syndrome. Because the majority of women with preeclampsia delivers by caesarean section, we aimed to collect control placentas from women who delivered by caesarean section as well. A second control group consisting of 21 placentas from pregnancies complicated by IUGR (defined as birth weight below fifth percentile for gestational age), but not by preeclampsia or hypertension, was included to confirm that our findings on thrombomodulin expression were preeclampsia-specific rather than a consequence of lower gestational age or birth weight in our case group. Paraffin-embedded samples were available for immunohistochemical staining from 11 patients in this control group; from the remaining ten patients, frozen samples were available for RNA isolation and PCR. This study was approved by the ethics committee of the Leiden University Medical Centre (P13.084) and subjects gave informed consent.

Histology

Specimens were stained with hematoxylin and eosin. 27 control samples and 21 preeclampsia samples were examined by a single pathologist blinded for cases and controls. The presence, extent, chronicity and composition of villous- and intervillous infiltrates were determined.

Immunohistochemistry

To investigate the placental protein expression of thrombomodulin and to investigate apoptosis and coagulation in the placentas, we performed immunohistochemical staining for thrombomodulin, caspase-3, and fibrin. Sections were deparaffinized, and antigen retrieval was performed. Sections were incubated with an anti-thrombomodulin mouse monoclonal antibody (1:200; Leica Biosystems, Danvers, MA, USA) or an anti-fibrin mouse

monoclonal antibody (1:200, Immunotech, Prague, Czech Republic) for 1 hour, or with an anti–cleaved caspase-3 rabbit antibody (1:300; Cell Signaling, Danvers, MA, USA) overnight at room temperature. Binding of the primary antibody was visualized with labelled anti-mouse IgG polymer or with labelled anti-rabbit IgG polymer (DAKO, Glostrup, Denmark) and diaminobenzidine as a chromogen.

Scoring of staining patterns

Slides were scored by two observers blinded with respect to cases and controls. The presence of thrombomodulin at the surface of viable syncytiotrophoblast was scored semiquantitatively as absent (present on <10% of viable syncytiotrophoblast), focal (present on 10–50% of viable syncytiotrophoblast), or overall (present on >50% of viable syncytiotrophoblast). Positivity for caspase-3 was scored quantitatively as the number of apoptotic cells per mm^2. Fibrin deposits were scored quantitatively as the number of villi with fibrin deposits divided by the total number of villi in ten 0.28 mm^2 sections per sample.

PCR

Quantitative PCR was performed to quantify placental mRNA expression of thrombomodulin, MMP 2 and 9, tumor necrosis factor alpha, intercellular adhesion molecule 1 and sFlt-1. RNA isolation was performed with TRIzol® (Lifetechnologies, San Francisco, CA, USA). Synthesis of cDNA was performed with AMV reverse transcriptase (Roche, Basel, Switzerland), and SYBR Green quantitative PCR was performed according to the manufacturer's protocol (Bio-Rad Laboratories Inc, Hercules, CA, USA). Primer sequences are described in Supplementary Table 1. Expression was measured by the comparative threshold cycle method and normalized to hypoxanthine phosphoribosyltransferase and GAPDH expression. A melting curve analysis was performed to verify the specificity of amplification.

In vitro experiments

Trophoblast cell lines BeWo (CCL-98, ATCC, Manassas, VA, USA) and Jeg-3 (HTB-36, ATCC, Manassas, VA, USA) were grown on 6-Multiwell plates. After 24 h, cells were transfected with a VEGF plasmid (pHIPPY-PGL3-VEGF). For transfection, plasmid DNA was diluted into growth medium at a concentration

of 1 g/L and X-tremeGene HP DNA Transfection Reagent (Roche, Basel, Switzerland) was added at a 3:1 ratio. 48 hours after transfection, RNA was isolated with TRIzol® (Lifetechnologies, San Francisco, CA, USA). cDNA synthesis was performed and expression of thrombomodulin, hypoxanthine phosphoribosyltransferase and GAPDH was determined with PCR as described above. The experiments were repeated three times.

Statistical analysis

Mean differences in normalized mRNA expression levels between two groups were analyzed with the unpaired *t*-test for normally distributed data or with the Mann–Whitney U test for skewed distributions. The distributions of staining patterns of thrombomodulin and histological scores in the preeclampsia and control groups were analyzed with the chi-square test or Fisher's exact test. Correlations between thrombomodulin mRNA levels, clinical parameters or mRNA levels of other proteins were calculated with the Pearson test or Spearman test. A *P* value of <0.05 was considered statistically significant. Mean differences in fibrin deposits and in caspase-3 positive cells between more than two groups were analyzed with the one-way ANOVA. Posthoc tests, Bonferroni and LSD, were subsequently performed to investigate differences between the preeclampsia group and the control groups. Standard deviations depict variance in the figures. All analyses were performed with the IBM SPSS statistics software package (version 20; IBM, Armonk, NY, USA).

RESULTS

Patient characteristics

Diastolic blood pressure and proteinuria were significantly higher in the preeclampsia group as compared to both control groups. The mean gestational age in healthy control subjects was 39 weeks and 4 days; in IUGR control subjects and in preeclampsia patients the mean gestational ages were was 33 weeks and 2 days and 30 weeks and 4 days, respectively, which were both significantly lower as compared to healthy control subjects. The mean birth weight in the healthy control group was 3609 g. Mean birth weights in the IUGR control group and in the preeclampsia group were 1521 g and 1167 g, respectively, which were both significantly lower compared to the mean birth weight in the healthy control group. The mean placenta weight was

significantly lower in preeclampsia patients and in growth-restricted control subjects as compared to healthy control subjects. Patient characteristics are illustrated in Table 1.

Immunohistochemical staining for thrombomodulin

In 29 of 30 placentas from healthy controls, thrombomodulin was observed in an overall staining pattern, with thrombomodulin present on the syncytiotrophoblast of >50% of villi, and in 1 control placenta a focal staining pattern was observed, with thrombomodulin being present on the syncytiotrophoblast of <50% of villi. In the IUGR group, thrombomodulin was present on the syncytiotrophoblast of >50% of villi in all 11 cases. In 12 of 28 placentas from patients with preeclampsia, thrombomodulin protein expression was decreased. In ten cases, we found a focal staining pattern, with thrombomodulin present on the syncytiotrophoblast of <50% of villi, and in two cases, thrombomodulin was nearly absent (present on the syncytiotrophoblast of <10% of villi). In the remaining 16 placentas from the preeclampsia group, thrombomodulin staining was present in an overall staining pattern. These findings are illustrated in Figure 1. Chi-square analysis indicated a strong association between decreased thrombomodulin expression and preeclampsia ($P = 0.001$). Thrombomodulin staining on fetal vessel endothelium was present in a similar pattern in preeclampsia and control groups .Supplemental Figure I shows thrombomodulin staining on fetal vessel endothelium in placentas from preeclampsia and control cases with most reduced thrombomodulin staining on the syncytiotrophoblast; fetal vessel thrombomodulin staining was present in these samples and consequently serves as an internal control for the adequacy of the staining procedure.

Placental mRNA expression of thrombomodulin

Placental mRNA levels of thrombomodulin were on average 3-fold lower in preeclamptic women compared to control subjects ($P = 0.001$), whereas in women with IUGR, the relative mRNA expression of thrombomodulin was not significantly different compared to healthy control placentas. These results are illustrated in Figure 2. In placenta samples where thrombomodulin protein expression was absent, thrombomodulin mRNA levels were 4-fold lower than

in placentas with a focal or overall staining pattern for thrombomodulin at the syncytiotrophoblast in the preeclampsia group (Supplemental Figure II).

Placental mRNA expression of thrombomodulin and clinical parameters in pre-eclampsia

Placental thrombomodulin mRNA levels were significantly lower in patients with mild preeclampsia and in patients with severe preeclampsia compared to healthy control subjects; levels were not significantly different between patients with mild and severe preeclampsia. Placental thrombomodulin mRNA levels were negatively correlated with maternal diastolic blood pressure and maternal body mass index in patients with preeclampsia. In controls, this correlation was not present. There were no correlations between gestational age or placental weight and thrombomodulin mRNA levels. These correlations are illustrated in Figure 2. Thrombomodulin mRNA levels were not associated with parity ($P>0.05$). When exclusively primiparous cases were analyzed, thrombomodulin mRNA levels were still significantly lower in preeclampsia cases compared to placentas from control cases.

Fibrin depositions

In placentas from healthy control subjects, villous fibrin deposits were present on 13 percent of the villi, and in IUGR control placentas on 7 percent. In pregnancies complicated by preeclampsia, fibrin depositions were present on 17 percent of villi, on average. One-way ANOVA analyses indicated an association between increased fibrin deposits and preeclampsia, but post-hoc analyses did not reveal any significant differences between pre-eclampsia cases and healthy or growth-restricted control cases. These data are shown in Figure 3. There was no significant association between fibrin deposits and thrombomodulin mRNA, or between fibrin deposits and the thrombomodulin protein staining pattern.

Immunohistochemical staining of cleaved caspase-3

In placentas from healthy subjects and IUGR control subjects, there were four and three apoptotic cells per mm^2, on average. In placentas from preeclamptic patients, there were on average 16 apoptotic cells per mm^2, which was significantly different from both control groups. These data are shown in Figure 3. There was no significant association between

thrombomodulin mRNA and the amount of apoptotic cells and a focal or absent thrombomodulin staining pattern was not associated with an increase in apoptotic cells.

Placental inflammation

Villitis was present in 14.8% of the control placentas and in 42.9% of the preeclampsia cases; this difference was significant ($P<0.05$). In all villitis cases, the infiltrate was multifocal and composed of mononuclear cells. Intervillositis occurred in 7.4% of control placentas and in 19% of preeclampsia placentas; the infiltrate consisted of histiocytes in the majority of cases. Neither villitis nor intervillositis was associated with a decreased thrombomodulin staining pattern or with decreased thrombomodulin mRNA expression. Placental mRNA levels of tumor necrosis factor alpha and intercellular adhesion molecule 1 were lower in placentas from preeclampsia patients as compared to healthy controls ($P < 0.001$ for both, Figure 3). There was no correlation between thrombomodulin mRNA expression and tumor necrosis factor alpha or intercellular adhesion molecule 1 expression in placentas from preeclamptic patients (Supplemental Figure III).

Placental mRNA expression of MMPs

On average, MMP2 mRNA levels were 2-fold lower in placentas from preeclamptic women compared to placentas from both control groups ($P < 0.01$). MMP9 mRNA levels were 5-fold lower in placentas from preeclamptic women compared to control subjects ($P < 0.01$). In IUGR control placentas, MMP2 and MMP9 mRNA levels were not significantly different compared to control placentas. In preeclamptic patients, MMP2 expression was positively correlated with thrombomodulin mRNA expression. The correlation between MMP9 and thrombomodulin did not reach statistical significance ($P = 0.095$). These findings are illustrated in Figure 3.

Placental expression of sFlt-1 and thrombomodulin

An inverse correlation between thrombomodulin protein expression and sFlt-1 expression was observed; in placentas from preeclampsia patients with a focal syncytiotrophoblast thrombomodulin staining pattern, mRNA expression of sFlt-1 was 3-fold higher compared to the expression in placentas with an overall thrombomodulin staining pattern at the syncytiotrophoblast

($P < 0.05$, Figure 4). This inverse correlation was not observed between thrombomodulin mRNA and sFlt-1 mRNA.

***In vitro* experiments**

In BeWo cells, vascular endothelial growth factor (VEGF) transfection resulted in a 1.5-fold upregulation of thrombomodulin mRNA expression. This increase was significant ($P<0.05$) compared to untreated control cells and control cells that had received the transfection reagent without plasmid DNA. In Jeg-3 cells, VEGF transfection was associated with a 1.3-fold upregulation of thrombomodulin mRNA expression, but this was not significantly different compared to both control cell groups. These results are illustrated in Figure 5.

DISCUSSION

Increasing evidence suggests that preeclampsia is caused by deprivation of factors essential for the placenta and endothelium, with consequent placental and endothelial dysfunction. This study demonstrates that thrombomodulin mRNA and protein expression are decreased in placentas of mildly and severely preeclamptic patients, and that this decrease correlates negatively with maternal body mass index and diastolic blood pressure. Additionally, thrombomodulin mRNA expression correlates directly with placental expression of MMPs and the decrease in placental thrombomodulin is accompanied by impaired villous cell survival.

In the preeclampsia group, parity, birth weight, placenta weight and duration of pregnancy were significantly different compared to the term control group. Therefore, we added an extra control group to our study, consisting of placentas from pregnancies complicated by growth restriction and not by hypertensive disorders. In this group, parity, birth weight, placenta weight and duration of pregnancy were comparable to the preeclampsia group. Thrombomodulin expression in the growth-restriction control group was not different from the expression in the term control group; therefore, we conclude that our findings on placental thrombomodulin loss in the preeclampsia group cannot be explained by, for example, the lower gestational age in the preeclampsia group.

A significant correlation was present between placental thrombomodulin expression and diastolic blood pressure in preeclampsia cases. This indicates that thrombomodulin expression is connected to the degree of endothelial

dysfunction in preeclampsia, which could be upstream, in the pathogenesis of endothelial dysfunction, or downstream, as a consequence of endothelial dysfunction. However, thrombomodulin expression was not significantly different between mild and severe preeclampsia cases, so placental loss of thrombomodulin alone does not seem to be a major contributor to the development of end-organ involvement in preeclampsia.

If thrombomodulin is involved in the pathogenesis of preeclampsia, one would expect mutations in the thrombomodulin gene to be associated with the syndrome. For example, hemolytic uremic syndrome, a disease also characterized by endothelial dysfunction and complement activation, is associated with mutations in the thrombomodulin gene.[19] However, a recent meta-analysis of genetic variants in preeclampsia revealed that thrombomodulin mutations are not associated with preeclampsia.[20] Our study shows that a decreased thrombomodulin protein expression pattern in the placenta is accompanied by an increase in placental expression of sFlt-1, and that VEGF transfection is associated with thrombomodulin upregulation in trophoblast cells *in vitro*. These results strengthen the hypothesis that the angiogenic imbalance of preeclampsia could cause the decreased thrombomodulin expression in the placenta that we found in our cohort.

The decreased thrombomodulin expression in the placenta in preeclampsia could have unfavorable downstream effects on the placenta through at least four pathways, which are illustrated in a hypothetical scheme in Figure 6. First, thrombomodulin inhibits coagulation through the APC pathway. Systemically, increased levels of the thrombin–antithrombin complex in the presence of increased thrombomodulin cleavage product have been reported in preeclampsia, [21] but we did not find an increase in placental fibrin deposits in preeclampsia or a correlation between thrombomodulin expression and placental villous fibrin deposits in our study; apparently, the placental decrease in thrombomodulin we observed in our cohort did not lead to increased fibrin at the fetomaternal interface. Second, we found that decreased placental thrombomodulin expression was accompanied by impaired placental cell survival in preeclampsia; these changes could also contribute to the impaired placentation seen in preeclampsia. However, a direct correlation between the amount of apoptotic cells detected with immunohistochemical staining and thrombomodulin expression was not present, possibly caused by sampling error due to the heterogeneous

nature of placental lesions. *In vitro* studies investigating the effects of thrombomodulin depletion on trophoblast cells would shed some light on this area. Third, although preeclampsia itself is associated with a hyper-inflammatory state,[4, 5] we found no increased mRNA expression of ICAM1 and TNFA in placentas from preeclamptic patients, and there was no correlation between thrombomodulin expression and these parameters. Further, thrombomodulin expression was not associated with villous- or intervillous infiltrates. Last, we found a positive correlation between thrombomodulin expression and expression of MMP2 and MMP9. These results indicate that thrombomodulin expression could lead to decreased MMP2 and MMP9 activity in the placenta, which is associated with impaired trophoblast invasion and preeclampsia.[22] Since our cohort contained exclusively third-trimester placentas obtained after delivery, early stages of placental development, and trophoblast invasion, were not studied. Further studies on thrombomodulin expression in the placenta at earlier stages of placental development are warranted.

In our cohort, we found a negative correlation between body mass index and placental thrombomodulin expression. Obesity is associated with endothelial dysfunction and increased levels of circulating thrombomodulin, suggestive for loss of endothelial thrombomodulin.[23] Obese pregnant women show increased levels of circulating markers of endothelial dysfunction and have an increased risk of developing preeclampsia. [24] Possibly, the highly inflammatory and hypoxic vascular environment of obesity leads to decreased expression of vasculoprotective factors, such as thrombomodulin, both on the fetomaternal interface and in the systemic vasculature, and contributes to the development of systemic endothelial dysfunction and preeclampsia. However, obesity in healthy control pregnancies was not associated with thrombomodulin loss. Apparently additional endothelial- or syncytiotrophoblast damaging factors are needed to cause placental thrombomodulin loss in preeclampsia.

Thrombomodulin has raised interest as a possible target in the treatment of preeclampsia; it could act beneficially by reducing both endothelial and placental dysfunction. Recently, administration of recombinant thrombomodulin was shown to rescue fetal tissue oxygenation and growth in a rat model of preeclampsia.[25] However, in rats, thrombomodulin did not improve the maternal systemic symptoms of preeclampsia such as hypertension. This could indicate that thrombomodulin administration

rescues fetal growth by attenuating placental dysfunction. Our findings, which link decreased thrombomodulin expression to impaired placental cell function, support this hypothesis. Further research exploring the safety and efficacy of thrombomodulin as a target for the prevention and treatment of preeclampsia are of interest.

To conclude, preeclampsia is characterized by placental and endothelial dysfunction, but the exact etiology is not completely understood. Our study shows that thrombomodulin is a new candidate role-player in the pathogenesis of preeclampsia that is associated with both clinical parameters and dysregulation of factors essential for placental function. These insights set the stage for further basic and clinical research on placental development, the development of placental pathology and on thrombomodulin as a target for the prevention and treatment of preeclampsia.

SUPPLEMENTAL MATERIAL

Supplemental material is available online at http://atvb.ahajournals.org/.

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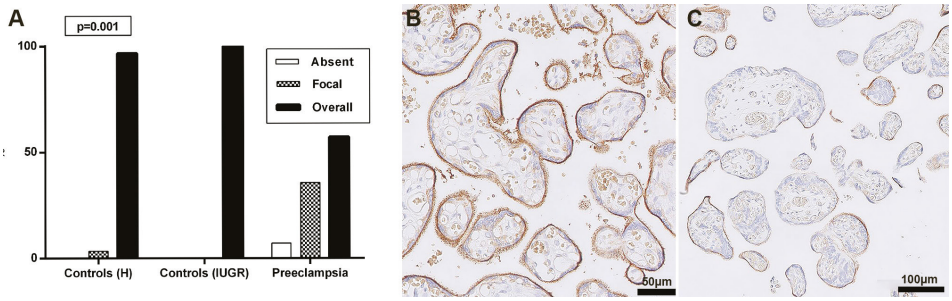
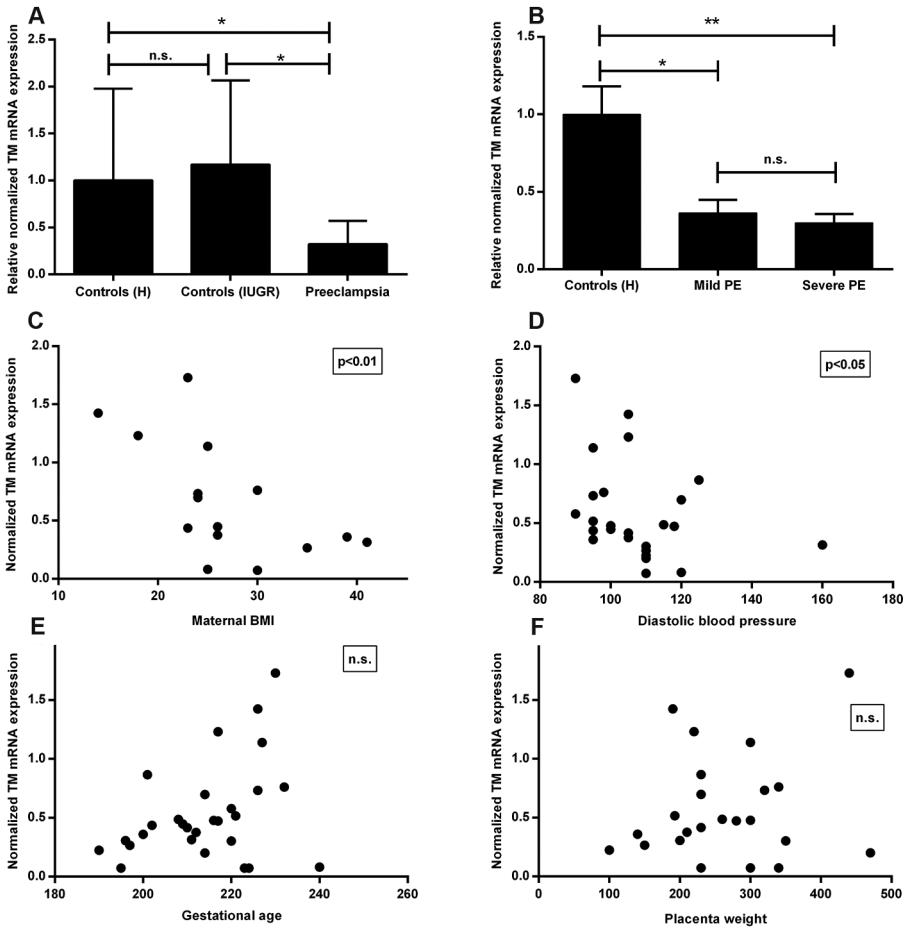
FIGURES

Figure 1 Placental thrombomodulin protein expression



Results of immunohistochemical and histological staining of placentas from preeclamptic patients and control subjects **A** Abundance of thrombomodulin protein expression in placentas from 30 placentas from healthy controls, from 11 control pregnancies complicated by IUGR and from 28 preeclampsia patients. ($P = 0.001$, Controls (H), healthy control subjects; Controls (IUGR), control subjects with pregnancies complicated by intra-uterine growth restriction). **B** Example of an overall staining pattern of thrombomodulin on the syncytiotrophoblast in control placenta. **C** Example of a focal staining pattern of thrombomodulin in a placenta from the preeclampsia group.

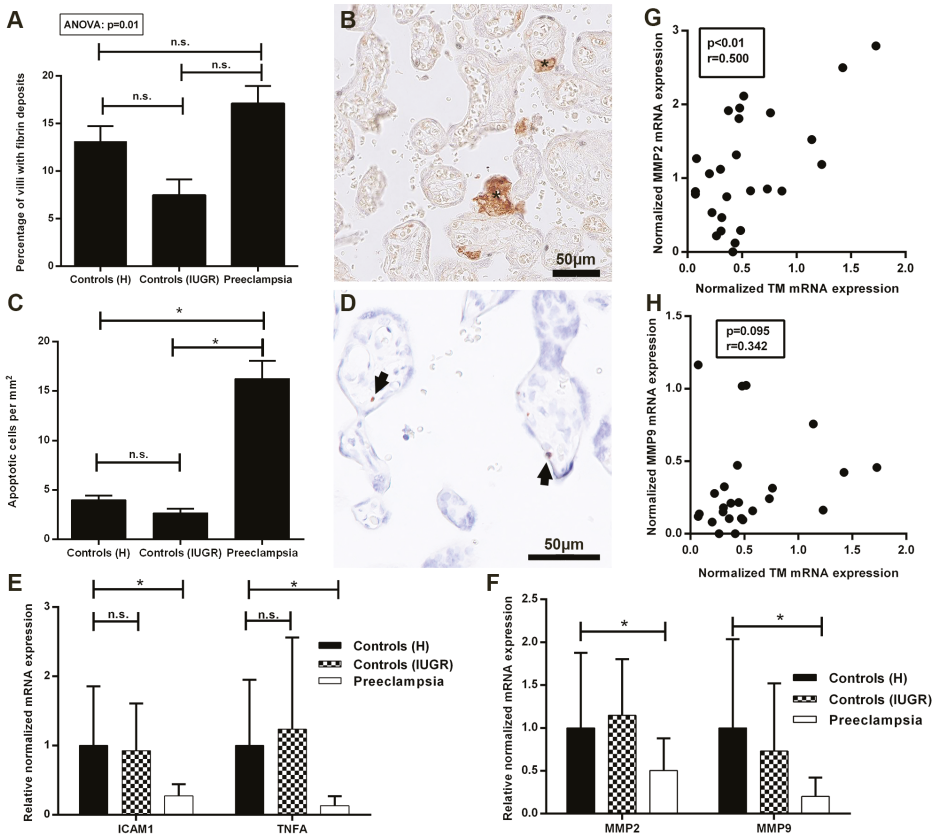
Figure 2 Placental mRNA expression of thrombomodulin



A Relative normalized placental mRNA expression of thrombomodulin in preeclampsia cases and controls; *, $P = 0.001$ Mann-Whitney U test comparing preeclampsia and growth-restricted or healthy control placentas. **B** Normalized placental thrombomodulin mRNA expression in healthy control subjects and patients with mild or severe preeclampsia. Expression was significantly lower in patients with mild or severe preeclampsia compared to healthy control placentas. Expression was not significantly different between patients with mild and severe preeclampsia ($p>0.05$, Mann-Whitney U test). *, $P < 0.05$; **, $P < 0.01$, calculated with Mann-Whitney U testing. **C** Maternal BMI and normalized placental thrombomodulin mRNA levels in the preeclampsia group; Spearman's rho=-0.671,

p<0.01. **D** Diastolic blood pressure and normalized placental thrombomodulin mRNA levels in the preeclampsia group; Spearman's rho=-0.431, $P < 0.05$. **E** Gestational age and normalized placental thrombomodulin mRNA levels in the preeclampsia group; Spearman's rho=0.361, $P > 0.05$. **F** Placenta weight and normalized placental thrombomodulin mRNA levels in the preeclampsia group; Spearman's rho=0.033, $P > 0.05$.
TM, thrombomodulin; Controls (H), healthy controls; Controls (IUGR), control subjects with pregnancies complicated by intra-uterine growth restriction; PE, preeclampsia.

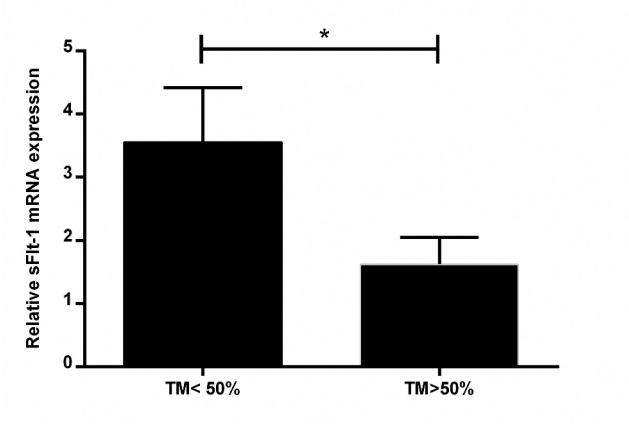
Figure 3 Downstream effects of thrombomodulin in placentas



A Percentage of villi with fibrin deposits as detected with immunohistological fibrin staining in placentas from preeclamptic patients compared to control subjects; $P = 0.01$, One-way ANOVA. **B** Placenta from a preeclamptic patient with

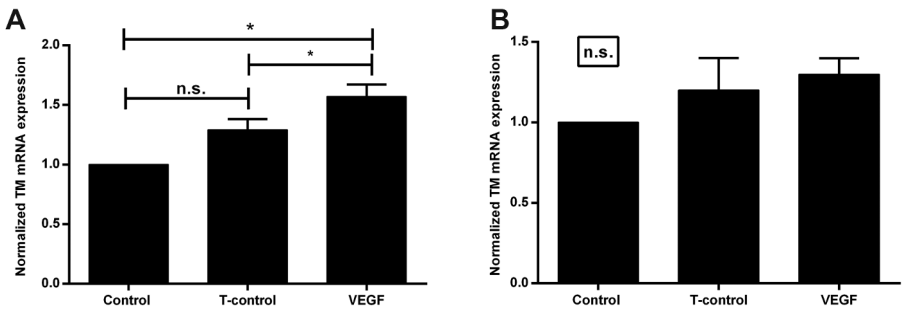
fibrin deposits. Asterisks indicate villous fibrin deposits. **C** Average number of apoptotic cells per mm^2 in placentas from preeclamptic patients and control subjects. *, $P < 0.001$. **D** Example of caspase-3 staining in a placenta from a patient with preeclampsia. Arrows indicate cells positive for caspase-3. **E** Relative mRNA expression of tumor necrosis factor alpha and intercellular adhesion molecule 1 in placentas from preeclamptic patients and control subjects. *, $P < 0.001$; n.s., not significant. **F** Relative mRNA expression of MMP2 and MMP9 in placentas from preeclamptic patients and control subjects; *, $P < 0.01$, Mann-Whitney U tests. **G** Correlation between thrombomodulin and MMP2 expression in placentas from the preeclampsia group; Spearman's rho=0.500, $P < 0.01$. **H** Correlation between thrombomodulin and MMP9 expression in placentas from the preeclampsia group; Spearman's rho=0.342, $P = 0.095$.
Controls (H), healthy controls; Controls (IUGR), control subjects with pregnancies complicated by intra-uterine growth restriction; TNFA, tumor necrosis factor alpha; TM, thrombomodulin; ICAM1, intercellular adhesion molecule 1; MMP, matrix metalloproteinase.

Figure 4 Placental sFlt-1 expression in preeclampsia cases with and without decreased thrombomodulin expression



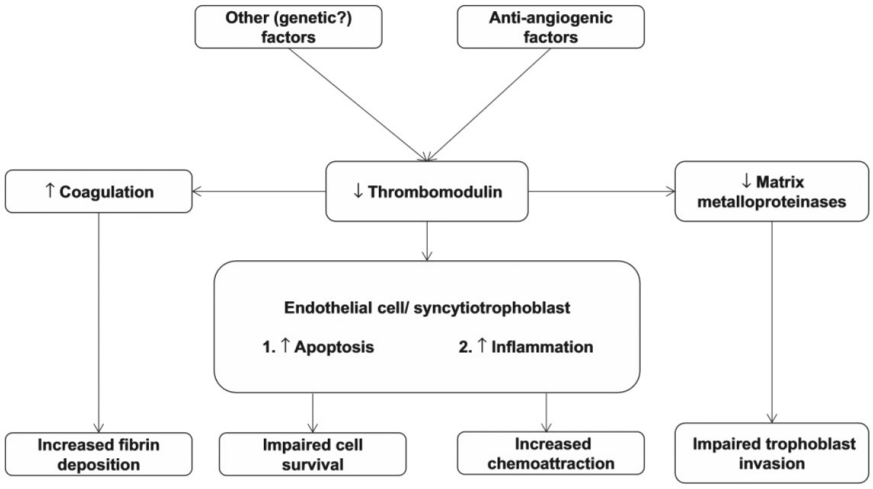
Relative mRNA expression levels of sFlt-1 in placenta samples from the preeclampsia group with decreased thrombomodulin protein expression (TM<50%) or without decreased thrombomodulin protein expression (TM>50%) are depicted. *, $P < 0.05$, Mann-Whitney U test.

Figure 5 Thrombomodulin expression in VEGF-transfected trophoblast cells



A Normalized relative thrombomodulin mRNA expression in BeWo control cells, cells treated with transfection reagent and cells treated with VEGF plasmid DNA. Thrombomodulin expression was significantly higher in the VEGF-transfected cells compared to both control groups ($P < 0.05$). **B** Normalized relative thrombomodulin mRNA expression in Jeg-3 control cells, cells treated with transfection reagent and cells treated with VEGF plasmid DNA. There were no significant differences between the three groups.
T-control, control cells treated with transfection reagent; VEGF, vascular endothelial growth factor; *, $P < 0.05$; n.s., not significant. .

Figure 6 Hypothetical scheme of thrombomodulin in the pathogenesis of preeclampsia



This figure shows the hypothetical role of thrombomodulin in the placenta in preeclampsia. Anti-angiogenic and possibly other (genetic) factors lead to thrombomodulin downregulation in the placenta, both on mRNA and protein levels. Since thrombomodulin inhibits coagulation, this downregulation could lead to increased fibrin deposition. Further, downregulation could increase apoptosis and inflammatory processes in endothelial cells and/ or syncytiotrophoblast. Lastly, thrombomodulin plays a role in activating matrix metalloproteinases, so its downregulation could result in impaired trophoblast invasion.

TABLES

Table 1 Patient characteristics

Characteristics	Healthy controls n=30	IUGR controls n=21	Preeclampsia n=28
Mean maternal age in years (SD)	33.9 (4.0)	31.4 (6.0)	31.6 (7.2)
Mean maternal BMI (SD)**	25.3 (4.2)	n.a.	26.9 (7.2)
Mean gravidity (SD)	2.9 (1.2)	1.9 (0.8)*	2.1 (1.9)*
Mean parity (SD)	1.3 (1.0)	0.5 (0.7)*	0.8 (1.2)*
Highest diastole (mmHg) (SD)	76.4 (5.1)	81.0 (8.3)*	107.5 (13.9)*†
Proteinuria (g/24h) (SD)	0.0 (0.0)	0.0 (0.0)	5.2 (5.1) *†
Severe preeclampsia (%)‡	0 (0)	0 (0)	19 (67.9)
Birth weight (g) (SD)	3608.5 (429.5)	1520.7 (854.0)*	1167.0 (320.0)*
Placenta weight (g) (SD)	645.7 (137.3)	302.4 (118.1)*	261.9 (90.2)*
Mode of delivery‡			
Caesarean section (%)	19 (63.3)	14 (66.7)	28 (100)
Vaginal delivery (%)	11 (36.7)	7 (33.3)	0 (0)
Gestational age at delivery (weeks + days; SD in days)	39 + 4 (9)	33 + 2 (35)*	30 + 4 (12)*

*, P < 0.05 compared to healthy controls; †, P < 0.05 compared to IUGR controls, ‡, P < 0.05 for overall comparison with Chi-square testing, **, data were available from 15 patients from the preeclampsia group.

Chapter 4

Thrombomodulin expression in the kidney is regulated by anti-angiogenic factors, *in vitro* and *in vivo*

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ABSTRACT

Background

Levels of soluble thrombomodulin are increased in preeclampsia. Whether this increase reflects a change in functional thrombomodulin on the glomerular endothelium, and if these changes affect anti-coagulative and cytoprotective signaling is unknown. Hence, we investigated thrombomodulin signaling and regulation in preeclampsia patients and experimental models of endothelial dysfunction.

Methods

Kidney tissue from eleven preeclampsia patients and 22 pregnant women was collected. Kidneys from five pigs with metabolic syndrome after a high-fat diet, five pigs with diabetes after streptozotocin injection and five control pigs and from twelve mice treated with endoglin or vascular endothelial growth factor A (VEGF-A) receptor inhibiting antibodies were collected. Human umbilical vein endothelial cells were treated with VEGF-A or a soluble VEGF-receptor (sFlt-1). Gene expression of thrombomodulin, endothelial protein C receptor and tissue factor was investigated with qPCR. Thrombomodulin protein expression was investigated with immunohistochemistry.

Results

In preeclampsia, kidney thrombomodulin was increased compared to pregnant control subjects and this increase was correlated with podocyte nephrin expression ($P<0.01$). Glomerular thrombomodulin was increased in diabetic pigs and in mice treated with anti-angiogenic compounds. In diabetic pigs, mRNA expression of thrombomodulin, endothelial protein C receptor, and VEGF-A increased (all $P<0,05$). sFlt-1 decreased endothelial thrombomodulin mRNA in vitro.

Conclusions

Renal endothelial thrombomodulin expression is increased during anti-angiogenic conditions, in vitro and in vivo. Increased thrombomodulin expression is accompanied by increased podocyte nephrin expression, indicative of a protective effect on the glomerulus. These results indicate an attempt of glomerular endothelial cells to maintain cytoprotection; investigating pathways through which thrombomodulin expression is

increased in endothelial cells could reveal clues to restore or prevent endothelial kidney damage.

ABBREVIATIONS

APC	Activated protein C
VEGF-A	Vascular endothelial growth factor A
VEGFR-2	Vascular endothelial growth factor receptor 2
soluble Flt-1	sFlt-1
DN	Diabetic nephropathy
HUVEC	Human umbilical vein endothelial cell

INTRODUCTION

Preeclampsia is a syndrome of pregnancy characterized by hypertension and proteinuria, with systemic endothelial dysfunction as the final common pathway leading to symptomatic disease.[1] Thrombomodulin is a transmembrane glycoprotein found on the endothelium of arteries, venules and capillaries.[2] It regulates endothelial cell survival, coagulation and endothelial activation through signaling via activated protein C (APC) and the endothelial protein C receptor (EPCR).[2, 3] Thrombomodulin is cleaved from the endothelial surface under inflammatory conditions, releasing a truncated, supposedly nonfunctional soluble thrombomodulin protein.[3, 4] In preeclampsia placental abundance of thrombomodulin is decreased.[5] This is accompanied by increased levels of cleaved thrombomodulin in the circulation.[6]

One of the first maternal organs symptomatically affected in preeclampsia is the kidney; proteinuria is one of the first symptoms of preeclampsia and microalbuminuria during early pregnancy is a predictor of the development of preeclampsia.[1] Kidney changes in preeclampsia are distinct; endothelial cells lose their fenestrations and become swollen, this is known as endotheliosis, and podocyte foot process effacement and podocyte loss in urine is observed.[7]

In preeclampsia, the placenta produces excessive levels of the anti-angiogenic factors soluble Flt-1 (sFlt-1) and soluble endoglin.[8, 9] sFlt-1 and soluble endoglin are soluble receptors, binding to vascular endothelial growth factor A (VEGF-A) and transforming growth factor beta, respectively, giving rise to an angiogenic imbalance when in excess.[10] VEGF-A and soluble endoglin play a pivotal role in maintaining an intact glomerular filtration barrier in the

kidney. Animal models with overexpression of sFlt-1 and soluble endoglin develop renal changes somewhat similar to those in preeclampsia, for example with glomerular endothelial cell swelling and podocyte changes.[11-13] Likewise, deviations in VEGF-A availability to the glomerulus are associated with a variety of kidney diseases in humans.[14-16] Patients treated with VEGF-A inhibitors develop similar renal lesions as in preeclampsia, including endothelial cell swelling, renal thrombi and proteinuria.[17, 18]

The angiogenic imbalance of preeclampsia might (partially) contribute to the renal lesions in preeclampsia through impairing glomerular thrombomodulin signaling; thrombomodulin is a mediator of glomerular endothelial homeostasis during endothelial dysfunction. In a mouse model for diabetic nephropathy, thrombomodulin-dependent APC signaling was decreased and impairment of APC signaling resulted in glomerular endothelial apoptosis.[19] VEGF-A increases thrombomodulin expression in endothelial cells *in vitro*.[20] Also, in preeclampsia, increased sFlt-1 production in the placenta is associated with placental loss of thrombomodulin.[5]

In summary, decreased thrombomodulin signaling and expression appear to be associated with renal endothelial dysfunction and preeclampsia. However, if thrombomodulin abundance changes in the kidney in preeclampsia, and if this is associated with the glomerular lesions of preeclampsia is unknown. We set out to investigate if kidney thrombomodulin expression is changed in preeclampsia, and if this change could be caused by the angiogenic imbalance of preeclampsia. Therefore, thrombomodulin abundance was studied in kidney tissue from human patients with preeclampsia and pregnant controls. Further, thrombomodulin expression and abundance were studied in disease models for endothelial dysfunction: porcine models for diabetes and metabolic syndrome and mice treated with anti-angiogenic compounds. Lastly, thrombomodulin expression was studied in an in-vitro model for glomerular endothelial cells under anti-angiogenic conditions.

MATERIALS AND METHODS

Patient samples

To collect renal tissue from women who died from the consequences of preeclampsia and control subjects, a nationwide search of the Dutch Pathology Registry was conducted. This is a network of all pathology laboratories in the Netherlands. The pathology data were linked with the

records of the National Maternal Mortality Committee of the Dutch Society of Obstetrics. Preeclampsia was defined according to the definition of the International Society for the Study of Hypertension in Pregnancy (ISSHP).[8] Two control groups were included; the first group consisted of pregnant women without a hypertensive disorder prior to or during pregnancy, who died from a cause unrelated to hypertension, and the second group consisted of non-pregnant women with a history of chronic hypertension. Paraffin-embedded kidney samples from 11 women with preeclampsia, 22 normotensive pregnant controls and 14 non-pregnant hypertensive controls were available for this study. The patient characteristics of these groups have been described before.[21] This study was approved by the Medical Ethics Committee of the Leiden University Medical Center (P12.107).

Porcine models for metabolic syndrome and diabetes

To further study the association between endothelial dysfunction, angiogenic imbalance and thrombomodulin expression and abundance, samples from porcine models for metabolic syndrome and diabetes were collected. The control group of five pigs received a diet consisting primarily of barley, wheat and soy bean oil. The metabolic syndrome group of ten pigs received a cafeteria diet with a high content of lard, fructose, sucrose and added cholesterol. Five of the cafeteria diet fed pigs were additionally treated with low-dose streptozotocin (STZ) after five months, with the dose of STZ being adjusted individually to induce non-insulin dependent diabetes to model type two diabetes. After seven months, paraffin and snap-frozen kidney tissue was collected as described by Jonker et al.[22]

sFLt-1/ endoglin mouse model

To study the effect of anti-angiogenic conditions on endothelial thrombomodulin expression in vivo, six C57/Bl6 mice were treated two times per week with VEGF-receptor inhibiting antibodies (10 mg/kg, DC101, BioXcell) or anti-endoglin antibodies (M1043, kindly provided by Tracon Pharmaceuticals, San Diego USA) or with a combination for five weeks. Six mice were treated with appropriate IgG control antibodies for five weeks. Afterwards, mice were sacrificed and kidney tissue was collected, processed and stained for thrombomodulin expression as described below. Animal

experiments were approved by the animal ethics committee of the Leiden University Medical Center, the Netherlands.

Histology and immunohistochemistry

Human renal sections were scored by a renal pathologist blinded with respect to cases and control subjects to evaluate histological changes, e.g. endotheliosis, glomerulosclerosis, podocyte count and parietal cell activation in human renal samples, as described before.[21] For immunohistochemistry, sections were deparaffinized, antigen retrieval was performed with citrate (for the thrombomodulin antibodies) or with Tris EDTA (for the fibrin antibody), and peroxidase was blocked by incubating the sections in a hydrogen peroxide solution for 20 minutes. Kidney samples from humans and pigs were incubated with a mouse monoclonal thrombomodulin antibody (1:200, Leica Biosystems, Danvers), a nephrin antibody, or a fibrin antibody (1:200) for one hour, and kidney samples from mice were incubated with a rabbit polyclonal thrombomodulin antibody (1:200 Santa Cruz) for one hour. Binding of the primary antibody was visualized with labeled anti-mouse or labeled anti-rabbit polymer (DAKO, Belgium) and diaminobenzidine as a chromogen. As positive and negative controls, human placenta and mouse skin samples were used.

Quantification of immunohistochemistry

In human patients and in mouse specimen, thrombomodulin, nephrin and fibrin staining were scored as being either present, when more than 10% of the capillary walls in the glomeruli were stained positively, or absent, when less than 10% of the capillary walls in the glomeruli was stained positively.

PCR

Quantitative PCR was performed to quantify placental mRNA expression of thrombomodulin, VEGF-A, Flt-1, tissue factor and endothelial protein C receptor. RNA was isolated with TRIzol® (Lifetechnologies, San Francisco, CA, USA). Synthesis of cDNA was performed with AMV reverse transcriptase (Roche, Basel, Switzerland), and SYBR Green quantitative PCR was performed according to the manufacturer's protocol (Bio-Rad Laboratories Inc, Hercules, CA, USA). Primer sequences are described in Supplementary Table 1. Expression was measured by the comparative threshold cycle method

and normalized to hypoxanthine phosphoribosyl transferase and GAPDH expression. A melting curve analysis was performed to verify the specificity of amplification.

Glomerular endothelial cell and podocyte cell culture experiments

Human umbilical vein endothelial cells (HUVECs) that were confluent for two days were incubated with VEGF-A (20 ng/ml; R&D Systems) for two, four, six and eight hours. To determine the effect of sFLT-1 on VEGF-A-induced endothelial activation, HUVECs were incubated for four hours with sFLT-1 (0, 10, 100 or 1000 ng/ml; R&D Systems) in the presence of VEGF-A (20 ng/ml). These experiments were performed three times. Cell lines were negative for mycoplasma contamination. [23] RNA isolation and PCR were performed as described above.

Statistical analyses

Categorical data were analyzed using the chi-square test. Mean differences in normalized mRNA expression levels between two groups were analyzed with the unpaired t-test for normally distributed data or with the Mann–Whitney U test for skewed distributions. A p-value below 0.05 was considered significant. All analyses were performed using the SPSS statistics software (IBM, version 20).

RESULTS

Thrombomodulin immunohistochemistry in human samples

Thrombomodulin abundance in glomeruli was studied in 11 kidney specimens from women with preeclampsia, from 22 pregnant control subjects and from 14 non-pregnant hypertensive control subjects. Thrombomodulin was present in 9 out of 11 (82%) kidney samples from preeclampsia patients and in 9 out of 22 (41%) kidney samples from the pregnant control subjects. In non-pregnant hypertensive control subjects, thrombomodulin was present in 3 out of 14 (21%) kidney samples. The staining pattern was significantly more frequently positive in preeclampsia patients as compared to pregnant controls (P = 0.026) and as compared to non-pregnant hypertensive controls (P = 0.003). These results are illustrated in Figure 1.

Thrombomodulin expression and renal lesions of preeclampsia

Thrombomodulin protein abundance was compared with the histopathological changes and fibrin- and nephrin abundance in the kidney specimen, as markers of excessive coagulation and podocyte viability, respectively. Thrombomodulin expression correlated with activation of parietal epithelial cells ($P = 0.023$), but not with endotheliosis, podocyte count or endothelial fibrin deposits ($P > 0.05$). Further, glomerular thrombomodulin expression correlated with podocyte nephrin expression ($P < 0.01$). Fibrin deposits were observed in similar quantities in specimen from patients with preeclampsia and from controls.

Thrombomodulin expression in endothelial dysfunction porcine model

Thrombomodulin protein abundance and mRNA expression were studied in porcine models for diabetes and metabolic syndrome, and controls; thrombomodulin protein abundance was increased in the diabetes model; staining in glomeruli was observed in 40% of control pigs, in 40% of pigs with metabolic syndrome and in 80% of pigs with diabetes ($P<0.05$). Examples of staining in glomeruli and negative glomeruli are depicted in figure 2. Kidney thrombomodulin mRNA expression was increased approximately 2-fold in pigs with metabolic syndrome ($P < 0.05$) and in pigs with diabetes mellitus as compared to control pigs. This increase was accompanied by an increase in mRNA expression of the co-receptor EPCR in the metabolic syndrome pigs ($P < 0.05$) and diabetes mellitus pigs as compared to controls. Tissue factor expression was not different between case and control groups. sFlt-1 mRNA was increased in kidneys from pigs with metabolic syndrome and diabetes mellitus as compared to controls (both $P < 0.05$), but VEGF-A mRNA was increased as well in the case groups and there was no difference between cases and controls with respect to the VEGF-A/sFlt-1 ratio. These results are illustrated in Figure 3.

Thrombomodulin immunohistochemistry in mice treated with anti-angiogenic compounds

Thrombomodulin abundance was investigated in kidney tissue from mice treated with VEGF-receptor inhibiting antibodies and anti-endoglin antibodies and control mice (Figure 4). Thrombomodulin expression was present in a diffuse and overall staining pattern among glomeruli in 50% of

treated mice and in 20% of control mice (P > 0.05). Thrombomodulin staining in peritubular capillaries was present in all treated and control mice.

In vitro experiments

To investigate the possible effect of the angiogenic imbalance on thrombomodulin expression in vitro, HUVEC cells were incubated with VEGF-A and sFlt-1. Adding sFlt-1 to HUVEC cells cultured in VEGF-enriched medium decreased thrombomodulin mRNA expression approximately twofold (Figure 5).

DISCUSSION

Soluble thrombomodulin is cleaved from the endothelial surface, e.g. by matrix metalloproteinases, under inflammatory conditions.[3] We have now shown that this increased thrombomodulin cleaving in preeclampsia and diabetes does not lead to decreased thrombomodulin abundance on the endothelium. Instead, increased thrombomodulin abundance was observed under these conditions, implicating a 'rescue-mechanism' initiated by endothelial cells to compensate for the pro-inflammatory, procoagulant environment. However, this 'rescue-mechanism' is not sufficient to prevent kidney damage. The above described mechanism has been proposed before in glomerulonephritis.[24]

Angiogenic factors play a substantial role in maintaining the glomerular filtration barrier. This study underlines that angiogenic factors regulate renal thrombomodulin expression, *in vitro* and *in vivo*. In vitro, sFlt-1 transfection of human umbilical vein endothelial cells resulted in downregulation of thrombomodulin expression. Contrastingly, glomerular thrombomodulin abundance increased in mice treated with anti-angiogenic compounds, and in pigs with diabetes and metabolic syndrome. Moreover, kidney thrombomodulin abundance was increased significantly in preeclampsia patients compared to pregnant controls.

Our results raise the question why sFlt-1 decreases thrombomodulin *in vitro*, but why thrombomodulin expression is increased under anti-angiogenic circumstances *in vivo*. Because thrombomodulin is known to be downregulated by hyperfiltration and shear stress, transforming growth factor beta and endotoxins, which are all increased in preeclampsia and diabetes, one would expect a decrease in glomerular endothelial

thrombomodulin expression, but our study revealed the opposite.[9] A possible explanation could be that one of the major isoforms of VEGF-A, VEGF-A 165, can be stored in the glomerular basement membrane through binding to heparin sulphate proteoglycans, so local levels of VEGF-A might even be increased under the endothelium despite increased levels of circulating sFlt-1.[25]

Interestingly, VEGF-A and sFlt-1 mRNA expression increased in diabetic pigs, resulting in a net unchanged VEGF-A/sFlt-1 ratio, implicating unchanged levels of freely circulating VEGF-A. However, thrombomodulin expression was still increased in these cases. Recently, sFlt-1 was revealed to have an effect on podocytes independent of VEGF-A.[26] sFlt-1 binds to lipid rafts on the podocyte cell surface and initiates nephrin phosphorylation and cytoskeleton changes.[26] We propose that the changes observed in preeclamptic and diabetic nephropathy do not merely result from decreased VEGF-A availability, but from independent sFlt-1 signalling to pericytes and podocytes as well.

Glomerular endothelial thrombomodulin expression is associated with increased expression of nephrin in podocytes and parietal epithelial cell activation in our study. This implies cross-talk across the glomerular basement membrane, between endothelial cells and podocytes. Known players in glomerular cross-talk are VEGF-A and endothelin-1.[10] VEGF-A produced in podocytes reaches the endothelial cells either through diffusion or transport through heparin sulphate proteoglycans, where it binds to the VEGFR-2 on the endothelium and stimulates survival and cell structure maintenance.[25] Lack of podocyte VEGF-A results in endothelial cell abnormalities. Endothelial cells become activated and produce endothelin-1, which binds to receptors on the podocyte's cell surface, resulting in podocyte damage. Thrombomodulin is a strong inhibitor of endothelial activation; enhanced thrombomodulin signaling under anti-angiogenic conditions could therefore suppress endothelin-1 expression and interfere with the glomerular damage loop.

Soluble thrombomodulin has been applied as therapy in humans during sepsis and results in decreased mortality.[27] Perhaps treatment with soluble thrombomodulin could have a beneficial effect on glomerular endothelium, and subsequently on glomerular cross talk and endothelin expression, under anti-angiogenic conditions as well. Illustrative, soluble thrombomodulin administration appears to be a promising way to restoring glomerular

function in mouse models of inflammatory kidney disease.[28] However, since thrombomodulin expression is already elevated in preeclampsia and diabetic nephropathy, it might not be the rate-limiting step in controlling the glomerular damage loop; effects of a further increase of thrombomodulin signaling in these pathologies still have to be investigated.

In sum, this research provides evidence for the hypothesis that angiogenic factors regulate renal endothelial thrombomodulin expression, *in vitro* and *in vivo*. Further, we illustrated that increased soluble thrombomodulin levels under inflammatory conditions are accompanied by increased endothelial thrombomodulin expression, and not by thrombomodulin loss. Investigating pathways through which thrombomodulin expression is increased in endothelial cells could reveal clues to restore or prevent endothelial damage in the kidney.

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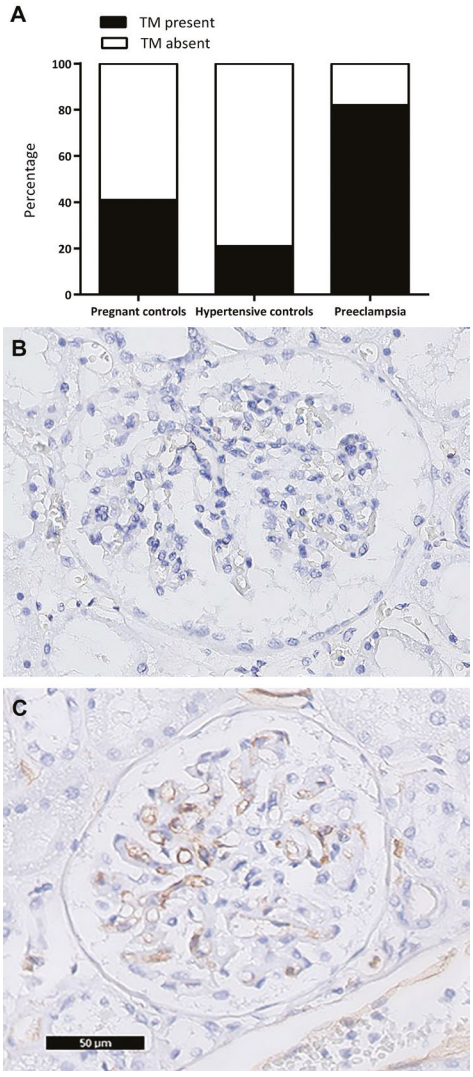
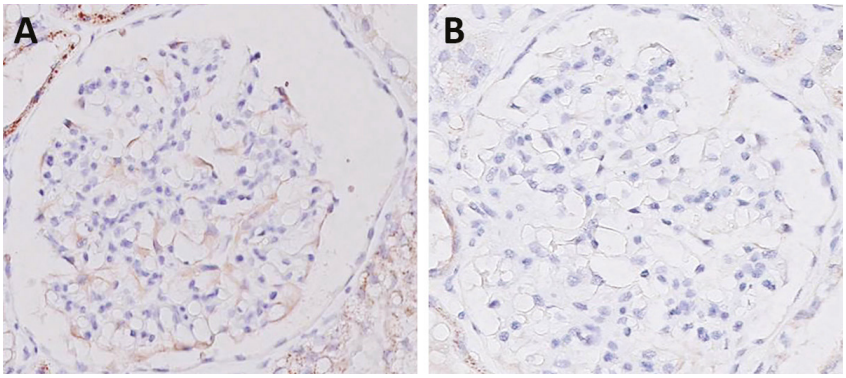
FIGURES & LEGENDS

Figure 1 thrombomodulin expression in kidney samples from human preeclampsia patients and controls



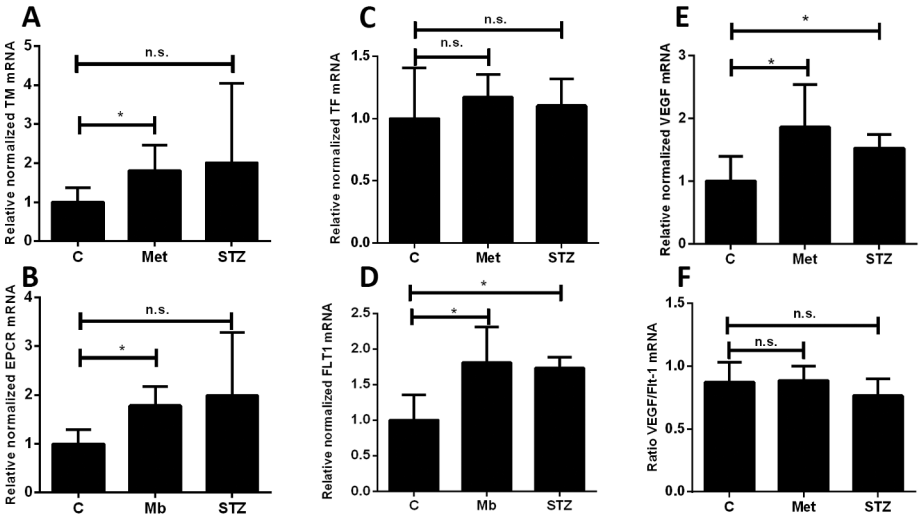
A) shows the percentage of kidney samples where thrombomodulin was absent or present in pregnant controls, hypertensive controls and preeclampsia patients.
B) shows a control kidney sample stained for thrombomodulin; there is no positive staining.
C) shows a preeclamptic kidney sample stained for thrombomodulin, there is positive staining along the capillary walls in the glomerulus and in the peritubular capillaries.

Figure 2 Thrombomodulin staining in porcine kidneys



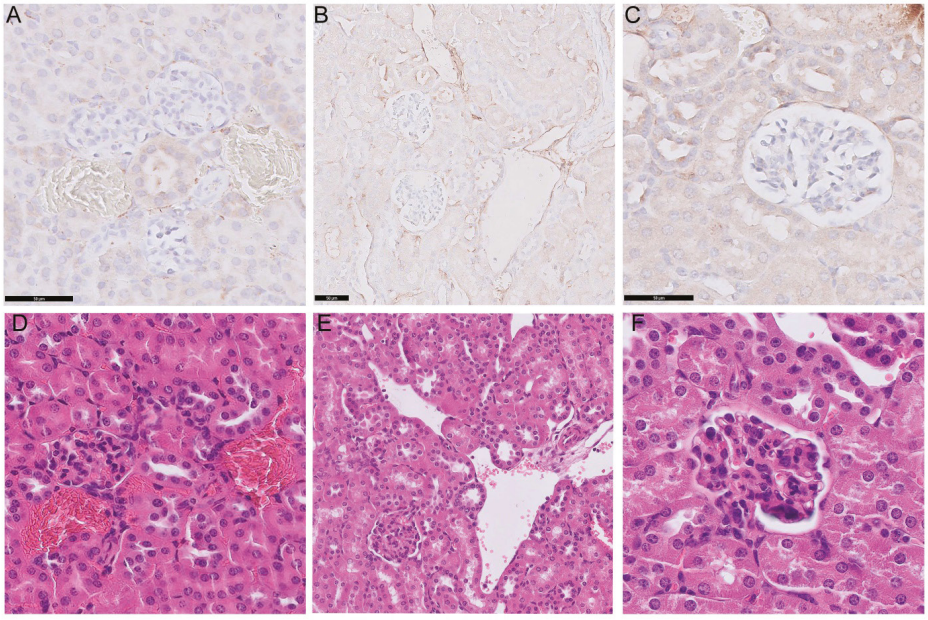
A) glomerulus from diabetic pig, immunohistochemically stained for thrombomodulin, with a positive staining pattern.
B) glomerulus from control pig, immunohistochemically stained for thrombomodulin, with a negative staining pattern.

Figure 3 mRNA expression in pig kidney tissue



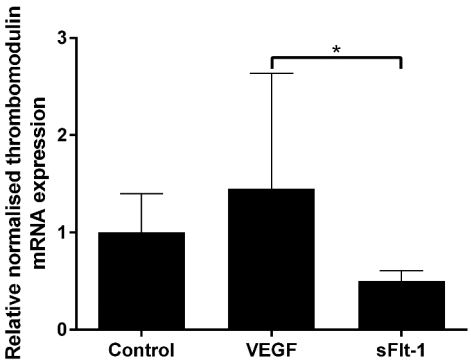
A) relative normalized thrombomodulin mRNA expression in cases and controls. B) relative normalized EPCR expression in cases and controls. C relative normalized tissue factor mRNA expression in cases and controls. D relative normalized Flt-1 mRNA expression in cases and controls. E relative normalized VEGF-A mRNA expression in cases and controls. F VEGF-A/Flt-1 mRNA ratio in cases and controls. C) control group; Mb: metabolic syndrome group; STZ: streptozotocin-treated group;*: P<0.05; n.s.: not significant; TM: thrombomodulin; TF: tissue factor.

Figure 4 Thrombomodulin expression in kidneys from mice treated with VEGF-receptor inhibiting antibodies and anti-endoglin antibodies



A) and B) thrombomodulin staining in big vessels of a mouse treated with anti-angiogenic compounds
C) thrombomodulin staining in parietal epithelial cells in a mouse treated with anti-angiogenic compounds.
D-F) HE-staining of the same regions as in A-C.

Figure 5 Thrombomodulin mRNA expression in HUVEC cells treated with VEGF and sFlt-1



Thrombomodulin relative mRNA expression in HUVEC cells incubated with VEGF, or with VEGF and sFlt-1.*: P<0,05

Chapter 5

From glomerular endothelium to podocyte pathobiology in preeclampsia: a paradigm shift

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ABSTRACT

Preeclampsia is a pregnancy-specific syndrome characterized by renal dysfunction and high blood pressure. When evaluated with light microscopy, the renal lesion of preeclampsia is marked by endothelial cell swelling and the appearance of bloodless glomeruli. However, regarding the pathobiology of renal damage in preeclampsia, attention recently has shifted from the glomerular endothelial cells to the podocytes. The angiogenic imbalance in preeclampsia plays a key role in the development of both podocyte and endothelial damage in the glomerular filtration barrier. Here we review the latest studies on the role of podocytes in the development of renal damage in preeclampsia and on podocytes as potential targets for diagnosis, treatment, and prevention of long-term complications of preeclampsia.

INTRODUCTION

Preeclampsia is a pregnancy-specific syndrome that complicates at least 2% of all pregnancies and contributes to up to 15% of maternal morbidity and mortality worldwide.[1,2] The condition is diagnosed by the presence of gestational hypertension (systolic blood pressure ≥140 mmHg and/ or diastolic blood pressure ≥90 mmHg) and proteinuria.[3] These symptoms are accompanied by underlying systemic endothelial dysfunction, inflammation, and vascular defects, which can lead to complications in different organ systems. The role of endothelial and vascular dysfunction in preeclampsia is illustrated further by the fact that women with pre-existing diabetes or hypertension are at greater risk of developing preeclampsia.[4]

The organ mainly affected in preeclampsia is the kidney. In a normal pregnancy, the renal blood flow and glomerular filtration rate increase significantly, but in preeclampsia, the glomerular filtration rate decreases.[5] Histologically, the renal lesion characteristic of preeclampsia is glomerular endothelial cell swelling with obliteration of the capillary lumen, a manifestation known as endotheliosis.[6] This endothelial involvement in the preeclamptic renal lesion is consistent with the systemic endothelial dysfunction, and endotheliosis thus has been viewed for decades as the characteristic lesion of preeclampsia.[7] However, electron microscopy evaluation of preeclamptic kidneys also reveals lesions in podocytes, the visceral epithelial cells of the glomerulus, in which the cell structure is disrupted and foot processes appear to be fused.[8]

In this review, we show that glomerular endothelial dysfunction alone does not explain renal pathology in preeclampsia. Rather, the podocyte is essential in the development of glomerular lesions and long-term renal damage in preeclampsia. In addition, we present the latest research on podocytes as promising targets for diagnostic tools and treatment in preeclampsia.

AN ANGIOGENIC IMBALANCE CAUSES RENAL DAMAGE IN PREECLAMPSIA

The exact etiology of preeclampsia and the development of renal lesions is still unknown, but the placenta plays an important role in the cause and maintenance of the syndrome. In preeclampsia, placental ischemia and ischemia reperfusion injury lead to the placental release of anti-angiogenic factors. Moreover, the placenta in preeclampsia sheds syncytial knots into the circulation, which are transcriptionally active for anti-angiogenic factors.[9] Specifically, the placental production of the anti-angiogenic factor soluble Flt-1 (sFlt-1) is increased.[10] sFlt-1 is the soluble splicing variant of Flt1, also known as vascular endothelial growth factor receptor 1 (VEGFR-1). This soluble form lacks a cytoplasmic domain and acts as a decoy receptor for vascular endothelial growth factor A (VEGF) and placental growth factor. Therefore, the increased production of sFlt-1 is associated with less freely available VEGF and placental growth factor in preeclampsia, an 'angiogenic imbalance'. Also, in preeclampsia, serum levels of a placenta-derived soluble transforming growth factor-beta co-receptor, soluble endoglin, are increased,[11] leading to impaired transforming growth factor-beta signaling and consequently altered vascular regulation.

This angiogenic imbalance is likely the cause of the renal pathology in preeclampsia. In vivo experiments of VEGF depletion in mice show renal lesions and symptoms similar to those in preeclampsia.[12,13] Mice injected with sFlt-1 or VEGF antibodies develop proteinuria, glomerular endothelial cell swelling, and loss of slit diaphragms, the cell junctions between podocytal foot processes that are required for physiological glomerular barrier function. Furthermore, patients treated with VEGF ablation therapy can develop a preeclampsia-like syndrome with high blood pressure, proteinuria, and renal lesions similar to those seen in preeclampsia.[14,15,16] These patients also show endothelial cell swelling and foot process effacement.

VEGF AND THE GLOMERULAR FILTRATION BARRIER

As the above-described findings illustrate, VEGF is important in maintaining healthy glomeruli and preventing development of preeclampsia-like renal lesions. Podocytes are the main source of VEGF in the glomerulus and contribute in this way to the maintenance of the glomerular filtration barrier. Several studies have shown that podocyte-specific VEGF knockout mice show glomerular endothelial cell swelling and podocyte foot process effacement.[17,18] Podocyte-derived VEGF regulates this maintenance of the glomerular filtration barrier in two ways: first through paracrine interaction with glomerular endothelial cells, and second through autocrine interaction with podocytes.

The paracrine interaction of VEGF in the glomerulus probably occurs through podocyte-derived VEGF that crosses the glomerular basement membrane in the opposite direction of the primary filtrate. It then binds to glomerular endothelial cells, where the potent VEGFR-2 is expressed at high levels.[19] Maintenance of fenestrations and the permeability of glomerular endothelial cells is VEGF-dependent, indicating that paracrine VEGF signaling by podocytes is essential for maintaining the normal glomerular endothelium.[20,21] Podocytes also promote survival of glomerular endothelial cells through VEGF signaling via VEGFR-2.[22]

Results regarding the autocrine VEGF loop in podocytes are contradictory. In detail, there are two transmembrane VEGF receptors. One of the two transmembrane receptors, VEGFR-1, has weak tyrosine kinase activity and acts as a decoy receptor; the other, VEGFR-2, is necessary for a normal biological response to VEGF. Sison et al. showed in a mouse model that VEGFR-2 expression levels are relatively low in podocytes and that podocyte-specific deletion of VEGFR-2 has no effect on glomerular development.[19] However, other studies using mouse models have shown that VEGFR-2 is expressed and active on podocytes.[23,24] In addition, VEGFR-2 is expressed in human podocytes, and changes in this VEGFR-2 expression are associated with renal pathology.[25,26]

Furthermore, different studies show autocrine effects of VEGF on podocytes. Therefore, the presence of an autocrine loop is generally accepted. For example, in vitro, VEGF regulates podocyte actin cytoskeleton rearrangement and foot process structure through a VEGFR-2–nephrin complex on podocytes;[27] nephrin is an essential component of slit diaphragms and

regulates foot process structure. Mutations in the nephrin gene result in proteinuria.[28] In diabetic nephropathy, VEGF production is also decreased and correlates with decreased nephrin and podocin levels and with podocyte numbers.[29] In addition, VEGF regulates TRP6 channels in podocytes, channels that co-localize with nephrin and play a role in maintaining slit diaphragm and foot process structure.[30] Furthermore, VEGF regulates matrix metalloproteinase secretion in podocytes,[31] and podocytal over- or under expression of matrix metalloproteinases causes disturbances in podocyte structure and function, leading to proteinuria. Apart from regulating podocyte structure, VEGF inhibits podocyte apoptosis directly through VEGFR-2 and Galpha-interacting vesicle-associated protein, independent of nephrin.[32]

In addition to the autocrine VEGF loop, an sFlt-1–mediated autocrine loop was also recently discovered in podocytes in a mouse model.[33] This study shows that deletion of the VEGF receptor Flt1 from podocytes causes cytoskeleton reorganization and proteinuria. Expression of a form of Flt1 lacking the cytoplasmic domain and thereby preserving sFlt-1 production rescues this phenotype, indicating that the podocyte damage is caused by loss of sFlt-1 rather than the lack of intracellular signaling through Flt1. Further research into this autocrine loop in the context of preeclampsia is of great interest because the existence of this loop indicates that high levels of sFlt-1 could lead to podocyte changes independent of VEGF.

DYSFUNCTION OF THE GLOMERULAR FILTRATION BARRIER IN PREECLAMPSIA

As these studies show, maintaining a balance between angiogenic and anti-angiogenic factors is essential for the maintenance of the glomerular filtration barrier. In preeclampsia the podocyte barrier-forming capacity is impaired because of this imbalance. Garovich et al. were the first to show that the podocytal foot process proteins nephrin and synaptopodin are downregulated in preeclampsia.[34] This downregulation correlates with an increase in sFlt-1 levels, with a decrease in VEGF levels and with proteinuria. Zhao et al. also found an altered expression of nephrin in podocytes from women with preeclampsia,[35] and this altered expression correlates with changes in polarity proteins.[36] Henao et al. further reported that sera from

preeclamptic women induced changes in important podocyte proteins such as podocin and CD2AP in cultured podocytes.[37] Also, they showed that preeclamptic sera disturb podocyte barrier formation in vitro and that VEGF supplementation restores this.[38]

Changes in podocyte slit diaphragm and foot process structure are associated with detachment and loss of podocytes.[39] As noted, less available podocytal VEGF in the glomerulus leads to glomerular endothelial cell damage, which in turn can result in further podocytal barrier disruption in preeclampsia. Collino et al. showed that endothelin-1 released from damaged glomerular endothelial cells incubated in preeclamptic sera leads to nephrin shedding by podocytes.[40] Also, endothelial cells under inflammatory conditions release microparticles that interfere with albumin endocytosis in podocytes.[41]

These findings indicate the existence of a damage loop, starting with damaged and detaching podocytes in preeclampsia, leading to glomerular endothelial cell damage through VEGF depletion and giving rise to more podocyte damage through endothelin.[42] The pathways leading to renal damage in preeclampsia are illustrated in Figure 1.

The native immune system is also dysregulated in preeclampsia, which may influence the angiogenic imbalance. As an example, activation of the complement system, part of the innate immune system, is increased in both placental tissue and the circulation in preeclampsia.[43, 44] In the kidney, there is an association with activation of the classical complement pathway in preeclampsia.[45] Furthermore, Burwick et al. recently showed that complement products are elevated in urine from women with severe preeclampsia and that complement markers correlate with kidney-injury marker 1.[46, 47] Wang et al. reported increased renal complement deposition of C3 in an auto-antibody–induced preeclampsia mouse model.[48] In the same study, complement receptor C3a inhibition improved hypertension and proteinuria and also lowered placental sFlt-1 production, indicating that complement inhibition might restore the angiogenic balance. However, whether complement activation is a cause or a consequence of preeclampsia remains elusive.

PODOCYTURIA

Podocyte detachment from the glomerular basement membrane leads in preeclampsia to the presence of both living podocytes and podocyte-specific proteins in urine, known as podocyturia. The increase in anti-angiogenic

factors in preeclampsia probably causes this podocyturia, as patients treated with bevacizumab, an anti-VEGF antibody, also develop podocyturia, and bevacizumab treatment and podocyturia show a dose-response relationship.[49] Currently, proteinuria and the protein:creatinine ratio are used as markers of renal injury in preeclampsia in clinical practice.[50] However, these markers reflect late stages of renal damage, and markers in the subclinical stage that predict preeclampsia are still lacking.[51] Because podocyturia is a more specific marker of ongoing glomerular damage, it might well be an interesting new diagnostic tool in preeclampsia.[52]

Garovic et al. in 2007 first described podocyturia as a marker for preeclampsia.[53] Since then, many studies have evaluated the detection of podocyturia as a predictive or diagnostic tool for preeclampsia, and an overview of this body of work has been published.[51] These studies all use different techniques to detect living podocytes as well as podocyte-specific proteins such as nephrin, and the specificity for diagnosing and predicting preeclampsia varies among them. For example, Garovic et al. found that all preeclampsia cases were associated with positive staining for podocin and all normal pregnant cases were negative.[53] Craici et al. performed a prospective study, detecting podocytes in urine samples by podocin staining to predict preeclampsia. All women who developed preeclampsia or gestational hypertension had podocyturia at the end of the second trimester.[54] In contrast, Kelder et al. found podocytal mRNA coding for nephrin, podocin, and VEGF in urine from normal pregnancy cases, although mRNA levels of these markers were significantly higher in preeclampsia cases.[55] Meta-analysis, validation, cost-effectiveness, and implementation studies on a larger scale should be conducted to determine which technique predicts preeclampsia most accurately and if podocyturia measurement is applicable in daily clinical practice.

THE ANGIOGENIC IMBALANCE AS A TARGET FOR THERAPY

As these findings suggest, screening for podocyturia could offer a diagnostic tool to detect preeclampsia and podocyte damage at earlier stages, which also creates possibilities for developing treatments for renal targets affected at an early stage of the syndrome. Ideally, to prevent renal damage in preeclampsia, the angiogenic imbalance originating from the placenta should be prevented or restored. However, as explained above, the autocrine

and paracrine VEGF and sFlt-1 system in the kidney is very complex. VEGF levels must be kept within very small boundaries to avoid inducing severe glomerular damage.[56] Also, VEGF levels must be regulated tightly for adequate placental development.[57] Thus, simply providing VEGF or anti-sFlt-1 therapy in preeclampsia is currently not possible.

Another interesting target in the treatment of preeclampsia would be the complement system; as mentioned above, complement inhibition can decrease placental sFlt-1 production in mice and improves hypertension and proteinuria. Further studies on the safety of complement-inhibitory drugs in pregnancy are needed.

TARGETING PODOCYTES IN PREECLAMPSIA

Another possible solution for preventing renal damage in preeclampsia is to target podocytes directly. Current preeclampsia guidelines recommend only symptomatic treatment for high blood pressure and eclampsia, but therapeutic options directly targeting podocytes in preeclampsia are lacking.[3] Therapeutic targeting of podocytes could prevent renal dysfunction in at least two ways. First, dysfunction of slit diaphragm molecules causes dysfunction of the glomerular filtration barrier and subsequently leads to proteinuria in preeclampsia. Second, damage to podocytes causes further damage to glomerular endothelial cells and gives rise to preeclampsia-like lesions in vivo. Some already available therapeutic drugs have been discovered to have previously unknown effects on podocytes.[58] For example, dexamethasone, a glucocorticoid, prevents apoptosis in podocytes and enhances intracellular trafficking of nephrin.[59, 60] Angiotensin-converting enzyme inhibitors also prevent and even restore podocyte damage, but cannot be used during pregnancy because of the risk of congenital abnormalities.[61, 62] Another widely-used group of drugs, the statins, protect podocytes by restoring podocytal slit diaphragm proteins and decreasing proteinuria in a model of HIV-associated nephropathy.[63] Statins recently also became of interest in preeclampsia because they suppress sFlt-1 and soluble endoglin release and are associated with anti-apoptotic and anti-inflammatory properties.[64] A recent case report described a patient with anti-phospholipid syndrome who presented with early preeclampsia at 23 weeks of pregnancy. After one month of treatment with pravastatin, her proteinuria and blood pressure returned to normal levels.[65] Further studies to assess the safety of statins in pregnancy

and their effectiveness during preeclampsia are needed and awaited.[64] Although there is no evidence that statins increase the risk of congenital abnormalities, thorough studies on this topic are lacking.[66]

In addition to using existing drugs for targeting podocyte-mediated renal diseases, developing new drugs specifically targeting podocytes has gained interest.[67] In recent decades, several new proteins in the podocytal slit diaphragm have been discovered, including nephrin, podocin, synaptopodin, CD2AP, NEPH1 and TRP6.[68] In preeclampsia and other proteinuric diseases, downregulation of these molecules and consequent disruption of the slit diaphragm structure contribute to the development of renal damage and proteinuria. Therefore, new drugs targeting these slit diaphragm molecules could perhaps prevent podocyte and glomerular filtration barrier damage in these conditions. Hinting at this possibility are results showing that inhibition of NEPH1 signaling with a transduction model in vitro and in zebrafish ameliorates podocytal damage in a model for minimal change glomerulonephritis.[69]

Because of their specific mode of action, an advantage of podocyte-specific drugs in comparison to currently-used drugs for renal diseases could be fewer side effects. However, thorough studies to explore the possibilities of slit diaphragm proteins as a target in renal diseases and their applicability in clinical practice still have to be conducted.

LONG-TERM EFFECTS OF RENAL DAMAGE IN PREECLAMPSIA

Women with a history of preeclampsia are at greater risk of developing renal pathology later in life. In 2010, a systematic review and meta-analysis showed that at 7.1 years postpartum, 31% of women who had preeclampsia had developed micro-albuminuria, a four-fold increased risk compared to women with uncomplicated pregnancies.[70] Furthermore, a large study from Wang et al. in 2013 showed that for women who had preeclampsia, the hazard ratio for developing end-stage renal disease is 14.0 compared to women who had normal pregnancies.[71] Studies using the Medical Birth Registry in Norway showed that preeclampsia itself, and not familial aggregation of common risk factors, leads to a relative risk of up to 15.5 for the development of end-stage renal disease.[72,73]

The cause of this increased risk of renal disease after preeclampsia could be podocyte loss and damage. Podocytes are terminally differentiated cells that

do not replicate;[74] therefore, critical podocytal loss can lead to permanent renal injury. For example, podocyte loss is associated with the development of focal glomerular sclerosis, as is preeclampsia.[75, 76] As described above, podocyturia is a key marker of preeclampsia. Recently, White et al. showed that podocyturia persists in women who had preeclampsia after delivery, whereas proteinuria normalizes.[77] This indicates ongoing, subclinical renal damage even after clinical symptoms of preeclampsia have resolved.
Although podocytes cannot replicate, the parietal epithelial cells (PEC) in the glomerulus can be recruited to replace lost podocytes.[78] Recently, interesting findings by Hakroush et al. showed that these PECs migrate to the glomerular basement membrane after extensive podocyte loss, but do not express VEGF, which results in impaired vascularization in the glomerulus and subsequent hypoxic cell death.[79] Activated PECs are even associated with the development of focal segmental glomerulosclerosis.[80] Penning et al. recently showed that in preeclampsia, activated PECs are increased compared to normal pregnant women and healthy controls and replace lost podocytes.[81] They also found a significant correlation between cellular bridges that connect the glomerular tuft and Bowman's capsule and focal segmental glomerulosclerosis. Although this is an observational study, these findings may indicate that podocyte loss and subsequent PEC activation contribute to the risk of developing focal segmental glomerulosclerosis after preeclampsia.

FUTURE STUDIES: PERSONALIZED MEDICINE

The emerging possibilities for early detection and treatment suggest opportunities for the development of personalized medicine in renal involvement in preeclampsia. Personalized medicine is defined as a strategy that seeks to improve stratification and timing of health care by utilizing biological information and biomarkers at the level of molecular disease pathways, genetics, proteomics, and metabolomics.[82] Recently, tests using both proteomics and metabolomics for the prediction and early detection of preeclampsia were developed,[83, 84] and a multi-center trial is ongoing to assess their clinical applicability.[85] However, these tests do not involve specific markers for renal involvement in preeclampsia.
As noted above, preeclampsia is associated with increased podocyte turnover, focal segmental glomerulosclerosis, and end-stage renal disease later in life. The rise of personalized medicine to detect patients at risk for developing

unresolvable renal damage in preeclampsia could possibly prevent serious renal complications. Biomarkers such as podocyturia and proteins indicative of podocyte damage open an interesting new field of research into predicting renal damage in preeclampsia more precisely. Currently, only patient characteristics such as nulliparity, pre-existing hypertension, and diabetes mellitus that put the mother into a high-risk group for the development of preeclampsia are taken into account in guidelines.[3]

CONCLUSION

In research on the pathobiology of renal damage in preeclampsia, attention has shifted from the endothelium to the podocytes. High levels of anti-angiogenic factors and VEGF depletion in preeclampsia cause podocyte damage and loss of slit diaphragm proteins, which in turn leads to loss of podocytes and podocyturia, a possible diagnostic tool in preeclampsia. Further research should explore podocyte-specific treatment options to prevent podocyte loss and subsequent permanent renal lesions.

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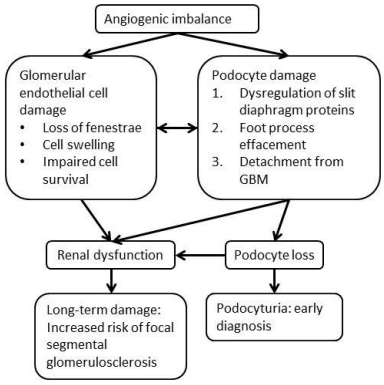
FIGURE & LEGEND

Figure 1 Overview of the pathways leading to renal damage in preeclampsia



GBM = glomerular basement membrane

The angiogenic imbalance in preeclampsia leads to damage to both glomerular endothelial cells and podocytes in the kidney. The damage to podocytes leads to podocyte loss and the presence of podocytes in urine (podocyturia). Crosstalk between podocytes and glomerular endothelial cells is essential for the maintenance of the glomerular filtration barrier. Therefore, podocyte damage leads to further glomerular endothelial cell damage and vice versa, resulting in renal dysfunction and even long-term renal damage in the form of focal segmental glomerulosclerosis.

Chapter 6

Stability and Species Specificity of Renal VEGF-A Splicing Patterns in Kidney Disease

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ABSTRACT

Vascular endothelial growth factor A (VEGF-A) is essential for maintaining the glomerular filtration barrier. Absolute renal levels of VEGF-A change in patients with diabetic nephropathy and inflammatory kidney diseases, but whether changes in the renal splicing patterns of VEGF-A play a role remains unclear. In this study, we investigated mRNA splicing patterns of pro-angiogenic isoforms of VEGF-A in glomeruli and whole kidney samples from human patients with kidney disease and from mouse models of kidney disease. Kidney biopsies were obtained from patients with acute rejection following kidney transplantation, patients with diabetic nephropathy, and control subjects. In addition, kidney samples were obtained from mice with lupus nephritis, mice with diabetes mellitus, and control mice. The relative expression of each VEGF-A splice variant was measured using RT-PCR followed by quantitative fragment analysis. The pattern of renal VEGF-A splice variants was unchanged in diabetic nephropathy and lupus nephritis and was stable throughout disease progression in acute transplant rejection and diabetic nephropathy; these results suggest renal VEGF-A splicing stability during kidney disease. The splicing patterns were species-specific; in the control human kidney samples, VEGF-A 121 was the dominant isoform, whereas VEGF-A 164 was the dominant isoform measured in the mouse kidney samples.

INTRODUCTION

Vascular endothelial growth factor A (VEGF-A) is a pro-angiogenic glycoprotein in the platelet-derived growth factor family. VEGF-A is essential for the survival, proliferation, and differentiation of endothelial cells.[1,2] In the kidney, VEGF-A is produced by visceral epithelial cells (podocytes) and transported across the glomerular basement membrane, ultimately reaching the glomerular endothelial cells, where VEGF-A plays a pivotal role in maintaining the glomerular filtration barrier by regulating the survival and structure of the endothelial cells.[3,4] VEGF-A also regulates the structure and survival of podocytes via an autocrine loop.[5,6] Many studies have highlighted the importance of VEGF-A under a variety of conditions; for example, in mice, both downregulation and upregulation of VEGF-A expression in developing glomeruli can result in severe glomerular abnormalities.[7] Moreover, overexpressing VEGF-A in the podocytes of adult mice causes

proteinuria and structural changes in the glomeruli.[8] Conversely, inhibiting VEGF-A expression in adult mice causes severe thrombotic glomerular injury.[9] Together, these results provide compelling evidence that VEGF-A expression must be tightly regulated in order to maintain the integrity of the glomerular filtration barrier.

In patients with kidney disease, renal levels of VEGF-A change during disease progression, particularly in patients with diabetic nephropathy (DN), in which a reduction in renal VEGF-A production is correlated with disease progression.[10] Also, changes in renal VEGF-A levels are seen in inflammatory-based renal diseases; patients with lupus nephritis have increased plasma levels of VEGF-A, but renal VEGF-A mRNA levels are decreased and correlate with deterioration of renal function.[11,12] During acute rejection of kidney allografts, the renal expression of VEGF-A and its receptors is increased.[13] As these studies show, quantitative VEGF-A levels change in kidney diseases. However, qualitative changes in VEGF-A expression—specifically, changes in mRNA splicing patterns—can also play a role in pathophysiology, for example in the placenta in preeclampsia.[14] Changes in the splicing of anti-angiogenic splicing variants of VEGF-A, the VEGF-A_b variants, are involved in the development of kidney damage; however, whether changes in the pro-angiogenic splice variants of VEGF-A occur during kidney disease is currently unknown.[15]

The VEGF-A gene contains eight exons that are alternatively spliced, yielding at least seven distinct pro-angiogenic splice variants. The splice variants VEGF-A 121, 165, and 189 are the most abundantly expressed isoforms in humans. The same isoforms are expressed in mice, but the murine splice variants yield isoforms that are one amino acid shorter (i.e., VEGF-A 120, 164, and 188, respectively). The proteins encoded by these three splice variants differ with respect to their transport, storage, and signaling efficacy. For example, only the longer isoforms (i.e., VEGF-A 165 and 189) contain exons 6 and 7, which encode a heparin-binding site that enables VEGF-A to bind with the extracellular matrix.[16] These two isoforms—but not the VEGF-A 121 isoform—are also essential for embryonic development, as they inhibit epithelial cell apoptosis.[17] In addition, the VEGF-A 165 isoform includes the portion of exon 7 that encodes a binding site for neurophilin, a co-receptor for VEGF-A that enhances intracellular signaling.[18]

Based on the studies discussed above, the various pro-angiogenic isoforms

of VEGF-A have distinct properties. Although total renal VEGF-A levels are altered in kidney disease, the role of each isoform is not currently known. To address this issue, we investigated qualitative changes in VEGF-A splicing patterns in a range of renal pathologies, including DN, acute renal transplant rejection, and lupus nephritis, with capillary electrophoresis. In addition, because the distribution of VEGF-A isoforms in the kidneys of healthy humans and rodents has not been examined with this method before, we also investigated VEGF-A splicing patterns in kidney samples obtained from healthy mice and humans.

MATERIALS AND METHODS

Samples

VEGF-A splice variants were measured in kidney tissues obtained from 14 human donor kidneys.[10] Laser microdissection was used to isolate the glomeruli for assessing the glomeruli-specific splicing patterns of VEGF-A. Kidney biopsies were collected from 28 patients with both diabetes type 2 and DN and used to measure the renal splicing pattern of VEGF-A. The clinical characteristics of this patient cohort have been described previously.[10] Laser microdissection was used to isolate the glomeruli. In addition, five kidney samples were obtained from mice with streptozotocin-induced diabetes (five weeks after induction), and five kidney samples were obtained from age-matched control mice; these samples were used to examine VEGF-A isoform expression in an early stage of DN.[19]

Kidney biopsies were obtained from 123 patients with acute rejection of a transplanted kidney;[20] these samples were used to investigate the renal splicing pattern of VEGF-A and were evaluated for acute and chronic lesions in accordance with the Banff classification system.[21]

Kidney samples were obtained from five healthy control mice and were used to examine the renal splicing pattern of VEGF-A. To assess the splicing patterns of VEGF-A in various organs, the lungs, lymph nodes, and spleens were obtained. Furthermore, kidney samples were obtained from a lupus nephritis mice model described and used to measure VEGF-A isoform expression at various time points. [22]

Ethics

Human samples were obtained from archived patient material from biopsies performed for routine clinical procedures. None of the transplant donors were from a vulnerable population and all donors or next of kin provided informed consent that was freely given; patients visiting Dutch hospitals are actively informed of the 'opt-out' system regarding research with their archived material, this 'Code of conduct for responsible use of residual material' applies to all studies in the Netherlands using archived patient material. Only biopsies from patients who did not opt-out have been included in this study. Samples were collected and processed anonymously and according to the medical ethics committee of the Leiden University Medical Center and the 'Code of conduct for responsible use of residual material'; approval from the institutional ethics board "Commissie Medische Ethiek LUMC" is given automatically to all studies following this code and correct adherence to the code is checked on a regular basis by national health care inspection.

Animal studies were approved by the DEC (Animal Experiments Committee) of the Leiden University Medical Center; the project code of this study is 13163. The care and use of all mice in this study was carried out in accordance with the Dutch law on animal testing. To ameliorate suffering, mice were anesthetized with isoflurane and sacrificed before collecting the kidneys.

Primer design

We designed primers that amplify all VEGF-A isoforms. The human and mouse primer sets were designed to anneal to exon 3 and exon 8, which are present in all VEGF-A mRNA isoforms. When amplified using these primers, each isoform produces a unique-length PCR product. The primer sequences are given in S1 Table.

PCR and capillary electrophoresis

RNA was isolated from the samples using Trizol (Life Technologies, San Francisco, CA). cDNA was synthesized using AMV reverse transcriptase (Roche, Basel, Switzerland). For PCR, FAM-labeled forward primers were used. The PCR products were subjected to capillary electrophoresis using an ABI 3100 fragment analyzer (Life Technologies). The ROX 500 dye was used as a size standard. The results were analyzed using GeneMapper, and the relative

level of each VEGF-A isoform was calculated by dividing the peak height of the isoform of interest by the total sum of all isoform peaks. An example of a GeneMapper analysis is shown in S1 Fig.

cDNA synthesis using specific primers

To test for a possible replication bias towards synthesizing cDNA from shorter isoform templates, we performed a one-step PCR using specific primers and mRNA isolated from glomerular samples obtained from three healthy humans. We also performed a serial dilution series of the resulting cDNAs.

Western blot analysis

Lysates from prepared from two human control kidney samples and from a HEK293 cell line. Sample buffer containing 2-mercaptoethanol was added to the lysates, and the samples were loaded on a 12.5% polyacrylamide gel. Electrophoresis was performed at 100 V for 1.5 hours. The gel was transferred to a membrane following semi-dry blotting overnight. VEGF-A was visualized on the membrane using a rabbit polyclonal anti-VEGF antibody (SC-152; Santa Cruz Biotechnology, Dallas, TX) and the Odyssey red anti-rabbit antibody (Li-Cor Biotechnology, Lincoln, NE).

Statistical analyses

Differences between groups were compared using either the unpaired *t*-test (for normally distributed data) or the Mann-Whitney *U* test (for data that were not distributed normally). Differences between >2 groups were analyzed using the one-way ANOVA. Differences with a p-value <0.05 were considered statistically significant. All analyses were performed using the SPSS statistics software package (version 20; IBM, Armonk, NY).

RESULTS

VEGF-A splicing patterns in healthy human glomeruli and whole kidney samples

We found that VEGF-A 121, 165, and 189 isoforms were all expressed in the kidneys and glomeruli of healthy human controls, and VEGF-A 121 was the most abundant isoform (Fig 1). Specifically, in the glomeruli, VEGF-A 121, 165, and 189 comprised 70%, 24%, and 5%, respectively, of the total VEGF-A pool; in the whole kidney tissue samples, VEGF-A 121, 165, and 189 comprised 75%,

22%, and 3%, respectively (Fig 1). In some samples, the 181 isoform represented <1% of the total VEGF-A pool.

VEGF-A splicing patterns in kidney disease

We next examined the VEGF-A splicing patterns in whole kidney tissue samples and microdissected glomeruli obtained from patients with moderate and advanced stages of DN. In these samples, we found a similar VEGF-A splicing pattern as in the healthy control samples. Specifically, VEGF-A 121, 165, and 189 comprised 78%, 20%, and 2%, respectively, of the VEGF-A pool in the whole kidney samples, and 72%, 22%, and 5%, respectively, of the VEGF-A pool in the glomeruli. As in the healthy samples, in a few of the disease samples, VEGF-A 181 represented <1% of the total VEGF-A pool. These patterns of VEGF-A splice variants were relatively stable, regardless of creatinine levels, proteinuria, or renal fibrosis.

Compared to healthy controls, the renal VEGF-A splicing patterns were slightly different in the renal transplant patients with acute rejection; in these patients, VEGF-A 121, 165, and 189 comprised 64%, 30%, and 5%, respectively, of the total VEGF-A pool ($p<0.01$ versus control for all three isoforms). In addition, the VEGF-A 145 and 181 isoforms were also detected in the patient samples, although both isoforms were present in extremely low levels (i.e., <1% of the total VEGF-A pool). The VEGF-A splicing patterns were stable in these patients, regardless of the Banff criteria or the degree of interstitial fibrosis. The distribution of the three principal VEGF isoforms in the kidneys of healthy controls, patients with DN, and patients with acute rejection is summarized in Fig 2.

VEGF-A isoform distribution in mouse kidney tissues

Next, we measured the VEGF-A splicing patterns in mouse kidney samples. Interestingly, we found that the renal splicing pattern in mice was significantly different than the pattern seen in human samples. In mice, VEGF-A 164 was the most abundant isoform, comprising approximately 48% of the total VEGF-A pool ($p<0.001$ versus human). The relative proportions of VEGF-A 120, 144, and 188 were 44% ($p<0.001$), <1%, and 7% ($p<0.001$), respectively (Fig 3). In kidney samples obtained from mice with diabetes, the VEGF-A splicing pattern was similar to healthy, age-matched control mice (Fig

4). The VEGF-A splicing pattern was similar in mice with lupus nephritis, and the pattern did not change over time (Fig 4).

cDNA synthesis using specific primers

Similar splicing patterns were obtained using specific primers in a one-step PCR compared to the experiments with random primers, and serial dilution of the cDNA had no effect on the VEGF-A splicing pattern (S2 Fig).

Distribution of VEGF-A isoforms in various mouse organs

We found different VEGF-A splicing patterns in tissue samples obtained from different organs from the same mouse (S3 Fig). For example, in lung tissue, VEGF-A 120, 164, and 188 comprised 53%, 28%, and 18%, respectively, of the total VEGF-A pool. In the thymus of the same mouse, these isoforms comprised 58%, 29%, and 13%, respectively, of the total VEGF-A pool.

Western blot analysis

Next, to confirm expression of the VEGF 121, 165, and 189 isoforms, we performed a western blot analysis using kidney samples obtained from two healthy human controls (S4 Fig). Both samples contained three bands that corresponded with the predicted molecular weight of the three VEGF-A isoforms. In addition, we performed a western blot analysis on HEK293 cell lysate and found bands corresponding to the VEGF-A 121 and 165 isoforms; VEGF 189 was not detected in the cell lysate (S4 Fig).

DISCUSSION

We demonstrate stability of the renal VEGF-A splicing pattern in renal pathology; specifically, splicing patterns were stable regardless of several histopathological parameters of disease progression in human patients with DN and acute renal transplant rejection, as well as in a mouse model of lupus nephritis. We also found that the pattern of VEGF-A splicing in the kidney is species-specific; notably, the VEGF-A 121 splice variant is the most abundant isoform in the human kidney, whereas the VEGF-A 164 splice variant is the most abundant isoform in mice.

In our study, we focused on the splicing pattern of VEGF-A in kidney disease. Changes in the splicing patterns of other alternatively spliced genes play a role in the development of kidney disease. For example, changes in

the splicing pattern of fibronectin can play a role in the pathogenesis of glomerulosclerosis and tubulointerstitial fibrosis by modulating the immune response and scar formation.[23] Further, Oltean et al. recently discovered that changes in splicing of anti-angiogenic VEGF-A isoforms are involved in the progression of diabetic nephropathy. Because the renal splicing pattern of pro-angiogenic VEGF-A remains stable irrespective of disease progression of DN or during acute transplant rejection, it is unlikely that a disruption in the distribution of pro-angiogenic VEGF-A isoforms plays a major role in the development of kidney disease.

On the other hand, changes in the distribution of these isoforms in the kidney could serve a protective role with respect to renal pathology. The kidney's inability to modify the distribution of VEGF-A isoforms to a pattern that protects endothelial cells under inflammatory conditions could facilitate the progression of kidney disease. For example, an upregulation of the VEGF-A 165 and VEGF-A 189 isoforms confers a robust, prolonged protective effect in glomerular endothelial cells.[18] However, because the pattern of VEGF-A isoforms does not appear to change in kidney disease, VEGF-A 121—which has relatively weak intracellular signaling strength—remains the predominant isoform, regardless of the underlying pathology.[18] Therefore, an increase in total VEGF-A production might not provide the sufficient conditions to rescue glomerular endothelial cells during renal pathology.

In our study, we quantified the pattern of VEGF-A splicing at the mRNA level. However, it is possible that the pathological changes in DN are not related to changes in VEGF-A mRNA expression and/or splicing, but may be related to post-translational modifications of VEGF-A. For example, the levels of plasmin-activator inhibitor are increased in patients with diabetes type 2; thus, plasmin-mediated post-translational cleavage of the VEGF-A 189 isoform could be dysregulated in the kidneys of patients with DN.[24,25] This dysregulation would lead to a decrease in the cleavage products of VEGF-A 189, which may play a role during disease phases with decreased angiogenic potential.[18]

Our findings in healthy human kidney tissue are comparable to previous findings by Whittle et al. and Simon et al., who detected VEGF-A 121 and 165 as the main isoforms expressed in kidney tissue with PCR and gel electrophoresis.[26,27] Our findings contradict the findings of a previous study by Bortoloso et al., in which a correlation was observed between albumin

excretion, histological changes in the glomerulus, and the levels of VEGF-A 121 and 165 levels in patients with DN.[28] This discrepancy might be due to the differences in methods used between the two studies; for example, Bortoloso et al. used PAGE followed by silver staining to quantify the various VEGF isoforms, whereas we used capillary electrophoresis. However, two lines of evidence suggest that our approach is both sensitive and robust. First, we synthesized cDNA using specific primers in order to exclude any bias for replicating the shorter isoforms. We found no difference in cDNA that was synthesized using specific primers or random primers. Second, we found distinct patterns of VEGF-A isoforms in different organs obtained from one mouse, demonstrating that our approach is sufficiently sensitive to detect differences in the VEGF-A splicing pattern. Based on these results, we conclude that regulation of VEGF-A splicing is tissue-specific, but is not altered under the pathological conditions investigated in our study.

In mouse kidney, VEGF-A 164 is the predominant VEGF-A isoform. Given the difference in splicing patterns between humans and mice, these species might differ with respect to the regulation of VEGF-A expression in the kidney. Therefore, the pathogenesis of VEGF-A-related diseases may differ between patients and mouse models. Mouse models that overexpress the VEGF-A 164 isoform have often been used to investigate kidney diseases;[8,29,30] however, because VEGF-A 121 is the predominant renal VEGF-A isoform in humans, these models may not fully represent the clinical situation in patients.

The results of our study may create opportunities to study the transport of VEGF-A in the kidney. In the kidney, VEGF-A is produced by podocytes, and it must cross the glomerular basement membrane in order to reach the glomerular endothelial cells. The glomerular basement membrane is comprised primarily of heparan sulfate proteoglycans, to which the longer VEGF-A isoforms (i.e., 165 and 189) can bind.[18,31] Therefore, these heparan sulfate proteoglycans may play a role in the storage of VEGF-A in the kidney and in the transport of VEGF-A across the glomerular basement membrane. However, because VEGF-A 121 does not bind to heparan sulfate proteoglycans, it is freely diffusible.[18] Therefore, the transport of VEGF-A across the glomerular basement membrane and the storage of VEGF-A in the human kidney may be regulated by other mechanisms.

ACKNOWLEDGEMENTS

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SUPPLEMENTAL FIGURES AND TABLES

Supplemental material can be found at http://journals.plos.org/plosone/.

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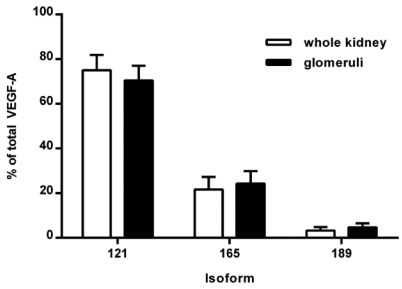
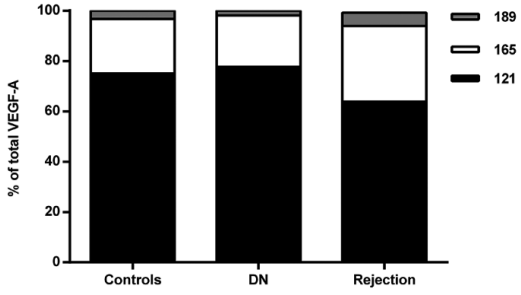
FIGURES & LEGENDS

Fig 1. VEGF-A splicing patterns in healthy kidneys and glomeruli.



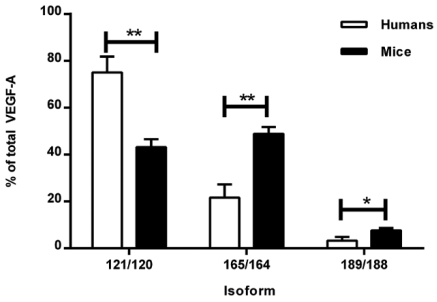
The VEGF-A 121, 165, and 189 splice variants (isoforms) were measured in total kidney tissue and glomeruli (isolated using laser microdissection) obtained from healthy human subjects.

Fig 2. VEGF-A splicing patterns in kidney tissue obtained from healthy subjects, patients with DN, and patients with acute renal transplant rejection.



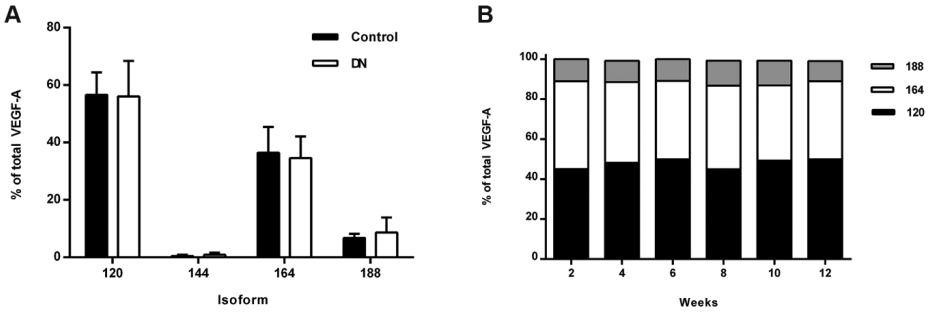
The VEGF-A 121, 165, and 189 isoforms were measured in the indicated samples. DN, diabetic nephropathy.

Fig 3. VEGF-A splicing patterns in kidney tissue obtained from healthy mice and humans.



The VEGF-A 121, 165, and 189 isoforms were measured in kidney biopsies obtained from healthy human subjects, and the corresponding VEGF-A isoforms (VEGF-A 120, 164, and 188, respectively) were measured in kidney tissue and obtained from healthy mice. *p<0.001.

Fig 4. VEGF-A splicing patterns in whole kidney tissue obtained from mice with DN, healthy age-matched control mice and mice with lupus nephritis.



A The VEGF-A 120, 144, 164, and 188 isoforms were measured in whole kidney tissue obtained from mice with early-stage DN and age-matched control mice. The two groups did not differ significantly with respect to any isoform measured (p>0.05). **B** Kidney samples were collected from mice at the indicated time points after lupus nephritis was induced. The glomeruli were isolated, and the VEGF-A 120, 164, and 188 isoforms were measured. The distribution of VEGF-A isoforms was unchanged over the duration of the experiment.

Chapter 7

Summary and general discussion

SUMMARY

As outlined in the introduction of this thesis, preeclampsia is a potentially devastating pregnancy complication with a complex, multifactorial aetiology. The risk factors lie within the genetic, vascular and immune domains. Genetic risk factors reported previously fall into two distinct categories: coagulation and vascular maintenance on one hand, and immunology on the other. Acquired contributing factors comprise vascular and immunologic components as well, with pre-existing diabetes, hypertension, obesity and older maternal age falling in the first, and oocyte donation, autoimmune diseases, nulliparity and new paternity falling into the latter category, respectively. These challenging vascular and immunologic states supposedly contribute to impaired placental development, placental oxidative stress and production of anti-angiogenic factors and subsequent systemic endothelial dysfunction. The complex interplay between these three pathways leading up to endothelial dysfunction in preeclampsia (coagulation, inflammation and angiogenesis) is studied in this thesis.

Chapter 2 - genetic variants in preeclampsia

Preeclampsia has a clear familial component, suggesting that the condition may be partly attributable to genetic susceptibility. Within the genetic susceptibility, clues on the causal pathways of the syndrome might remain. However, many genetic association studies on preeclampsia have been performed, but they have yielded inconsistent results. Therefore, **chapter 2** focuses on identifying the pooled effect of each genetic variant that is reproducibly associated with preeclampsia through meta-analyses. 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.

Seven variants 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). Interestingly, four of these mutations (in F2, F5, and SERPINE1) are found within genes involved in the coagulation and fibrinolysis systems, reinforcing the association between inherited thrombophilia and preeclampsia. Two genes (ACE and LPL) are directly

involved in endothelial biology; ACE is a key regulator of blood pressure through activation of the renin-angiotensin-aldosterone system, and LPL facilitates lipid uptake in endothelial cells. Dysregulation of these pathways causes endothelial activation and a subsequent inflammatory response. Interestingly, these variants are also risk factors for developing cardiovascular disease, revealing that preeclampsia and cardiovascular disease have shared genetic risk factors. Many association studies have reported links between mutations in genes involved in the immune system and preeclampsia, but only a mutation in CTLA4 remained significant after meta-analysis.
These results reveal coagulation and inflammation as key elements in the genetic background of preeclampsia. Studying these pathways in depth might provide new insights on how placental dysfunction and the angiogenic imbalance of preeclampsia develop. However, the role of the genetic component in preeclampsia should not be overestimated: not all women with these mutations develop preeclampsia, and not all women who suffer from preeclampsia carry these mutations. Pre-existing endothelial damage or immunologically challenging pregnancies could provide the other 'hits' necessary for the development of placental dysfunction and the syndrome.[1, 2]

Chapters 3 and 4 - thrombomodulin and the angiogenic imbalance of preeclampsia
A possible link between endothelial damage and dysregulation of coagulation and inflammation as seen in preeclampsia could be thrombomodulin. This is an endothelium- and syncytiotrophoblast-bound protein that regulates coagulation, inflammation, apoptosis, and tissue remodelling. Thrombomodulin can be cleaved from the endothelium under inflammatory conditions, and in preeclampsia, levels of this non-functional soluble thrombomodulin are increased, indicating loss of functional thrombomodulin from the endothelium. Therefore, we hypothesized that thrombomodulin loss and subsequent loss of its mediating effects on coagulation and inflammation could be involved in preeclampsia. **Chapter 3 and 4** of this thesis are focused on the role of thrombomodulin in preeclampsia. First, we investigated placental thrombomodulin dysregulation and consequent downstream effects in the pathogenesis of preeclampsia; these experiments are described in **chapter 3**.
Thrombomodulin protein and mRNA expression were investigated in placentas from women with preeclampsia, from women with a normal, term

pregnancy and from women with a pregnancy complicated by intrauterine growth restriction matched for placenta- and birth weight. Both protein and mRNA expression were significantly decreased in preeclampsia as compared with both control groups, indicating loss of thrombomodulin production and abundance in the preeclamptic placenta. Further, thrombomodulin mRNA expression correlated with maternal body mass index and diastolic blood pressure in preeclampsia, suggestive of a link with the extent of vascular dysfunction.
The pathways mediated by thrombomodulin, including coagulation, inflammation, apoptosis and tissue remodelling, were investigated in this subsets of placentas. An increase in placental apoptotic cells was associated with preeclampsia. Thrombomodulin expression correlated positively with matrix metalloproteinase expression in preeclampsia, but not with fibrin deposits, inflammatory markers or the influx of inflammatory cells.
Studies in endothelial cells have shown before that VEGF is a stimulator of thrombomodulin expression.[3] Therefore, we hypothesized that the angiogenic imbalance of preeclampsia could be the cause of the low placental thrombomodulin levels found in our group of patients. Indeed, placental soluble Flt-1 mRNA expression correlated with decreased thrombomodulin expression. To further study the possibility of a causal mechanism, thrombomodulin mRNA expression was determined in VEGF-transfected trophoblast cell lines; VEGF induced significant upregulation of thrombomodulin mRNA expression in these cells.

Altogether, the experiments described in **chapter 3** stress the link between the angiogenic imbalance and endothelial pathology preeclampsia. Concurrently, they stage thrombomodulin as a new role-player in the development of placental and endothelial pathology in preeclampsia. In **chapter 4**, these mechanisms are investigated more in-depth in the organ most affected during preeclampsia, the kidney.
Glomerular thrombomodulin was studied in human patients with preeclampsia and deceased pregnant women without hypertensive complications as control subjects. Kidney thrombomodulin abundance was increased significantly in preeclampsia patients compared to pregnant control subjects. Interestingly, glomerular endothelial thrombomodulin expression was associated with parietal epithelial cell activation and nephrin

expression in podocytes, indicative of a protective effect on the glomerular filtration barrier.
Subsequently, we hypothesized that sFlt-1 might be responsible for this increase in glomerular thrombomodulin expression, but in vitro, sFlt-1 addition to human umbilical vein endothelial cells resulted in downregulation of thrombomodulin expression. Contrastingly, glomerular thrombomodulin abundance increased in mice treated with the anti-angiogenic factors sFlt-1 and sEng. Results from **chapter 3** hinted towards an association between obesity and thrombomodulin loss; therefore, glomerular thrombomodulin expression was investigated in porcine models for diabetes and metabolic syndrome; glomerular thrombomodulin was increased in these models as well.
In sum, **chapter 4** provides evidence for the hypothesis that angiogenic factors regulate renal endothelial thrombomodulin expression, in vitro and in vivo. Possibly, the increased endothelial thrombomodulin expression in preeclampsia indicates an attempt of glomerular endothelial cells to maintain cytoprotection during the challenging, anti-angiogenic environment of preeclampsia. Such a mechanism has been proposed before in glomerulonephritis.[4] Investigating pathways through which thrombomodulin expression is increased in endothelial cells could reveal clues to restore or prevent endothelial damage in the kidney. Interestingly, thrombomodulin expression is associated with increased expression of podocyte markers; this stresses the importance of cross-talk across the glomerular basement membrane, between endothelial cells and podocytes.

Chapter 5 - the interplay between endothelium and podocytes in preeclampsia
The role of this cross-talk in preeclampsia is investigated further in **chapter 5.** In this chapter, current literature concerning the kidney in preeclampsia was reviewed for studies concerning endothelium and podocyte biology and their cross-talk.
Early studies mainly describe endothelial cell swelling or 'endotheliosis', and the appearance of apparently bloodless glomeruli as trademarks of preeclampsia.[5] However, since endothelial pathology is not observed in all patients with preeclampsia and proteinuria, these lesions cannot explain the renal pathology in preeclampsia on their own. Podocyte foot process effacement in preeclampsia has been described as early as 1980,[6] but the

hypothesis for direct podocyte involvement in the pathogenesis of the syndrome had not been proposed until 2007.[7] Since then, numerous studies on the development of podocyte damage and the use of podocytes in urine as a diagnostic tool have been published.

The angiogenic imbalance in preeclampsia plays a key role in the development of both podocyte and endothelial damage in the glomerular filtration barrier and is presumed to be responsible for a glomerular *damage loop*. VEGF is a crucial survival factor for both cell types in the glomerular filtration barrier. Glomerular VEGF is predominantly produced in the podocytes; high levels of sFlt-1 prevent glomerular VEGF from binding to its receptors on the podocytes and glomerular endothelial cells. Consequently, podocytes get damaged and detach from the glomerular basement membrane, leading to even less available VEGF through diminished production. Endothelial cells, deprived of VEGF, get swollen and produce endothelin, which damages the podocytes even further.

An appropriate treatment for preeclamptic nephropathy might be established by interrupting this *damage loop*. During the last decade, major advances in the field of podocyte biology have been achieved through studying the structure of their foot processes. Numerous new slit diaphragm and cytoskeletal proteins have been discovered, and treatment strategies targeting these proteins in vitro have shown promising results. However, restoring levels of single podocyte structure proteins on their own in kidney disease will probably not be effective; the architecture of the podocyte foot processes and their slit diaphragms is particularly complicated and involves hundreds of different proteins, and their levels and interactions are delicately balanced. A gain- or loss of function in one of these proteins might even result in disruption of podocyte architecture and irreversible glomerular damage.

Chapter 6 - VEGF splicing variants in the kidney

Overall, **chapter 5** highlights the importance of VEGF in the cross-talk between podocytes and endothelial cells in the healthy glomerulus. Remarkably, it still remains an enigma how this cross-talk is established; somehow, VEGF manages to cross the glomerular basement membrane in the opposite direction of the filtrate flow, and reaches the endothelial cells. Clues might lie within the composition of the renal splicing pattern

of VEGF; the VEGF mRNA can be spliced alternatively, resulting in at least 8 different isoforms. Some of these isoforms are short and can diffuse freely; others possess heparin binding sites and are stored and transported in extracellular matrix until release.[8] To gain more insight on VEGF crosstalk in the glomerulus in kidney pathology, **chapter 6** focuses on renal VEGF splicing patterns in health and disease.

First, mRNA splicing patterns of pro-angiogenic isoforms of VEGF in glomeruli from human and murine control subjects were determined with capillary electrophoresis. Strikingly, the splicing patterns were revealed to be species-specific; in the control human kidney samples, the short and freely diffusible VEGF 121 was the dominant isoform, whereas the longer and more potent VEGF 164 was the dominant isoform measured in the mouse kidney samples. This suggests different mechanisms of VEGF transport in human and murine kidneys, and brings to question if studies on glomerular VEGF in murine models can be extrapolated to the human situation.

Subsequently, VEGF splicing patterns during endothelial dysfunction and under inflammatory conditions were measured in kidney samples from patients with diabetic nephropathy and from patients with acute rejection following kidney transplantation, respectively. In addition, kidney samples from mice with lupus nephritis and mice with diabetes mellitus were studied. The pattern of renal VEGF-A splice variants was unchanged in diabetic nephropathy and lupus nephritis and was stable throughout disease progression in acute transplant rejection and diabetic nephropathy; these results suggest renal VEGF-A splicing stability during kidney disease. This could, on the one hand, imply that renal splicing is not affected by inflammatory or metabolic kidney damage. On the other hand, these results could show inability of the podocyte to change its splicing pattern towards a more favourable one for survival during challenging circumstances.

GENERAL DISCUSSION

The work described in this thesis underlines the complexity of the aetiology of preeclampsia. In the placenta, loss of cytoprotection, increased coagulation and increased inflammation may give rise to placental dysfunction and subsequent production of anti-angiogenic factors, as strengthened in **chapters 2 and 3**. However, the predisposing factors for preeclampsia are so heterogeneous, that preeclampsia and its placental dysfunction might merely

reflect a phenotype resulting from various syndromes or diseases, composed of more confined aetiologies. Below, this hypothesis is explored and the results from this thesis are considered in this setting.

THE AETIOLOGY OF PLACENTAL DYSFUNCTION IN PREECLAMPSIA: A SPECTRUM RANGING FROM COAGULATION TO IMMUNITY?

Although an imbalanced inflammatory environment in the placenta during early development has been proposed as the primary mechanism for placental dysfunction in preeclampsia,[9] **chapter 2** of this thesis mainly provides support for inherited thrombophilia as a genetic contributor to preeclampsia. These results suggest that the immune component of the aetiology of preeclampsia is probably composed of acquired risk factors, which offers perspective for the targeting of these risk factors in prevention of the syndrome. These observations gave rise to a hypothesis where preeclampsia comprises of a spectrum of pregnancy complications with the placenta as the central role player, but with different underlying aetiologies of the placental dysfunction, ranging from inherited thrombophilia to extreme immune dysregulation (Figure 1). This is of particular interest, because treatment strategies targeting downstream effects of preeclampsia have not been effective so far; targeting processes upstream from endothelial dysfunction might be a more effective approach. Each of these possible factors contributing to placental dysfunction and suggestions for their individual approach will be discussed below.

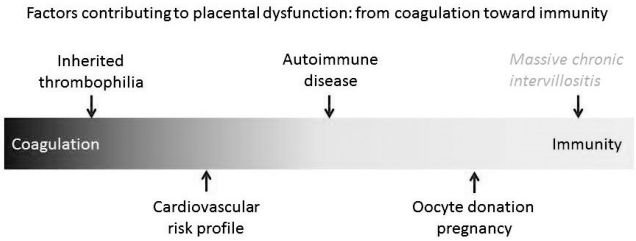


Figure 1 Factors contributing to placental dysfunction: from coagulation towards immunity

It is hypothesized that the underlying aetiologies of placental dysfunction in preeclampsia lie within a spectrum, ranging from coagulation towards immunity

Inherited thrombophilia

A clear, distinct contributor to the development of placental dysfunction in preeclampsia would appear to be inherited thrombophilia; e.g. with mutations in genes encoding for factor V, prothrombin and SERPINE1, as illustrated in **chapter 2**. Women with these mutations have increased activation of the coagulation cascade, putting them at risk for developing deep venous thrombosis and preeclampsia.[10] A rationale for this might lie within the communication between embryonic cells and the mother's coagulation system through thrombin, thrombomodulin and the PAR-1 receptor;[11] excessive coagulation and disturbed PAR-1 signalling could subsequently lead to impaired trophoblast function and subsequent placental dysfunction, as illustrated by our own results in **chapter 3.**

However, the thrombophilic mutations described in **chapter 2** have a low penetrance regarding the preeclampsia phenotype; a 'second hit' and other risk factors clearly are essential, perhaps from the lifestyle category discussed below.[12] Although these variants remained associated with preeclampsia after meta-analyses, others still question the association between these mutations and preeclampsia completely, mentioning possible publication bias favouring small studies reporting high relative risk ratios.[13] Nevertheless, inhibiting the coagulation and complement systems with heparins in women with inherited thrombophilia after their first adverse pregnancy outcome decreases the risk of foetal growth restriction, implying a positive effect on the placenta.[12, 14] All in all, the contribution of inherited thrombophilias to the aetiology of preeclampsia seems present but not substantial without other contributing factors. Other, more effective ways of targeting the coagulation system have to be investigated before genetic screening for and treatment of inherited thrombophilia becomes effective for the prevention of preeclampsia in pregnant women.

Cardiovascular risk factors

This brings us to the second etiological pathway, which is particularly exciting, because it might provide us with the most accessible handles for preventing preeclampsia. This second pathway consists of adverse cardiovascular circumstances predisposing for preeclampsia. The greatest contributors in this category are obesity, metabolic syndrome, hypertension, and diabetes,[15, 16] and the share of these contributors is probably only going

to grow in the next decade due to the obesity-epidemic. In Europe and the United States of America, more than half of the population is overweight today.[17] Interestingly, the incidence of preeclampsia has grown along with the obesity incidence.[18]

Obesity and its comorbidities are associated with dysregulation of both coagulation and inflammation, so this group lies more towards the middle of the spectrum. All these risk factors namely contribute to endothelial dysfunction prior to pregnancy, reducing the endothelial reserve to cope with challenging circumstances such as pregnancy. For example, women with chronic hypertension prior to pregnancy develop preeclampsia more often than normotensive pregnant women, but when they develop preeclampsia, the sFlt-1/ placenta growth factor ratio is smaller than in normotensive subjects, indicating increased sensitivity to small changes in this ratio.[19]

More in-depth, obesity, metabolic syndrome and diabetes cause endothelial dysfunction through various pathways, including hyperlipidaemia, increased inflammation and insulin resistance.[20] Interestingly, **chapter 2** of this thesis strengthened this hypothesis by underlining the link between lipoprotein lipase mutations and preeclampsia. The contribution of this predisposing mechanism is illustrated further illustrated by **chapter 4** from this thesis, where models of diabetes and metabolic syndrome show increases in glomerular thrombomodulin expression similar to those seen in preeclampsia.

Adverse cardiovascular circumstances could even contribute to the pathophysiology of preeclampsia during the placental development stage; they have been proposed to enhance vascular dysfunction of the decidual arteries, possibly through increased inflammation and disrupted remodeling,[21] which could contribute to diminished placental blood supply. Also, interference with the normal coagulation-trophoblast interaction through PAR-1, as mentioned above, could contribute to impaired trophoblast invasion. This thought is strengthened by the results from **chapter 3** from this thesis, where the extent of placental thrombomodulin loss was correlated to maternal BMI and blood pressure, and to placental dysfunction.

The great impact of these metabolic disturbances on the endothelium even prior to pregnancy might provide an explanation for the modest effect of treatment targeting downstream effects of coagulation and inflammatory pathways, such as aspirin and heparin. Treatment strategies targeting the dysfunctional endothelium itself and diminishing the endothelial activation

might have a more potent effect. Perhaps activating PAR-1 signalling through thrombomodulin to enhance anti-inflammatory and anti-coagulative signalling, as in more severe examples of endothelial dysfunction, could be of use here.

Autoimmune disease and preeclampsia

Slightly further towards the immunological end of the spectrum we find preeclampsia in the setting of autoimmune diseases. Some autoimmune diseases which are associated with renal involvement themselves are associated with a particular high risk of preeclampsia, for example, the risk of preeclampsia in systemic lupus erythematosus can increase to up to 40 percent.[22] However, the presence of autoantibodies, in particular antiphospholipid antibodies, independent of disease activity is associated with preeclampsia.[23, 24] The imbalanced immune system and in particular the T-cells have been proposed to be involved in the development of preeclampsia in the setting of autoimmune disease.[22] Nevertheless, heterogeneity in the extent of the autoimmune disease prior to pregnancy appears to be of importance as well when calculating the risk for obstetric complications, pointing towards the 'endothelial reserve' hypothesis described above.[22] So far, maximizing care and minimizing symptoms prior to pregnancy still appear to be the most feasible options for preventing obstetric complications in autoimmune disease.

Oocyte donation

A distinct form placental dysfunction occurs with preeclampsia in the setting of an oocyte donation pregnancy. Oocyte donation is a technique that involves harvesting of oocytes from a donor, subsequent fertilization of the ovum with sperm in vitro, and transfer of the embryo in the recipient mother's uterus. If implantation succeeds, logically, this results in a foetus that is completely allogeneic to the mother, creating a challenging environment for the mother's immune system, comparable with the setting of organ transplantation. This allogeneic foetus appears to be the main 'hit' for the development of preeclampsia after oocyte donation. Further, women receiving oocyte donation are generally older than women with an autologous pregnancy, putting them at an increased risk of developing

pregnancy complications and experiencing 'second hits' such as diabetes or hypertension.[25]

The immunomodulating mechanisms of early pregnancy described in the introductory chapter of this thesis do not suffice in oocyte donation pregnancy, and characteristic changes can be found in the oocyte donation placenta, such as villitis of unknown aetiology and chronic deciduitis.[26] These changes are much more prominent than those in the preeclamptic placenta from naturally conceived pregnancies; for instance, in **chapter 3** of this thesis, no differences were found in placenta infiltrates between cases and control subjects, despite loss of the inflammation-modulating thrombomodulin. Further, direct evidence of differences in regulation of the innate and adaptive immune systems between naturally conceived and oocyte donation pregnancies reveals reduced regulation of the complement system and increased T-cell activation in oocyte donation pregnancies.[27, 28]

These changes in immunoreactivity are likely contributors to the development of placental dysfunction in preeclampsia; as described in the introductory chapter, delicate balances between pro- and anti-inflammatory subtypes of immune cells have to be established in the developing placenta to facilitate assistance for the trophoblast cells during invasion and remodelling of the placenta. Disturbances in the composition of these macrophage, NK-cell or T-cell populations are associated with impaired placentation. So far, mainly term placentas from women with oocyte donation pregnancies have been studied for their immune population compositions. In-depth studying of the early changes in placental immune cell composition during preeclampsia in oocyte donation pregnancies might provide therapeutic targets for prevention of immune-mediated forms of preeclampsia.

Massive chronic intervillositis

An extreme variant of placental dysfunction due to immune dysregulation is massive chronic intervillositis of the placenta. Massive chronic intervillositis is characterized by a wide-spread intervillous infiltrate composed of T-cells and macrophages, somewhat resembling a form of chronic rejection, however, the macrophages make up over 80% of the infiltrate.[29] Malaria infection during pregnancy can be associated with a similar placental infiltrate, but to date, no infectious agent has been identified in massive chronic intervillositis.[30] Chronic intervillositis has been reported to be associated

with foetal growth impairment, intra-uterine foetal death, and, unfortunately, has a high recurrence rate.[31] Interestingly, the syndrome is associated with antiphospholipid syndrome, suggestive of an aetiology involving autoimmunity.[32]

To date, it remains a mystery what attracts the macrophages in massive chronic intervillositis. In preeclampsia, excessive amount of M1-type, pro-inflammatory macrophages are attracted to the placenta in reaction to oxidative stress.[33] In massive chronic intervillositis, an adverse event such as large decidual artery infarction or placental dysfunction during early stages of pregnancy might provoke a similar, but enhanced reaction. This severe placental syndrome does not give rise to maternal symptoms and therefore does not fit into the spectrum of preeclampsia completely, but studying an extreme variant of immune dysregulation in the placenta might provide new, confined mechanisms regarding the regulation of inflammation in the placenta that can be extrapolated to the setting of preeclampsia, and vice versa.

GLOMERULAR VEGF CROSS-TALK

All in all, different pathways appear to contribute to the development of placental dysfunction in preeclampsia, ranging from dysregulation of coagulative to inflammatory processes. So far, kidney involvement in preeclampsia appears to have a more confined aetiology, and appears to be mainly caused by the angiogenic imbalance. This is supported by findings in animal models of anti-angiogenic circumstances, which show glomerular changes similar to preeclampsia. Further, removal of sFlt-1 from the circulation in women with preeclampsia has shown to reduce proteinuria significantly in a small trial recently.[34] Also, other hallmarks of preeclamptic nephropathy, such as complement and fibrin deposition,[35] appear to lie downstream of the angiogenic imbalance: both appear after treatment with anti-angiogenic agents.[35, 36]

Below, the impact of an angiogenic imbalance on the kidney and key points for future research are discussed.

Future: interference with glomerular crosstalk

Anti-angiogenic factors interfere with the delicate balance in the cross-talk between podocytes and endothelium, which leads to a damage loop, as supported in **chapters 4, 5 and 6**. Further research into glomerular VEGF cross-talk should comprise the exact role and transportation of sFlt-1 and VEGF in the kidney. As described in chapter 6, the freely diffusible VEGF121 is the dominant VEGF isoform in the human kidney. This isoform lacks heparan binding sites and could therefore, theoretically, freely pass the glomerular basement membrane to reach the endothelial cells. However, the filtration pressure over the glomerular basement membrane approaches 20 mmHg and subsequently results in shear stress with pressures reaching 8 Pa on the podocytes near the basement membrane.[37] The enigma remains how podocytal VEGF 121 could reach the glomerular endothelial cells in the opposite direction of these forces; it would appear more logical for VEGF121 to be washed away with the urine. Perhaps VEGF 121 is involved in local or autocrine VEGF signalling between podocytes, and the longer isoform VEGF 165 is transported over the glomerular basement membrane through binding to heparans. Still, in the setting of preeclampsia, this seems illogical: the glomerular filtration rate diminishes in preeclampsia and glomerular blood flow decreases, presumably leading to decreased filtration pressure and shear stress on the podocytes, logically making it easier for VEGF to pool and reach the endothelial cells.[38]

Obviously, the excessive sFlt-1 levels in preeclampsia prevent the podocytal VEGF from binding to its receptors on the endothelium and podocytes; sFlt-1 can freely pass the glomerular basement membrane and can be measured in urine from women with preeclampsia.[39] However, results from thesis **chapter 4**, together with previous results from other groups,[40] show increases of renal VEGF expression under anti-angiogenic circumstances. This raises the question if this increase in angiogenic factors would not be enough to compensate for the increased sFlt-1 levels. Of course, the anti-angiogenic imbalance of preeclampsia leads to a distinct renal phenotype, but a recent study shed new light on the role of sFlt-1 in kidney disease. Jin et al revealed that podocytes possess an sFlt-1 receptor which regulates their actin cytoskeleton arrangement.[41] Further, this sFlt-1 receptor seems to be present on pericytes in the rest of the vasculature as well. Possibly, the increased

sFlt-1 levels in preeclampsia act on these pericyte receptors and promote the hypertensive phenotype this way.
Interfering with VEGF cross-talk in the glomerular damage loop may not be the only promising option for targeting preeclampsia; recent studies have revealed promising results by interfering with glomerular cross-talk on the level of the endothelium. The endothelin pathway appears to be a promising target: blocking endothelin signalling in a mouse model of diabetic nephropathy ameliorated proteinuria.[42] Endothelial endothelin expression is increased in preeclamptic women as well, making it appear as a promising target.[43] However, endothelin expression is a downstream effect of endothelial activation, and targeting the endothelial activation of preeclampsia as a whole would probably more effective and subtle, as endothelin is an important vasoconstrictor and blocking it completely will not be possible for humans. An inhibitor of endothelial activation is thrombomodulin; as described in **chapter 4**, levels of glomerular thrombomodulin increase as a counter-mechanism under anti-angiogenic circumstances, but this increase is not sufficient. Soluble thrombomodulin administration appears to be a promising way to restoring glomerular function in mouse models of inflammatory kidney disease;[44] perhaps treatment with soluble thrombomodulin could have a beneficial effect on glomerular endothelium, and subsequently on glomerular cross talk and endothelin expression, under anti-angiogenic conditions as well.

Irreversible damage after preeclampsia?

After delivery of the placenta, levels of anti-angiogenic factors in serum return to normal and proteinuria resolves in most women with preeclampsia. However, persistent podocyte loss after resolution of preeclampsia has been described,[45] and women who have had preeclampsia remain at an increased risk for developing chronic kidney disease.[46] The ongoing and increasing damage even after resolution of the anti-angiogenic environment suggest an irreversible and continuing process in the glomerulus after preeclamptic nephropathy.
Recently, Kriz et al proposed an interesting paper on a hypothesis where loss of several podocytes leads to increased mechanical stress on other podocytes, leading to irreversible podocyte damage and loss.[37] In contrast to the glomerular endothelium, which can replicate and expand easily,

and the glomerular basement membrane, which has never been reported to break under physiologically possible ranges of pressure, the podocytes are particularly sensitive to increased filtration through the glomerulus. Podocytes have a limited capacity to expand with increasing GBM areas due to the definite width of the slit diaphragm, and cannot be replaced effectively when they are damaged. Loss of podocytes, as in preeclampsia, leads to an increased working load for the remaining podocytes, and subsequent increased mechanical challenges. According to Kriz, this leads, in the long term, to glomerulosclerosis.
In patients who experienced preeclampsia, this process might be accelerated by the unfavourable cardiovascular circumstances associated with preeclampsia; diabetes or hypertension increase glomerular stress and podocyte working load even further. Therefore, physicians should be cautious when treating a patient with cardiovascular problems and preeclampsia in their patient history; these women might be at an increased risk for developing renal failure as well.[46]

Preeclampsia: a 'model' disease?

Kidney disease in most women with preeclampsia runs relatively mild. However, preeclamptic nephropathy is of particular interest for the rest of the field of kidney research; preeclampsia can be viewed as an extreme example of the disruption of glomerular VEGF crosstalk during a short period of time. Observing the changes and mechanisms involved in restoring this cross-talk mild provide suggestions for research into other forms of proteinuric kidney disease. Milder, but chronic forms of angiogenic imbalance play a role in for example diabetic nephropathy and FSGS, which is also associated with disrupted TGF-beta signaling.[47, 48] Recently, different polymorphisms of the VEGF gene have even been reported to be associated with hypertension-related chronic kidney disease.[49-51] Since VEGF cross-talk appears to act differently in the human and murine kidney, as suggested in **chapter 6**, patients with preeclampsia might be the best 'in vivo' example of an anti-angiogenic state. However, this might be unpractical because biopsy specimen from patients with preeclampsia are rare or even non-existent, since kidney biopsy is a relatively hazardous procedure, especially during pregnancy and the cause of kidney disease in preeclampsia usually is evident, so the need for a biopsy is often absent.

FUTURE PERSPECTIVES

In conclusion, preeclampsia appears to be a two-stage disease, starting with disrupted placental formation through both vascular and immunological challenges early during pregnancy, and resulting in maternal systemic involvement, with the vascular system and the kidney as the first organs targeted. However, the reverse is also true, as noted in this discussion; pre-existent vascular damage and kidney disease prior to pregnancy also put women at risk for developing preeclampsia, probably by decreased reserves for coping with the increased demands of pregnancy. So, cause and consequence appear to be intertwined in the aetiology of preeclampsia, resulting in a complex, multifactorial pathogenesis. This can also possibly explain why treating single targets from the immune or coagulation system at once, during later stages of the disease, does not resolve the symptoms of preeclampsia.

In the discussion of this thesis, a new way of unravelling this multifactorial aetiology has been proposed; the different risk factors for preeclampsia, such as thrombophilia, cardiovascular risk factors, oocyte donation and autoimmune disease might all result in different aetiologies of the same syndrome, requiring an individual approach. The role of the placenta, and its early changes, might differ between these different etiological pathways leading up to preeclampsia. The late changes in the placenta in preeclampsia have been studied extensively, but the early stages of the placenta in preeclampsia in human patients have remained underexposed. However, such material is, of course, hard to obtain; placenta biopsies cannot be performed for research purposes, and with materials from first-trimester miscarriages it remains unknown whether the patient would have developed preeclampsia. A feasible alternative for detecting changes during early stages of pregnancy is proteomics analysis. With proteomics, all proteins expressed in a tissue or blood sample are measured and their levels can be compared with other samples.[52] This “drag-net” like method might reveal new serum markers that reflect placental changes in certain types of patients; for preeclampsia in general, several new role players such as complement factor C1 and clusterin, involved in apoptosis, have been discovered recently with proteomics.[53, 54] New biomarkers might allow us to distinguish between primary placenta-mediated preeclampsia, with major involvement of immune dysregulation, from preeclampsia with a major role for decreased vascular reserve. This

might also set the stage from patient-tailored therapy; some patients might benefit from anti-inflammatory or anti-coagulative drugs targeting the systemic response, and others might benefit from immunomodulating drugs early during pregnancy, during placental formation.

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Chapter 8

Summary in Dutch (Nederlandse Samenvatting)

INTRODUCTIE

Pre-eclampsie, doorgaans zwangerschapsvergiftiging genoemd, is een syndroom dat één tot vier procent van de Nederlandse zwangere vrouwen treft. Vrouwen met pre-eclampsie hebben een hoge bloeddruk en eiwitverlies in de urine als gevolg van nierschade. Bij de meeste vrouwen met pre-eclampsie verloopt het ziektebeeld mild, maar het syndroom kan zich plots ontwikkelen tot een ernstig beeld met complicaties voor moeder en kind. Diverse risicofactoren voor het ontwikkelen van pre-eclampsie zijn al aangetoond. Uit studies naar genetische risicofactoren voor pre-eclampsie bleken associaties met verschillende genen die coderen voor factoren uit de stolling van bloed en het onderhoud van de bloedvaten aan de ene kant, en genen die coderen voor immunologische factoren aan de andere kant. Uit studies naar verworven risicofactoren voor pre-eclampsie is gebleken dat enerzijds vasculaire invloeden, zoals diabetes, hypertensie, obesitas en een oudere maternale leeftijd met het syndroom geassocieerd zijn. Anderzijds zijn ook immunologische factoren, zoals een auto-immuunaandoening, een zwangerschap na eiceldonatie en het hebben van een eerste zwangerschap risicofactoren.

Deze verschillende risicofactoren dragen bij aan de uiteindelijke uitingsvorm van pre-eclampsie, waarbij de placenta te veel anti-angiogene stoffen produceert. Deze stoffen komen in het bloed van de moeder terecht, waar zij stoffen blokkeren die belangrijk zijn voor het onderhoud van organen en bloedvaten, zoals 'vascular endothelial growth factor' (VEGF), dat onmisbaar is voor het onderhoud van de nier en het endotheel, de bekleding van de bloedvaten.

Ondanks al het onderzoek naar risicofactoren en veranderingen in de placenta bij pre-eclampsie dat tot nu toe is verricht, is het nog onduidelijk wat precies het mechanisme van het ontstaan van pre-eclampsie is. Ook is de reden waarom sommige vrouwen een verhoogd risico hebben op het ontwikkelen van pre-eclampsie niet duidelijk. Wellicht zijn dit de redenen dat het nog niet gelukt is een geschikte methode voor preventie en genezing van pre-eclampsie te ontwikkelen. Verder onderzoek naar pre-eclampsie zou nieuwe aangrijpingspunten voor therapie kunnen onthullen.

In dit proefschrift wordt onderzoek naar de samenhang tussen de drie verschillende hierboven genoemde mechanismen, stolling, immunologie

en anti-angiogene stoffen, en het mogelijke effect van ontregeling van deze mechanismen op de placenta en de nier en de rol ervan bij pre-eclampsie.

HOOFDSTUK 2

In hoofdstuk twee worden de genetische risicofactoren van pre-eclampsie verder onderzocht. Omdat is gebleken dat pre-eclampsie een belangrijke familiaire component heeft, zijn er veel studies uitgevoerd om te ontdekken welke genen zouden kunnen bijdragen aan de ontwikkeling van pre-eclampsie. De meeste studies naar genen in pre-eclampsie betroffen genetische associatiestudies, waarbij een zogeheten kandidaatgen wordt gekozen op basis van beschikbare kennis over het ziektebeeld. De frequentie van voorkomen van dit kandidaatgen wordt dan vergeleken tussen vrouwen met pre-eclampsie en gezonde vrouwen. Echter betreft dit vaak kleine studies, en worden studies waarin geen associatie gevonden wordt tussen een kandidaatgen en pre-eclampsie meestal niet gepubliceerd of gerapporteerd, gewoonweg omdat een negatief resultaat minder interessant is voor een wetenschappelijk tijdschrift om te publiceren. Er is dan ook een vrij grote kans dat een deel van de studies waarin een associatie significant bleek, bij toeval een positief resultaat lieten zien. Dit fenomeen wordt ook wel 'publicatiebias' genoemd.

Om uit te zoeken voor welke genetische varianten een robuust bewijs voor een associatie met pre-eclampsie bestaat, werd een meta-analyse uitgevoerd. Bij een meta-analyse worden alle tot nu toe bekende onderzoeksresultaten over een onderwerp verzameld en met statistische technieken samengevoegd, zodat een 'totaal effect' ondersteund door alle studies geschat kan worden. Voor deze meta-analyse naar genetische varianten in pre-eclampsie werden alle artikelen die dit onderwerp betroffen verzameld, en alleen genetische varianten waarvan de associatie gereproduceerd was in de literatuur werden geïncludeerd.

Dit betrof 22 genetische varianten, maar na het samenvoegen van de effecten gerapporteerd in alle studies, bleven slechts zeven genetische varianten geassocieerd met pre-eclampsie. Dit waren vier genen betrokken bij het stollingssysteem, twee genen betrokken bij endotheelschade en bloeddrukregulatie, en een gen betrokken bij ontsteking. Deze resultaten zijn een aanwijzing dat stolling, inflammatie en endotheelschade een rol spelen bij het ontwikkelen van pre-eclampsie; dit past ook bij de bekende associaties

tussen *cardiovasculaire risicofactoren* en pre-eclampsie, en de centrale rol van *endotheelschade* in pre-eclampsie. Echter is het niet duidelijk hoe groot de rol van deze genetische varianten in het ontwikkelen van pre-eclampsie precies is: niet alle vrouwen die deze mutaties dragen ontwikkelen pre-eclampsie, en niet alle vrouwen met pre-eclampsie dragen deze mutaties. Voor het ontwikkelen van pre-eclampsie is waarschijnlijk een 'second hit' nodig, zoals bijvoorbeeld ongunstige immunologische omstandigheden zoals een auto-immuun ziekte of het hebben van cardiovasculaire risicofactoren. In hoofdstuk 2 werd bevonden dat ontregeling van het stollingssysteem en inflammatie van endotheel waarschijnlijk een belangrijke rol spelen in pre-eclampsie. Een eiwit dat betrokken is bij de regulatie van allebei deze systemen is trombomoduline. Dit eiwit is aanwezig op de bekleding van de placenta, de syncytiotrofoblast, en op de endotheelcellen in de bloedvaten. Trombomoduline zorgt daar voor remming van stolling, inflammatie en celdood van de endotheelcellen, en speelt ook een rol bij het in stand houden van de structuur van de endotheelcellen. Het is bekend dat tijdens ontsteking trombomoduline wordt gekliefd van het endotheel, waarna het zijn functie verliest. Dit zou een nadelig effect kunnen hebben, want dat betekent dat er dan meer stolling en ontsteking op zou kunnen treden. Ook is het bekend dat meer van dit gekliefde trombomoduline aanwezig is in het bloed van de moeder in pre-eclampsie, maar het is niet bekend of er dan ook minder trombomoduline aanwezig is op het endotheel of op de bekleding van de placenta tijdens pre-eclampsie. Het is tevens nog niet bekend wat voor effecten trombomoduline op de placenta en organen van de moeder heeft bij de ontwikkeling van pre-eclampsie. In hoofdstuk drie en vier van dit proefschrift wordt daarom de rol van trombomoduline in de placenta en nier in pre-eclampsie onderzocht.

HOOFDSTUK DRIE

In hoofdstuk drie werd de aanwezigheid van het trombomoduline-eiwit en trombomoduline mRNA, dat in de cel zorgt dat het trombomoduline-eiwit geproduceerd wordt, onderzocht in placenta's van vrouwen met pre-eclampsie, en als controle in placenta's van vrouwen met een goed verlopen zwangerschap en van vrouwen met een zwangerschap gecompliceerd door groeivertraging. Deze laatste groep werd toegevoegd omdat deze meer vergelijkbaar zijn met de pre-eclampsie groep wat betreft

zwangerschapsduur, placentagewicht en geboortegewicht; zo kon uitgesloten worden dat deze de oorzaak waren van een gevonden verschil tussen de gezonde controlegroep en de patiënten met pre-eclampsie.
Zowel trombomoduline-eiwit als trombomoduline mRNA waren minder aanwezig in de placenta's van vrouwen met pre-eclampsie in vergelijking met de controlegroepen. Ook bleek er een negatieve associatie tussen bloeddruk en lichaamsgewicht met de hoeveelheid trombomoduline mRNA in de vrouwen met pre-eclampsie, wat suggereert dat de hoeveelheid vasculaire disfunctie in de moeder invloed heeft op het functioneren van de placentacellen. Tevens was de afwezigheid van trombomoduline gecorreleerd met meer celdood (apoptose) en verminderde aanwezigheid van eiwitten betrokken bij regulering van de placentastructuur.
Om te onderzoeken of de angiogene disbalans van pre-eclampsie wellicht de oorzaak kon zijn van de verminderde trombomodulineproductie in pre-eclampsie werd de aanwezigheid van het anti-angiogene eiwit soluble Flt-1 (sFlt-1) in de placenta's onderzocht. Verminderde aanwezigheid van trombomoduline in de placenta was geassocieerd met een hogere productie van soluble Flt-1. Op basis hiervan werd de hypothese voorgesteld dat VEGF een regulator van trombomoduline in de placenta zou kunnen zijn. Inderdaad werd in een celkweekexperiment met placentacellen gevonden dat het toevoegen van VEGF aan de cellen leidde tot meer productie van trombomoduline.
Deze bevindingen illustreren wederom de relatie tussen de angiogene disbalans van pre-eclampsie en het disfunctioneren van het endotheel en de placenta. Ook suggereren ze dat trombomoduline wellicht een interessant aangrijpingspunt zou kunnen zijn voor het onderscheppen van het causale pad van pre-eclampsie, aangezien de angiogene disbalans zelf een lastig mechanisme is om op aan te grijpen (zie onder, hoofdstuk vijf).

HOOFDSTUK VIER

In hoofdstuk vier werd de aanwezigheid van trombomoduline-eiwit in de nieren van vrouwen met pre-eclampsie en de relatie met de (immuno) histologische kenmerken van de nier onderzocht. Omdat er bij het nemen van een nierbiopt een grote kans is op complicaties, zoals interne bloedingen, wordt dit bij zwangere vrouwen vrijwel nooit uitgevoerd. Daarom werd voor dit onderzoek gebruik gemaakt van niermateriaal verkregen van vrouwen

overleden tijdens de zwangerschap. Via het Pathologisch-Anatomisch Landelijk Geautomatiseerd Archief (PALGA) werden alle vrouwen met pre-eclampsie die overleden waren tijdens de zwangerschap opgezocht en niermateriaal uit pathologische archieven werd opgevraagd. Ook werd niermateriaal van overleden zwangere vrouwen zonder pre-eclampsie opgevraagd als controlemateriaal. De aanwezigheid van het trombomoduline-eiwit in het niermateriaal werd onderzocht, en histologische veranderingen in de nieren werden beoordeeld door een patholoog.
Uit dit experiment bleek dat de aanwezigheid van trombomoduline-eiwit verhoogd is in de nieren van vrouwen met pre-eclampsie in vergelijking met vrouwen zonder pre-eclampsie. Ook was deze verhoogde expressie geassocieerd met expressie van het eiwit nefrine in de nier, en met de activatie van pariëtale epitheelcellen; dit zijn aanwijzingen voor een beschermend effect van trombomoduline. Dit is een verrassende bevinding in het licht van de resultaten van hoofdstuk drie, waarin werd gevonden dat trombomoduline-expressie juist verminderd is in de placenta tijdens pre-eclampsie.
Om te onderzoeken of de angiogene disbalans van pre-eclampsie deze verhoging van trombomoduline in de nier zou kunnen veroorzaken, werden een aantal vervolgexperimenten uitgevoerd. In een celkweekexperiment werd sFlt-1 toegevoegd aan cellen representatief voor endotheelcellen uit de nier; dit resulteerde in een verlaagde productie van trombomoduline door de cellen. In tegenstelling hiermee, werd in nieren van muizen die behandeld waren met anti-angiogene factoren een verhoogde aanwezigheid van trombomoduline in de nier gevonden. Omdat in hoofdstuk drie een associatie was gevonden tussen lichaamsgewicht (en hiermee, indirect, een inflammatoire staat) en trombomoduline-expressie in de placenta, werd trombomoduline onderzocht in nieren van varkens met diabetes en controle varkens. In de varkens met diabetes was de trombomoduline expressie in de nier verhoogd.
De resultaten van hoofdstuk vier laten zien dat trombomodulineproductie in de nier verhoogd is onder anti-angiogene condities, terwijl er in celkweek een direct verminderend effect van sFlt-1 op trombomodulineproductie werd geobserveerd. Een verklaring hiervoor zou kunnen zijn dat de endotheelcellen van de nier een defensieve strategie tegen schade inzetten

door trombomodulineproductie te verhogen; zo een mechanisme is eerder beschreven bij een ander ziektebeeld van de nier, glomerulonefritis.

HOOFDSTUK VIJF

De associatie van trombomoduline-expressie op het endotheel van de bloedvaten in de nier met de aanwezigheid van nefrine op de podocyten in de nier, gevonden in hoofdstuk vier, illustreert de 'cross-talk' tussen de verschillende lagen in het filterapparaat van de nier, de glomerulus.
De belangrijkste functie van de nier is het filteren van stoffen uit het bloed, en daarmee het behouden van de homeostase in het lichaam. Dit filteren gebeurt in de 'glomeruli' van de nier, waar het filtraat langs drie lagen geleid wordt. Eerst passeert het filtraat de endotheelcellen, die fenestraties hebben waar kleine moleculen en ionen kunnen passeren, vervolgens passeert het de glomerulaire basaalmembraan, waar negatief geladen moleculen (met name eiwitten) worden tegen gehouden, en vervolgens passeert het filtraat de podocyten, een laag van zeer gespecialiseerde cellen met een complexe vorm. Deze podocyten spelen verder een belangrijke rol bij het in stand houden van de andere lagen van de glomerulus door de productie van VEGF.
In hoofdstuk vijf werd de rol van de verschillende lagen in de glomerulus in pre-eclampsie en hun cross-talk verder onderzocht middels een systematische review. Hieruit bleek dat vroege studies naar de nier in pre-eclampsie vooral focusten op de veranderingen in de endotheelcellen, welke opzwellen in pre-eclampsie, een verandering die goed zichtbaar is met de microscoop. Echter treden veranderingen in het endotheel niet zichtbaar op bij veel van de nierbiopten van vrouwen met pre-eclamspie, dus zijn de endotheelveranderingen op zich niet voldoende de nierpathologie in pre-eclampsie te verklaren. De laatste paar decennia is de aandacht verschoven naar de rol van podocyten in pre-eclampsie: met elektronenmicroscopie is aangetoond dat de structuur van podocyten beschadigd raakt, en in de urine kunnen loslatende podocyten al aangetoond worden voordat er eiwitverlies in de urine optreedt.
Er wordt verondersteld dat aan de nierpathologie in pre-eclampsie een vicieuze cirkel van schade in de glomerulus optreedt, veroorzaakt door de angiogene disbalans. Het excessieve sFlt-1 bindt aan VEGF, waardoor minder VEGF kan binden aan de endotheelcellen en podocyten in de glomerulus. Hierdoor raakt de complexe structuur van de podocyten beschadigd, laten ze

los en vermindert de VEGF-productie in de glomerulus. De endotheelcellen raken ook beschadigd door het gebrek aan VEGF, waarna zij endotheline, een inflammatie-eiwit, produceren, dat de podocyten nog verder kan beschadigen. Er is de laatste jaren veel onderzoek gedaan naar een punt om aan te grijpen in deze vicieuze cirkel van schade; dit is echter erg lastig door de delicate balans van de cross-talk eiwitten die nodig is voor een gezond functionerende glomerulus. Zowel te lage, als te hoge niveaus van VEGF brengen schade toe aan de nier.

HOOFDSTUK 6

In hoofdstuk vijf werd het belang van VEGF in het samenspel tussen de podocyten en endotheelcellen voor het behoud van het functioneren van de nier benadrukt, maar het is nog niet duidelijk hoe dit samenspel precies tot stand komt. Het filtraat in de nier passeert lagen van de glomerulus van de endotheelcellen naar de podocyten onder een relatief hoge filtratiedruk. Toch bereikt het VEGF geproduceerd door de podocyten tegen deze stroom in de endotheelcellen.

Aanwijzingen voor het antwoord op deze vraag liggen misschien bij de structuur van het VEGF-eiwit. Het mRNA van VEGF, dat codeert voor de productie van het VEGF-eiwit in de cel, kan op verschillende manieren gesplitst worden. Op deze manier kunnen van hetzelfde VEGF-gen minstens acht verschillende vormen van VEGF geproduceerd worden, acht verschillende *isovormen*, welke verschillen in eigenschappen. Sommige isovormen kunnen zich vrij verspreiden via de bloedvaten en het interstitium, terwijl andere isovormen een regio bevatten om te gebonden te worden door extracellulaire matrix. Ook zorgen de langere isovormen voor een sterker activerend effect dan de kortere. In hoofdstuk zes van dit proefschrift worden het voorkomen en de verdeling van de verschillende isovormen van VEGF in de nier, onder fysiologische en pathologische omstandigheden, onderzocht. Met capillaire elektroforese werd de relatieve mRNA-expressie van de VEGF-isovormen onderzocht in nierbiopten van gezonde mensen en niermateriaal van muizen. In mensen was de korte, vrij verspreidbare VEGF-121 isovorm het meest voorkomend, terwijl in muizen de langere isovorm VEGF-164 het meest voorkwam. Dit suggereert wellicht dat het transport van VEGF in de nier bij de mens op een andere manier verloopt dat in muizen.

Ook werd de verdeling van VEGF isovormen onder diverse pathologische

omstandigheden in de nier onderzocht; humane nierbiopten van patiënten na niertransplantatie en van patiënten met diabetische nefropathie, en niermateriaal van muizen met diabetes en lupus nefritis werd onderzocht. De verdeling van de isovormen was voor diabetische nefropathie en lupus nefritis vergelijkbaar met de verdeling in de controle samples, en in het niermateriaal afkomstig van verschillende stadia van acute rejectie van een niertransplantaat en diabetische nefropathie liet ook vergelijkbare isovorm verdelingen zien. Deze resultaten suggereren dat de verdeling van VEGF-isovormen in de nier stabiel blijft tijdens diverse pathologische omstandigheden. Dit zou kunnen betekenen dat het splitsen van het VEGF-mRNA niet beïnvloed wordt door inflammatoire of metabole nierschade, of dat de podocyten in de nier niet in staat zijn de verdeling van isovormen aan te passen tot een meer geschikt patroon onder pathologische omstandigheden. De bevindingen in dit proefschrift ondersteunen zowel vasculaire als immunologische oorzakelijke wegen voor de ontwikkeling van pre-eclampsie. Aan pre-eclampsie kan een breed gebied van oorzaken ten grondslag liggen, en het samenspel tussen deze oorzaken is complex. Aan de ene kant lijkt de pathogenese te beginnen bij de placenta, welke door verstoorde ontwikkeling vroeg in de zwangerschap te veel anti-angiogene stoffen produceert, die vervolgens leiden tot endotheelschade bij de moeder. Echter zijn ziektebeelden die gepaard gaan met endotheelschade, zoals hypertensie en diabetes, weer risicofactoren voor het ontwikkelen van pre-eclampsie; oorzaak en gevolg lijken verweven, en de oorzaak van pre-eclampsie lijkt multifactorieel te zijn.

Dit maakt het ziektebeeld voor iedere vrouw met pre-eclampsie uniek en ingewikkeld om op aan te grijpen; het ontwikkelen van een strategie die aangrijpt op dezelfde causale factor bij iedere vrouw lijkt niet vruchtbaar. Het direct aangrijpen op de angiogene disbalans lijkt nu nog te riskant, gegeven de delicate balans van anti-angiogene en angiogene factoren die nodig is voor het behoud van het endotheel en de glomeruli. Toekomstig onderzoek zou zich kunnen richten op het verder identificeren van de verschillende aanleidingen van pre-eclampsie in een vroeg stadium in de placenta; echter is placentamateriaal van vroege stadia van de zwangerschappen lastig te verkrijgen. Wellicht zou het op grote schaal onderzoeken en combineren van *biomarkers* uit het serum en de urine van zwangere vrouwen gedurende de zwangerschap hier een oplossing voor kunnen zijn, en zal zo therapie gericht

op de specifieke patiënt ontwikkeld kunnen worden. Sommige patiënten zullen misschien gebaat zijn bij het vroeg aangrijpen op de systemische symptomen, zoals hypertensie en inflammatie, en anderen bij het aangrijpen op immunologische factoren die de ontwikkeling van de placenta verstoren.

Chapter 9

CV, publications and acknowledgements

CURRICULUM VITAE

Rosanne Jane Turner werd 7 juli 1992 geboren te Castricum. In 2010 behaalde zij cum laude haar gymnasiumdiploma aan het Bonhoeffercollege te Castricum. Datzelfde jaar begon zij met de studie Geneeskunde aan de Universiteit Leiden in het Leids Universitair Medisch Centrum. In 2011 werd zij toegalaten tot het Excellente Studententraject van het Honours College van de Universiteit Leiden. In het kader van dit traject startte zij in 2011 naast haar studie geneeskunde met onderzoek naar preeclampsie en angiogenese onder begeleiding van Dr. Hans Baelde, Prof. Kitty Bloemenkamp en Prof. J.A. Bruijn. Ook volgde zij een deel van haar studie in Engeland aan de Peninsula Medical School.
Naast haar studie was zij ook actief als violist, onder meer in Leids studenten koor- en orkest Collegium Musicum als concertmeester, en in het Nederlands Strijkersgilde, waar zij ook bestuurslid was (2014).

Na het behalen van haar bachelor geneeskunde met honours certificaat (2013) en het afsluiten van haar bestuursjaar verrichtte Rosanne fulltime promotieonderzoek naar endotheelschade in preeclampsie aan de afdeling Pathologie van het Leids Universitair Medisch Centrum (hoofd: prof. dr. V.T.H.B.M. Smit); hiervoor ontving zij in 2014 een beurs voor een tweejarige aanstelling in het kader van het Excellente Studententraject. De resultaten van dit onderzoek zijn beschreven in dit proefschrift. Tijdens haar promotieonderzoek bezocht Rosanne verschillende congressen waar zij haar onderzoek presenteerde, onder meer de American Society of Nephrology Kidney Week in San Diego (2012) en Atlanta (2013), en het jaarlijkse congres van de Society for Reproductive Investigation (2016). Hiervoor ontving zij diverse beurzen, waaronder de Kidney Stars grant en de Women's Renal Health grant uitgereikt door de American Society of Nephropathy.

Tijdens haar promotietraject ontdekte Rosanne dat de wetenschap en methodologie haar het meest aan het hart lagen, en besloot zij haar masterstudies na haar aanstelling als promovendus te continueren in deze richting. In 2017 is zij gestart met de masterstudie Statistical Science aan de Universiteit Leiden. In 2019 hoopt zij deze studie af te ronden, om daarna als statisticus werkzaam te zijn.

LIST OF PUBLICATIONS

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5. **Turner RJ**, Bloemenkamp KW, Penning ME, Bruijn JA, Baelde. From glomerular endothelium to podocyte pathobiology in preeclampsia: a paradigm shift. HJ.Curr Hypertens Rep. 2015 Jul;17(7):54.
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