Endothelial pathology in preeclampsia

Turner, R.J.

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.

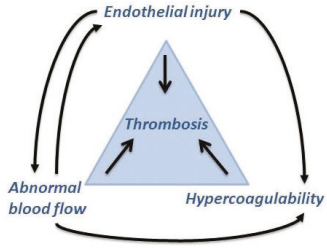

Figure 1, Virchow's triad
Virchow's triad depicts the interplay between the three factors leading to thrombosis.

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.

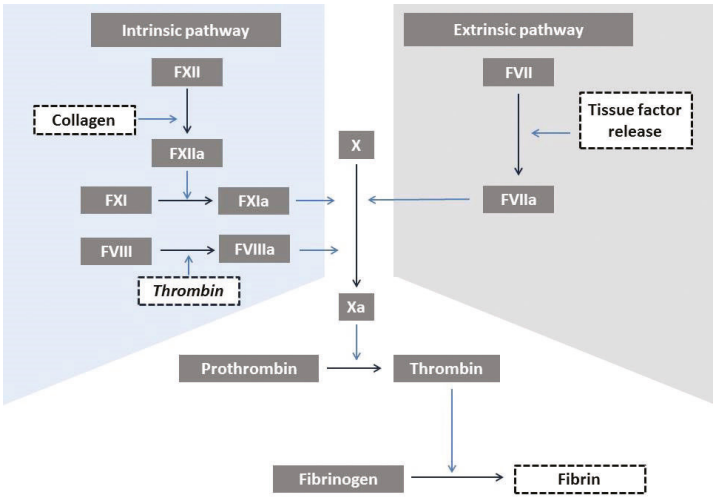

Figure 2, two pathways leading to coagulation

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.

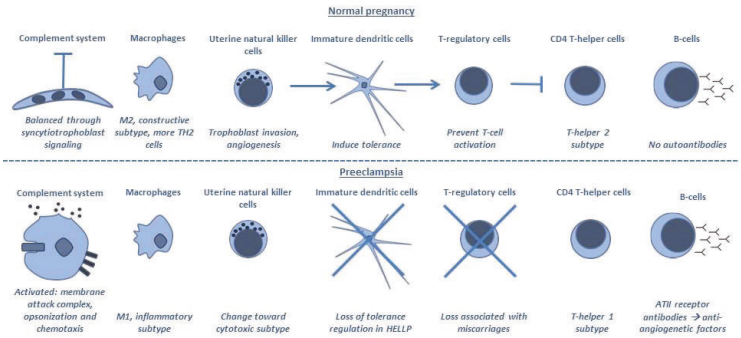

Figure 3, the inflammatory response in normal pregnancy compared to preeclampsia

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]

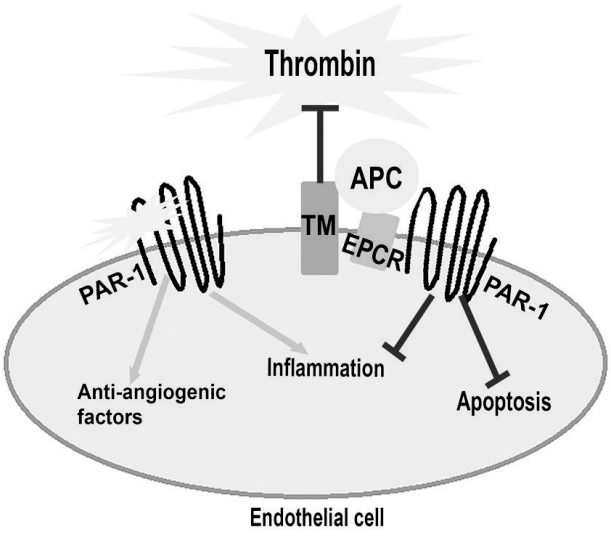

Figure 4, effects of thrombin and thrombomodulin signalling in endothelial cells
When thrombin (star figure) initiates signalling through PAR-1 in endothelial cells, intracellular pathways resulting in the release of anti-angiogenic factors and activation of inflammation are activated. In contrast, when 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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