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

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**CHAPTER VII**  
**SUMMARY AND**  
**GENERAL DISCUSSION**

## Summary

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

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

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

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

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

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

and systemic lupus erythematoses.

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

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

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

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

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

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

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

Regardless the cause of preeclampsia, the end result is a dysfunctional placenta that releases excessive amounts of anti-angiogenic factors. Pregnancy—and preeclamptic pregnancy in particular—is characterized by a systemic increase in the levels of anti-angiogenic factors, including sFlt-1.<sup>7</sup> The placenta is an important source of circulating sFlt-1. However, it remains disputable how sFlt-1 is released from the placenta, as its heparin-binding site causes sFlt-1 to have a strong avidity for extracellular matrix.<sup>8</sup> A putative mechanism by which sFlt-1 reaches the maternal circulation is through the dissociation of placental material—called syncytial knots.<sup>8</sup> **CHAPTER 6** explores this mechanism. The experiments described in this chapter show that syncytial knots are the main production site of sFlt-1. To study the systemic spread of this placental material, we investigated the presence of placental material in lungs of women who died during pregnancy. Syncytial aggregates were frequently observed in maternal lungs and importantly, preeclampsia was associated with a significantly higher number of syncytial aggregates within maternal lung tissue. In these syncytial aggregates, we demonstrated co-localization of the syncytiotrophoblast-specific marker hCG, the anti-angiogenic protein sFlt-1 as well as the Y-chromosome. These



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

## *Questions arising from this thesis*

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

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

variants contribute to the pathogenesis of preeclampsia?

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

Genetic variants could also predispose to placental dysfunction, thereby contributing to the development of early-onset preeclampsia. Genetic variants in the coagulation and fibrinolysis system may increase the risk for developing preeclampsia by affecting trophoblast growth and differentiation. This idea is supported by the observation that activated coagulation factors induce cell death and growth inhibition of placental trophoblast cells.<sup>12</sup> In a thrombomodulin knock-out mouse model, activation of the blood coagulation cascade at the fetal-maternal interface leads to embryonic lethality, indicating that a delicate balance between coagulation and fibrinolysis is essential for the maintenance of pregnancy.<sup>12</sup> These observations support the idea that genetic variants in the coagulation and fibrinolysis system can contribute to development of pregnancy complications, including preeclampsia. However, it remains largely unknown through which mechanism(s) excessive activation of the blood coagulation cascade causes pregnancy complications. Importantly, activation of the coagulation cascade has been linked to complement activation<sup>13</sup>, also in the setting of pregnancy complications. Mice injected with antiphospholipid-antibodies show increased fetal loss as well as excessive amounts of complement component C3b, neutrophil infiltration and increased tissue factor expression in their deciduas. However, no increases in fibrin and no thrombi are observed in these deciduas, suggesting that it is not thrombosis that mediates antiphospholipid-antibody induced fetal loss. In this murine model, binding of antiphospholipid-antibodies to trophoblast leads to the generation of the complement component

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

### **COMPLEMENT IN PREECLAMPSIA; CAUSE OR CONSEQUENCE?**

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

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

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

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

#### **PLACENTAL COMPLEMENT ACTIVATION: EXCESSIVE**

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

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

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

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

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

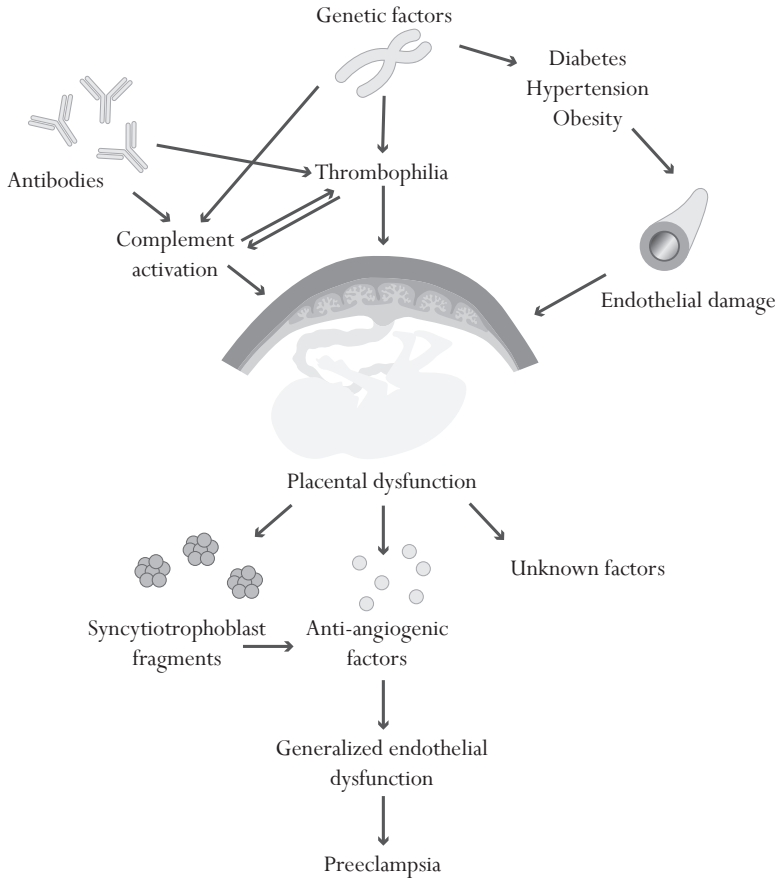
is inadequately regulated due to lower factor H levels.<sup>27</sup> In this thesis, an association between placental complement activation and the presence of increased syncytial knots was described. Syncytial knots are the main placental production site of sFlt-1.<sup>8</sup> Although it remains uncertain how placental complement activation is related to the development of syncytial knots, the association indicates another link between the complement system and angiogenic dysregulation.

### **THE ROLE OF (PREEXISTENT) ENDOTHELIAL DYSFUNCTION**

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

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

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



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



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

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