On renal pathophysiology in preeclampsia

Colophon

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"Education is the most powerful weapon which you can use to change the world"

Nelson Mandela
1918 – 2013

Voor mijn ouders en grootouders

Table of Contents

General introduction

Introduction

Preeclampsia is a pregnancy complication, which can suddenly change from a relatively mild phenotype into a life-threatening situation. Hypertensive disorders represent a variety of conditions associated with hypertension during pregnancy, including preeclampsia. This thesis focuses on the renal pathophysiology of preeclampsia, and on the challenges regarding the prediction of preeclampsia.

Preeclampsia complicates 2-8% of pregnancies, and remains a significant cause of fetal and maternal morbidity and mortality, both in the Netherlands[1, 2] and worldwide.[3] This pregnancy specific disorder is defined as new onset hypertension (diastolic blood pressure >90 mmHg) and proteinuria (>300 mg in 24h) at or after 20 weeks' gestation.[4] Importantly, this condition may affect multiple organ systems causing varying clinical features in the mother, such as HELLP syndrome (Haemolysis, Elevated Liver enzymes, and Low Platelet count), and eclampsia. In addition, preeclampsia is also a major cause of adverse perinatal outcomes, such as prematurity and intrauterine growth restriction. Poor early placentation and generalized endothelial dysfunction are thought to contribute to the pathogenesis of preeclampsia.[5] Perhaps because the exact mechanisms causing preeclampsia have still not been fully elucidated, the only definitive cure for preeclampsia is removal of the placenta. In addition, although prediction models based on maternal factors and biophysical and biochemical markers during early pregnancy are continuously improving, prediction of preeclampsia remains challenging.6 In the long run, the effect of preeclampsia is not only restricted to pregnancy. Increasing evidence suggests that maternal health is affected decades after a pregnancy complicated by hypertensive disorders (including preeclampsia) by an increased prevalence of cardiovascular and metabolic disease.[7, 8] The first part of this introductory chapter (*Part 1 – Renal consequences of preeclampsia)*, focuses on the consequences of preeclampsia in one of the major target organs during this syndrome: the kidney. In particular, the role of endothelial dysfunction, and angiogenic imbalance will be discussed. In the second part (*Part 2 – Placental dysfunction in preeclampsia)*, the disturbed development and function of the placenta, which underlie renal damage during preeclampsia, will be explained, as well as a putative role for the complement system in renal injury during preeclampsia. The third part *(Part 3 – Prediction of preeclampsia)* describes the challenges in the prediction of preeclampsia, and subsequently focuses on the promising use of urinary biomarkers. The fourth and last part of this chapter will focus on the research questions that form the basis for the experiments and the studies described in this thesis (*Part 4 – This thesis*), followed by an outline of the chapters.

Part 1 Renal consequences of preeclampsia

The kidney is one of the major target organs that are severely injured during pregnancies complicated by preeclampsia, as demonstrated by the presence of proteinuria and abnormalities in renal histology. Because a large part of this thesis deals with the renal consequences of preeclampsia, an introduction to the anatomy of the kidney is given below.

ANATOMY

The kidneys are the main excretory organs of the human body. They control extracellular volume by regulating water and salt balance, and acid-base homeostasis. In addition, the kidneys have hormonal and metabolic functions. With these functions they regulate blood pressure, erythropoiesis, and calcium metabolism.[9] The kidney is a bean-shaped organ that weighs approximately 150 grams in adults, and is situated in the retroperitoneum. Each kidney has three major components: the cortex, the medulla, and the collecting ducts. The outermost layer, the cortex, consists of glomeruli and convoluted

tubules. The medulla is situated more towards the center, consisting of pyramidal structures of parallel arranged tubular formations with apical papillae. The collecting ducts merge in the medulla to form larger ducts. From these ducts, the urine goes from the minor calyces to the major calyces, and then flows down the ureter to the bladder where it is stored until voided via the urethra.

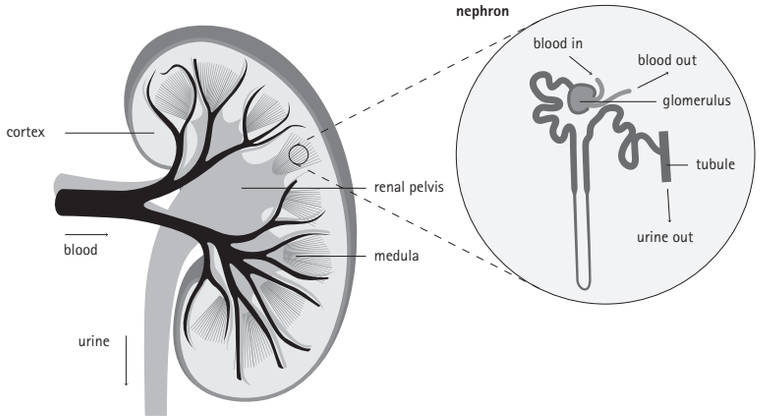

Figure 1: The kidney and a nephron.

Within the kidney, blood is filtered. The urine is then further modified through functional units, called nephrons. Each kidney contains around one million nephrons. A nephron consists of a glomerulus and its attached tubules.

Blood is supplied to each kidney by a renal artery, which arises from the aorta and divides into two main branches in the hilum. Each of these gives rise to several interlobar arteries. These arteries ascend between the pyramids to the cortico-medullary junction and give rise to arcuate arteries. Eventually, afferent arterioles branch off, each directing to a single glomerulus. A glomerulus represents an anastomosing network of capillaries, arranged in several lobules.

The efferent vessel draining blood from the glomerulus is called the efferent arteriole. The efferent arterioles give rise to another network of capillaries; the peritubular capillaries which surround the renal tubules.[9] A nephron, the functional unit of the kidney, consists of a glomerulus with attached tubules. Each kidney contains approximately one million nephrons. The glomerulus consists of different cell types, namely mesangial cells, endothelial cells, visceral epithelial cells (called podocytes), and parietal epithelial cells. Mesangial cells, together with their mesangial matrix, form the mesangium. Mesangial cells have an important role in regulating glomerular function and blood pressure.[9]

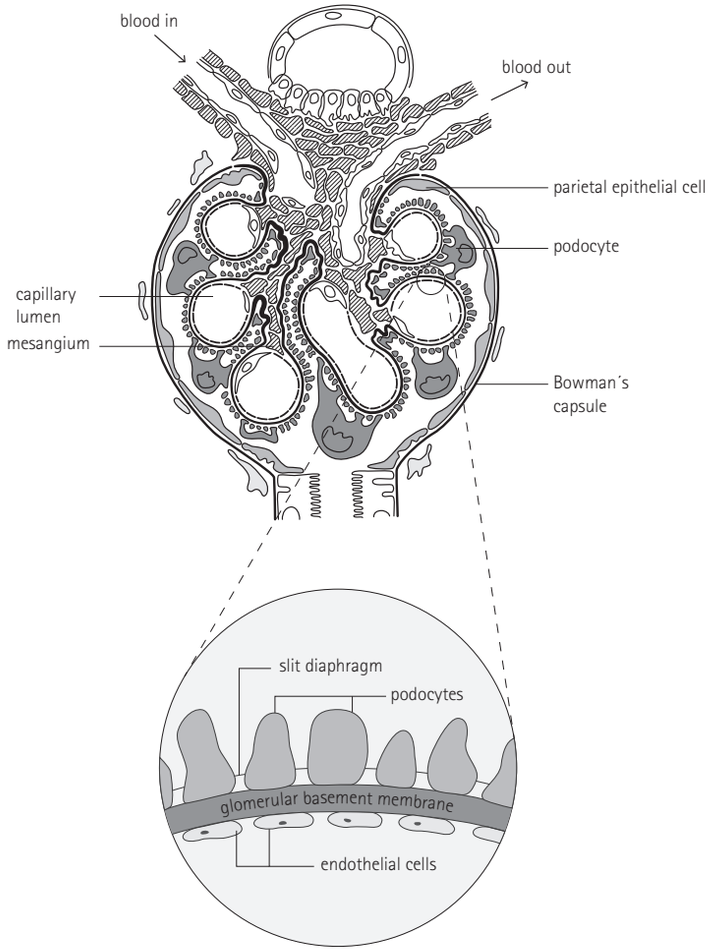

Figure 2: The glomerulus and the glomerular filtration barrier.

The glomerulus is a network of capillaries that performs the first step of filtering blood. The glomerulus consists of different cell-types, such as mesangial cells, endothelial cells, podocytes, and parietal epithelial cells. Within the glomerulus, the blood is filtered from the capillaries. The glomerular filtration barrier consists of endothelial cells, the glomerular basement membrane, slit diaphragms, and podocytes. This figure was modified from Kriz et al [10], and Deen[11] with permission from authors and publishers.

The glomerular filtration barrier consists of endothelial cells, the glomerular basement membrane, slit diaphragms, and podocytes. The endothelial cells in the glomerulus are thin and fenestrated. The glomerular basement membrane surrounds the glomerular capillaries. The outer aspects of the glomerular capillaries are covered with podocytes. Each podocyte has a large body with small foot-like processes that interdigitate with foot-processes of adjacent cells. The gaps between foot processes, called filtration slits, are bridged via slit diaphragms. The slit diaphragm is composed of specific proteins, including nephrin. Blood is filtered through these slits, and the slit diaphragm—together with endothelial cell layers and the glomerular basement membrane—restricts the passage of any large or negatively charged molecules. Water, the remaining small proteins, and electrolytes now enter Bowman's space, which is aligned by visceral and parietal epithelial cells. As Bowman's space is continuous with the lumen of the renal tubule, the pre-urine now first enters the proximal tubules. The pre-urine is further concentrated after selective reabsorption of water and essential molecules in the loop of Henle and the distal tubule.

THE KIDNEY DURING PREECLAMPSIA: EFFECT OF ANGIOGENIC IMBALANCE

During preeclampsia, a characteristic glomerular lesion is observed called 'glomerular endotheliosis', which represents swelling of endothelial cells, and may result in a 'bloodless' appearance.[12-14] Electron microscopy shows loss of endothelial fenestration. Although glomerular endotheliosis can also be observed in healthy pregnancies,[15] it is more prominent and widespread in preeclampsia.[16] Endothelial swelling usually disappears within eight weeks following delivery, together with hypertension and proteinuria.[17]

Swelling of endothelial cells, similar to that seen in preeclampsia, develops in podocyte-specific vascular endothelial growth factor–A (VEGF-A) knockout mice, which suggests that VEGF-A—produced

by podocytes—plays a role in the maintenance of the glomerular endothelium.[18] Human preeclampsia is characterized by significantly decreased concentrations of VEGF-A,[19, 20] which suggests that loss of VEGF-A is one of the major factors causing glomerular injury during this pregnancy complication.

VEGF-A-signaling between podocytes and endothelial cells is thought to be crucial for the maintenance of the glomerular filtration barrier. VEGF-A has many physiological functions, including regulation of proliferation, angiogenesis, lymphogenesis, endothelial cell permeability, and vasodilation.[21, 22] VEGF-A signaling is mediated via two receptors, namely VEGF-R1/ Flt-1 and VEGFR2/Flk-1.[22] VEGF-A is expressed in practically every tissue in the human body. Tissues with fenestrated endothelium, such as the kidney, have the highest density of VEGF-A-expressing cells.[21, 22] Both VEGF-A and VEGF Receptors (VEGFRs) are abundantly present in the kidney. VEGFRs are expressed on endothelial, mesangial, and peritubular capillary cells.[23]

The anti-angiogenic factor soluble fms-like tyrokinase (sFlt-1) is the soluble form of the vascular endothelial growth receptor 1 (VEGF-R1), and the preeclamptic placenta releases excessive amounts of sFlt-1.[19, 24, 25] Since sFlt-1 neutralizes VEGF-A, sFlt-1 prevents VEGF-A from binding to its membrane-bound receptor. Adenoviral overexpression of sFlt-1 in mice causes proteinuria, hypertension, and swelling of glomerular endothelial cells.[25] These symptoms also develop in patients with malignant tumours treated with VEGF-A-inhibitors, such as bevacizumab.[18] Taken together, these studies underscore the notion that lack of VEGF-A—via inactivation of VEGF-A through high sFlt-1 concentrations—plays an important role in the development of endotheliosis in the glomerulus during preeclampsia.

VEGF-A inhibition leads to endotheliosis and proteinuria in preeclampsia, but the underlying mechanisms are not yet fully elucidated, and multiple factors seem to play a role. The glomerular endothelial injury during preeclampsia might not exclusively result

from increased sFlt-1 concentrations. For example, hypertension and its accompanying high shear stress during preeclamptic pregnancy might result in injury of the glomerular endothelium as well. Perhaps, hypertension-induced injury makes the glomerular endothelium even more sensitive to the high concentrations of sFlt-1, which will aggravate the endothelial injury.

Injury to the endothelial cell might also affect the nearby podocytes via different mechanisms. Firstly, as mentioned before, the VEGF-A-signaling between podocytes and endothelial cells is thought to be crucial for the maintenance of the glomerular filtration barrier. When this 'crosstalk' between podocytes and endothelial cells is interrupted during preeclampsia through increased sFlt-1 concentrations, podocytes may become injured.[23] Secondly, neutralization of VEGF-A induces the release of endothelin-1 (ET-1) from endothelial cells. ET-1 triggers shedding of nephrin (an important podocyte-specific protein which is involved in the maintenance of the glomerular permeability) from podocytes.26 Considering these observations, it is highly likely that podocytes are injured in the setting of preeclampsia. Injured podocytes may detach from the glomerular basement membrane, and consequently be shed into the urine.[27] Indeed, increased loss of podocytes in urine—from here on referred to as 'podocyturia'—was found in women with preeclampsia.[27] Podocyturia in the setting of preeclampsia is discussed in detail in the section '*Urinary footprints: podocyturia'* on page 29 in this Introduction.

Because knowledge on the function of the placenta during preeclampsia is essential to understand the renal pathophysiology, an introduction on placental dysfunction during preeclampsia is given below.

Part 2 Placental dysfunction in preeclampsia

IMPAIRED PLACENTAL IMPLANTATION IN PREECLAMPSIA

The pathophysiology of preeclampsia is usually divided into two stages.[28] The preclinical stage is characterized by abnormal placental development, whereas the clinical stage is associated with dysregulation of angiogenic factors and systemic endothelial dysfunction. The abnormal placental development that characterizes the first stage of the pathophysiology of preeclampsia is associated with impaired remodeling of spiral arteries.[5, 29] In normal pregnancy, the remodeling of the uterine spiral arteries occurs via different steps.[30] Around implantation, vascular changes arise in the endo- and myometrium and, subsequently, trophoblast invasion of the uterine vessels starts. Initially, endovascular trophoblast cells migrate down the lumen of spiral arteries. In the first period, these cells occlude these arteries. This early plugging by trophoblast cells protects the embryo against a high oxygen environment.[31] When embryogenesis is complete after 10-12 weeks, these plugs are gradually resolved by intravascular migration of trophoblast cells. Consequently, the intervillous circulation is fully established, and oxygen concentrations within the placenta increase. In preeclampsia, the process of endovascular trophoblast invasion is impaired, leading to placental flow defects. In the majority of patients who develop preeclampsia, placental flow defects can be detected as early as 12 weeks of gestation.[32-34] Flow defects may result in placental oxidative stress, which may predispose a woman to preeclampsia.[35] It has been suggested that an important underlying cause of the impaired placentation is an aberrant interaction between the maternal and fetal (trophoblast) immune system.[36]

ANGIOGENIC IMBALANCE AS A CONSEQUENCE?

The second stage of preeclampsia is characterized by dysregulation of angiogenic factors followed by the clinical manifestations of the syndrome. The aforementioned placental flow defects in the first stage are believed to result in placental dysfunction. The preeclamptic placenta releases excessive amounts of the anti-angiogenic factors sFlt-1, and soluble endoglin (sEng).[19, 24, 25, 37] By neutralizing VEGF-A and placental growth factor (PlGF), sFlt-1 prevents these factors from binding to its membrane-bound receptors. Endoglin is a co-receptor for transforming growth factor B (TGF-β). The soluble form, sEng, binds and neutralizes TGF-β. The factors VEGF-A, PlGF, and TGF-β are essential for normal endothelial function and maintenance. As a consequence, low concentrations of these factors in the setting of preeclampsia are thought to result in endothelial dysfunction.[38] The important role of sFlt-1 and sEng in the pathophysiology of preeclampsia has been demonstrated in animal models, in which administration of these anti-angiogenic factors induces the manifestations of preeclampsia.[25, 37]

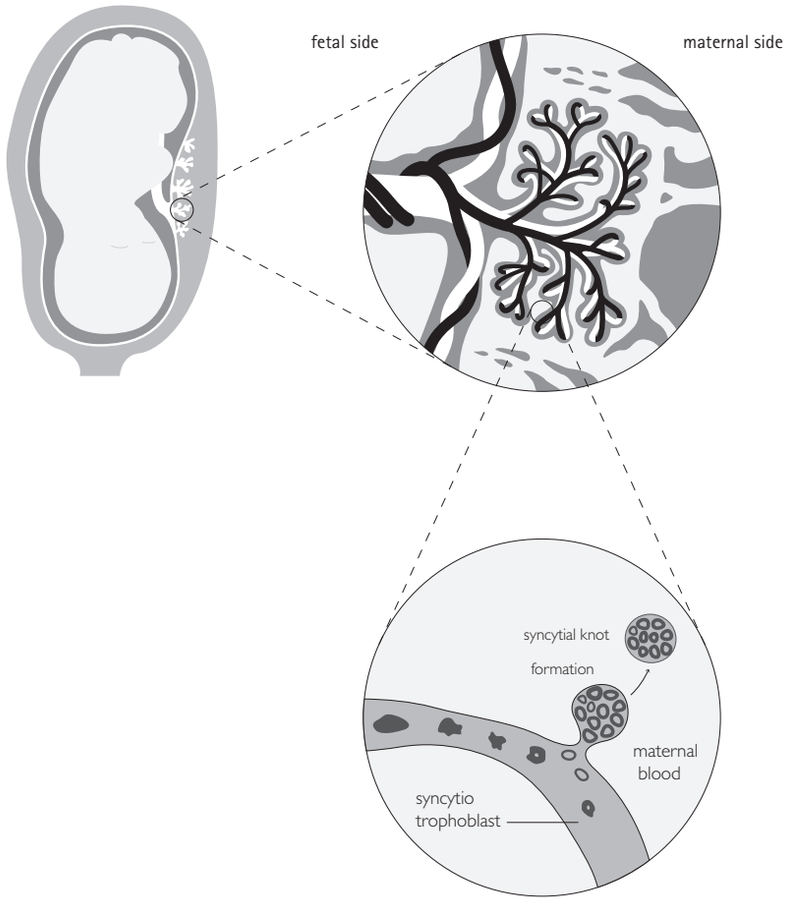

Figure 3: The placenta and syncytial knot formation.

The placenta provides the fetus with oxygen and nutrients. The outermost layer of the placenta is called the syncytiotrophoblast. The syncytiotrophoblast forms multinucleated placental fragments called 'syncytial knots'. The knots—called syncytial aggregates upon their release from the placenta —are shed into the maternal circulation.

SHEDDING OF PLACENTAL FRAGMENTS CONTRIBUTES TO ENDOTHELIAL DYSFUNCTION

Within the placenta, the anti-angiogenic factors are produced by the outermost layer of the placental villi.[39] This outermost layer of the placenta is a multinucleated structure called the syncytiotrophoblast. During both normal pregnancy and preeclampsia, the syncytiotrophoblast forms so called 'knots', which are multinucleated placental fragments. These syncytial knots—called syncytial aggregates upon their release from the placenta—are shed into the maternal circulation. Importantly, a recent study showed that these syncytial knots contain high amounts of the anti-angiogenic protein sFlt-1, particularly in women with preeclampsia.[40] Moreover, circulating syncytial aggregates remain transcriptionally active for at least 48 hours.[40] The formation of these knots is markedly increased in the preeclamptic placenta.[40] In addition, in preeclampsia in particular, these syncytial knots are released into the maternal circulation.[41, 42] This makes it is possible that these aggregates may serve as an autonomous source of sFlt-1 protein within the maternal circulation, thereby contributing to the generalized endothelial dysfunction that characterizes preeclampsia. This idea has been further supported by *in vitro* experiments showing that syncytiotrophoblast fragments interfere with endothelial cell growth.[43, 44]

ENDOTHELIAL DYSFUNCTION

Altogether, the angiogenic imbalance that characterizes preeclampsia leads to generalized endothelial dysfunction. Numerous studies have shown that preeclampsia is characterized by endothelial injury. Women with preeclampsia are characterized by decreased flow-mediated vasodilatation.[45, 46] Furthermore, *in vitro* experiments have shown that serum from women with preeclampsia is capable of altering endothelial function.[47, 48] Importantly, the clinical findings of severe preeclampsia may be explained by endothelial dysfunction in the different target organs including the

brain (eclampsia or seizures), the liver (the hemolysis, elevated liver function, and low platelet count (HELLP) syndrome), and the kidney (proteinuria).

IS THERE MORE?

Preeclampsia is believed to result from an imbalance in pro- and antiangiogenic factors, and it is evident that reduced VEGF-A-signaling plays an important role in the pathogenesis of the renal lesions in preeclampsia. However, not all women with preeclampsia have increased sFlt-1 serum concentrations,[19] which suggests that other factors may be involved. Recently, increasing evidence points towards the involvement of complement activation in the pathogenesis of preeclampsia.

COMPLEMENT SYSTEM

The complement system is part of the innate immune system. Where the adaptive immune system is acquired during life, the innate immune system acts in a non-specific manner and is present at birth. The innate immune system provides immediate defense against infections, therefore it is called the 'first line of defense'. The complement system defends the host against infection, but it also provides a bridge between the adaptive and the innate immune system. In addition, it is involved in the clearance of necrotic and apoptotic cells.[49] The complement system consists of more than 30 proteins in plasma and on cell surfaces, of which a schematic overview is shown in Figure 4. Activation of the system is highly regulated, as it needs to be ready for action at all times.[50, 51] Specific triggers, like an invading pathogen, can initiate a cascade of amplifying enzymatic reactions and positive feedback loops which are also known as complement activation pathways. There are three different pathways that can activate the complement system, namely the classical, the lectin, and the alternative pathway. Activation of any of the three pathways is triggered by specific initiators, but they do share a final common pathway as all three pathways converge at the level of C3.

The classical pathway becomes activated when its recognition molecule C1q binds to immune complexes, i.e., antibodies bound to an antigen, or to apoptotic cells. After an antibody response has developed, this pathway links innate immunity to B-cell mediated immune activity. However, activation of the classical pathway can also lead to tissue injury and graft rejection via deposition of antibodies. Deposition of auto- and alloantibodies might occur in the setting of auto-immune diseases and organ transplantation, which illustrates that the complement system can act as friend or foe.[52]

The lectin pathway is initiated by binding of mannose-binding lectin or ficolin to mannose (carbohydrates) on the surfaces of pathogens.[50, 51] After recognition of the pathogen, the lectin pathway follows the same steps as the classical pathway, via C4 and C2, leading to activation of the final common pathway. This overlap between the classical and lectin pathway makes it difficult to distinguish between these two routes.

The last pathway, the alternative pathway, mainly amplifies the reactions initiated by the classical- and the lectin pathway, thereby providing a positive feedback loop. To achieve this function, the alternative pathway is constantly being activated by the hydrolysis of C3. Whenever C3 is activated by the classical or lectin pathway, Factor D and B immediately bind to it. This complex is stabilized by the presence of properdin, leading to the formation of an enzyme that leads to more C3 activation, resulting in a positive feedback loop. At the same time, this pathway is strictly regulated to prevent excessive activation.

Activation of the complement system has various effects. Firstly, it leads to the release of anaphylatoxins, C3a and C5a, thereby attracting leukocytes. Secondly, opsonization of the target with specific membrane-bound fractions allows phagocytosis. Lastly, activation of the complement system leads to the generation of the lytic Membrane Attack Complex (MAC).

This complex of enzymes creates pore-like structures on cell membranes, leading to cell lysis and, eventually, cell death. Given the multitude of effects that the complement system can exert, there are mechanisms in place to limit complement activation where and when it occurs.[53] Probably for this reason, almost half of all complement components have regulatory functions.[54] These complement regulatory proteins are either membrane-bound or soluble, and protect the host tissue by inhibiting activation, amplification, and the formation of the MAC. Membrane-bound complement regulatory proteins are abundantly present on tissues that are in direct contact with blood, which contains complement components. Illustratively, complement regulatory proteins are highly expressed on endothelial cells, and on placental- and renal tissues.[55, 56] Soluble regulators, such as factor H, I, and C4-binding protein, regulate the alternative pathway by preventing amplification of the cascade, which results in blockade of the positive feedback loop.

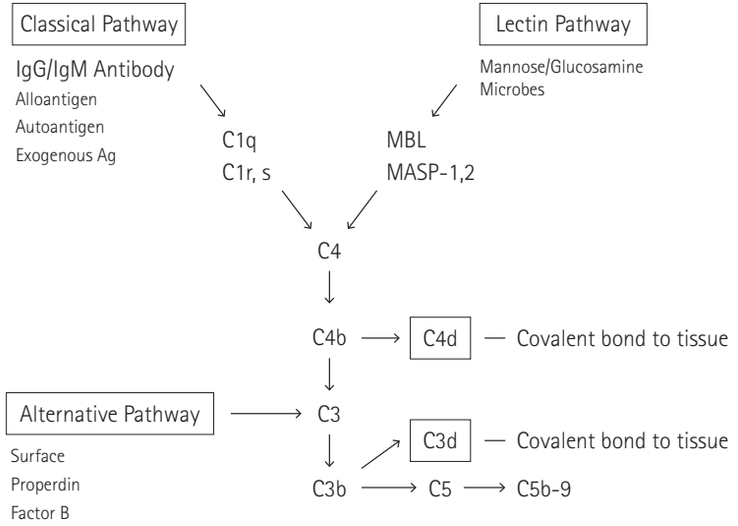

Figure 4: A schematic overview of the complement system.

THE ROLE OF COMPLEMENT IN PREECLAMPSIA

Increasing evidence suggests that complement dysregulation is involved in the development of preeclampsia. Firstly, within the placenta, preeclampsia is associated with excessive complement activation at the fetal-maternal interface.[57-59] Secondly, elevated concentrations of complement degradation products in both serum and urine samples are found in women with preeclampsia.[60, 61] Finally, mutations in genes encoding for complement regulatory proteins have been found in women with preeclampsia,[62] and in HELLP syndrome.[63] In people with either inherited or acquired mutations in their genes encoding for complement regulatory proteins, one would perhaps expect systemic complement activation. Clinically, however, these mutations are primarily associated with atypical Hemolytic Uremic Syndrome (aHUS), which is often affecting the kidney. This finding suggests that particularly the kidney is vulnerable to excessive complement dysregulation.[64]

COMPLEMENT ACTIVATION AND THE KIDNEY

Within the kidney, the fenestrated endothelium that lines the glomerular basement membrane is particularly vulnerable to complement activation as demonstrated in a variety of renal diseases.[65] The anatomic composition of the renal endothelium is thought to cause this susceptibility, as the fenestrated structure allows immune complexes to deposit more easily than in the normal inner layer of a vessel.[66] High vascular stress and hypertension might result in additional injury of the glomerular endothelium. During preeclampsia—characterized by hypertension—excessive sFlt-1 concentrations may provide the additional hit which aggravates endothelial injury. It has been shown that injury to the renal endothelium can lead to complement activation.[67] So the observation that preeclampsia is characterized by complement dysregulation, combined with severe endothelial injury within the kidney during preeclampsia suggests that complement activation might play an important role in the renal complications of preeclampsia.

CONSEQUENCES OF RENAL INJURY: FOOTPRINTS IN URINE

Regardless of which mechanism is responsible for renal injury during preeclampsia, it is evident that extensive kidney injury is present, as shown by—sometimes severe—proteinuria. During preeclampsia not only proteins are lost in urine, but also podocytes, and—most likely—other smaller molecules. The combination of these markers could result in a 'urinary footprint' specific for preeclampsia. Whether specific urinary biomarkers might help to distinguish preeclamptic patients from control subjects early in pregnancy is discussed below.

Part 3 Prediction of preeclampsia

PREDICTION IS CHALLENGING

Predicting who will develop preeclampsia remains challenging. The ability to predict preeclampsia is currently of limited benefit because in most patients neither the development of the disorder nor its progression from mild to severe disease can be prevented, and there is no cure except delivery. Nevertheless, the accurate identification of women at risk, early diagnosis, and appropriate management (such as antenatal corticosteroids for fetal lung maturation, treatment of severe hypertension, and early delivery) may improve maternal outcome, and possibly fetal outcomes as well. A good test for predicting who will develop preeclampsia should be rapid, simple, non-invasive, inexpensive, and easy-to-perform.[68] In particular, a test should adequately predict whether a condition, such as preeclampsia, will develop. For this purpose likelihood ratios are used, of which two versions exist: one for positive, and one for negative test results. The 'likelihood ratio positive' is defined as the probability of a person who has the disease testing positive (sensitivity) divided by the probability of a person who does not have the disease testing positive (1-specificity). The 'likelihood ratio negative' is defined as the probability of a person who has the disease testing negative (1-sensitivity) divided by the probability of a person who does not have the disease testing negative (specificity). A good test for preeclampsia should have a likelihood ratio positive >15, and a likelihood ratio negative <0.1.[69] Ideally, early prediction of preeclampsia should provide an opportunity for preventive strategies and close surveillance. Many tests to predict preeclampsia have been assessed for their relation to vascular resistance, endothelial dysfunction, fetal-derived products, and hormones. However, in a systematic review only a few tests reached a specificity above 90%, and no single test met the clinical standards for a predictive test.[70] Although no single marker seems to be able to reliably predict such a heterogeneous syndrome as preeclampsia, prediction models based on combined information on maternal factors, biophysical,- and biochemical markers during early pregnancy are continuously being improved and show promising high sensitivity and specificity.[6] However, these models still need optimization, and the use of urinary biomarkers for the prediction of preeclampsia might be worthwhile.

URINARY FOOTPRINTS: PODOCYTURIA

The role of podocyte injury in proteinuric kidney disease has been the subject of extensive research in diverse settings. Evidence was found that urinary excretion of viable podocytes is confined to a phase of active ongoing glomerular damage.[71] Interestingly, previous studies have reported significantly higher levels of podocytes in urine of patients with atypical HUS,[72] IgA-nephropathy,[73] diabetes mellitus,[74] lupus nefritis,[75] and focal segmental glomerulosclerosis (FSGS),[75] which were shown by either immunohistochemistry on cultured podocytes or increased podocyte mRNA levels using quantitative Polymerase Chain Reaction (qPCR). These results suggest that podocytes are shed from the glomerulus in disease states. Interestingly, podocytes appear to be lost—although to a much lesser extent—in urine of healthy subjects as well.[75] Although it remains debatable what the underlying mechanism for this

phenomenon is, it could be speculated that podocytes are shed because they are senescent, and that their loss is compensated for by other glomerular cells. In preeclampsia, Garovic et al[27] showed for the first time that the presence of podocyturia, detected with podocyte specific immunohistochemical stainings on cultured podocytes, is a highly sensitive and specific method for the detection of this syndrome at disease onset. The method used in this study is labour-intensive, and time-consuming. Part of the work described in this thesis has therefore been focused on another method to detect podocytes in urine of women with preeclampsia.

URINARY FOOTPRINTS: METABOLOMICS

In addition to cells, small molecules are probably voided in the urine of women with preeclampsia as well. These small molecules are called metabolites. The set of metabolites that reflects the organism under a particular set of conditions is called the *metabolome*.[76] The metabolome is the product of genetic and environmental conditions, and provides a new logical framework to elucidate disease etiology. Analyzing metabolites in body fluids, such as plasma and urine, provides information that cannot directly be obtained from the genotype or gene expression profiles. More importantly, the metabolome putatively contains crucial information on disease development.[77]

High-tech procedures allow analysis of the metabolome, in which many molecules are identified in one single experiment.[77] One of the techniques to discriminate between different metabolites, is analyzing their magnetic properties. This technique is called nuclear magnetic resonance spectroscopy, and allows identification and quantification of many metabolites in one sample. To identify the origin of the metabolites, their magnetic characteristics are matched to a metabolite database. This analysis may provide a deeper understanding of interactions between different systems involved in the pathophysiology of a particular disease. It is therefore imaginable that a large-scale analysis of metabolites would provide—in addition to prediction of a disease—much more insight in disease etiology.

In the setting of preeclampsia, metabolomics analysis has recently been performed in plasma. In the first trimester of pregnancy, plasma samples were collected from women who later during pregnancy developed preeclampsia.[78-80] The metabolomic profiles of the women who later developed preeclampsia were distinct from those of pregnant control subjects in all studies. In contrast to plasma samples, the technique for obtaining urine samples has the benefit of being non-invasive, and relatively easy. In disease settings other than preeclampsia, predictive metabolomic profiles have been successfully defined in urine samples.[81, 82] Given the fact that the kidney is one of the major target organs which is severely injured during preeclamptic pregnancy combined with the non-invasive character of the technique for obtaining urine samples, in the work described in this thesis we investigated whether urinary metabolomics analysis can be used for the prediction of preeclampsia.

Part 4 This thesis

In preeclampsia, not only the fetus and its growth are affected, but severe maternal morbidity can occur as well, even leading to maternal mortality. Further unraveling the pathophysiologic mechanisms underlying this systemic syndrome could contribute to the development of diagnostic tools and the identification of therapeutic targets. This thesis focuses on the prediction and the systemic—in particular the maternal renal—consequences of preeclampsia.

AIMS OF THE WORK DESCRIBED IN THIS THESIS

- To explore whether placenta-derived fragments are retained in the maternal lung, and whether they still retain the anti-angiogenic factor sFlt-1
- To investigate the relation between complement dysregulation in the kidney and preeclampsia
- To determine whether the podocyte number within the glomeruli in preeclampsia is altered and whether this is compensated for by recruitment of other cells
- To investigate the relationship between urinary podocyte loss and preeclampsia
- To explore whether a distinctive urinary metabolomic profile in the first trimester is predictive for preeclampsia.

THESIS OUTLINE

In the studies described in chapters 2, 3, and 4 we investigated autopsy samples from women who died during pregnancy, either with or without preeclampsia. The placental expression of sFlt-1 is explored in the studies described in **chapter 2**, which furthermore focus on the systemic spread of placenta-derived fragments in preeclampsia, and their contribution to sFlt-1 expression. Complement activation plays an important role in the pathophysiology of preeclampsia, as is shown by genetic mutations and increased concentrations of complement activation products in both serum and placentas from preeclamptic women. Given the fact that the complement system is involved in many renal diseases, combined with the observation that the kidney is one of the major target organs in preeclampsia, the studies described in **chapter 3** were designed to explore complement activation in kidneys of women with preeclampsia. In the studies described in **chapter 4**, we further investigated the renal injury in women with preeclampsia, with a special focus on changes in podocyte numbers, and possible compensating mechanisms for podocyte injury and loss. With so many systemic and renal changes occurring during preeclampsia, changes in urine may be found, providing 'urinary biomarkers' of preeclampsia. Therefore, the studies described in **chapters 5 and 6** were devoted to changes observed in urine of women with preeclampsia. A new technique to detect loss of podocytes in urine of women with preeclampsia was investigated in the studies described in **chapter 5**. To further explore the pathophysiology of preeclampsia, the studies described in **chapter 6** were devoted to a urinary metabolomics analysis in the first trimester of pregnancy to predict preeclampsia. In **chapter 7**, the general discussion, the findings of the work described in this thesis are summarized and placed in a more general perspective. The general discussion is followed by a summary in Dutch.

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II Preeclampsia is associated with the presence of transcriptionally active placental fragments in the maternal lung

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Abstract

Preeclampsia is associated with increased levels of the circulating anti-angiogenic factor sFlt-1 and with an excessive shedding of placenta-derived multinucleated syncytial aggregates into the maternal circulation. However, it remains unclear whether these aggregates are transcriptionally active in the maternal organs and can therefore contribute to the systemic manifestations of preeclampsia.

In this study, we measured placental sFlt-1 mRNA levels in preeclamptic- and control placentas and performed RNA in situ hybridization to localize the main placental expression site of sFlt-1 mRNA. Because the maternal lung is the first capillary bed that circulating syncytial aggregates traverse, we studied the presence and persistence of placental material in lungs of preeclamptic and control subjects. To confirm the placental origin of these aggregates in maternal lungs, immunohistochemistry for the placenta-specific marker hCG and Y-chromosome in situ hybridization were performed.

Using human placental tissue, we found that syncytial knots are the principal site of expression of the anti-angiogenic factor sFlt-1. In addition, autopsy material obtained from women with preeclampsia (n=9), showed significantly more placenta-derived syncytial aggregates in the lungs than in control subjects (n=26). Importantly, these aggregates still contained the anti-angiogenic factor sFlt-1 following their entrapment in the maternal lungs.

The current study confirms the important role of syncytial knots in placental sFlt-1 mRNA production. Additionally, it shows a significant association between preeclampsia and larger quantities of sFlt-1 containing syncytial aggregates in maternal lungs, suggesting that the transfer of syncytial aggregates to the maternal compartment may contribute to the systemic endothelial dysfunction that characterizes preeclampsia.

Introduction

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

The widespread endothelial dysfunction that characterizes preeclampsia is believed to be due to an imbalance between pro- and anti-angiogenic factors.[2, 3] The placenta is a major source of circulating anti-angiogenic factors during both normal and preeclamptic pregnancies.[3-6] In preeclampsia in particular, the outermost layer of the placenta—the syncytiotrophoblast—forms "knots" that contain high amounts of the anti-angiogenic protein sFlt-1.[7] These syncytial knots are released into the maternal circulation, thereby becoming syncytial aggregates that can become lodged in maternal organs.[8-10] Importantly, a recent study showed that upon their release, circulating syncytial aggregates remain transcriptionally active and likely serve as an autonomous source of sFlt-1 protein within the maternal circulation.[7]

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

Methods

PATIENT SELECTION AND TISSUE COLLECTION

Placentas were obtained from preeclamptic[11] (n=32) and control (n=37) subjects who delivered at the Leiden University Medical Center (LUMC), the Netherlands from 2007 through 2010. All women gave written informed consent. Parallel to the collection of placenta material, autopsy samples from women who died during pregnancy were obtained via a nationwide search using the Dutch PALGA system, a histopathology and cytopathology network and archive that includes all pathology laboratories within the Netherlands.[12] The paraffin-embedded lung samples obtained from nine preeclampsia patients and 26 pregnant control subjects were provided by collaborating laboratories. The control subjects were women who died due to a cause other than a hypertensive disorder of pregnancy. The cause of death in each case was confirmed using the records of the National Maternal Mortality Committee of the Dutch Society of Obstetrics and Gynecology. To investigate the effect of pregnancy on maternal aggregates, an additional control group (n=11) comprised of non-pregnant, non-hypertensive women was included. All tissues were coded and handled anonymously in accordance with the Dutch National Ethics Guidelines (Code for Proper Secondary Use of Human Tissue, Dutch Federation of Medical Scientific Societies). This study was approved by the ethics committee of the LUMC.

PLACENTAL sFlt-1 mRNA EXPRESSION

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

IMMUNOHISTOCHEMISTRY

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

Y CHROMOSOME IN SITU HYBRIDIZATION

A DIG-labeled DNA probe that specifically recognizes the Y chromosome[13] was used to determine whether the putative syncytiotrophoblast aggregates in the maternal lungs were of fetal origin. Sections of lungs from women who had carried a male fetus were incubated with the DIG-labeled probe. To visualize the probe, the sections were incubated first with a mouse-anti-DIG monoclonal antibody (Sigma-Aldrich) followed by goat-anti-mouse IgG Alexa-647 (Invitrogen).

QUANTIFICATION OF STAINING

The number of sFlt-1 mRNA positive syncytial knots was counted by two independent observers who were blind with respect to the groups. Two observers also scored the lung sections for the absence

or presence of hCG. When hCG-positive multinucleate aggregates were present, the sequential sections were tested for the co-localization of hCG with Flt-1 protein and the Y chromosome.

Results

CLINICAL DATA

Placentas were investigated from women with preeclampsia (n=32) and pregnant controls (n=37). Gestational age in the women with preeclampsia (mean 30.6 weeks, SD 1.3 weeks) was significantly lower than the controls (mean 39.6 weeks, SD 1.7 weeks; $p<0.05$). Clinical data of the women whose lungs were investigated is provided in Table 1. Furthermore, in these women, the presence of pulmonary edema was investigated at the clinical, gross and microscopic levels. Neither the presence of clinical symptoms of pulmonary edema nor evidence of pulmonary edema on either gross or microscopic examination differed significantly between the groups.

INCREASED PLACENTAL sFlt-1 mRNA EXPRESSION IN PREECLAMPSIA

To compare the levels of sFlt-1 mRNA in the preeclamptic and control placentas, quantitative PCR was used to measure sFlt-1 mRNA. On average, the placental sFlt-1 mRNA levels were six-fold higher in the preeclamptic placentas than in the placentas obtained from control subjects ($p<0{\cdot}001$, Mann-Whitney test). The preeclamptic placentas had more intense sFlt-1 staining (measured using *in situ* hybridization) than control placentas, particularly in the syncytial knots (Figure 1). In addition, the number of syncytial knots was significantly higher in the preeclamptic women than in the control subjects ($p<0{\cdot}05$, Figure 1). As expected, the sense control probe was negative in all samples (Figure 1).

THE PRESENCE OF HCG POSITIVE AGGREGATES IN MATERNAL LUNGS IS SIGNIFICANTLY ASSOCIATED WITH PREECLAMPSIA

Because hCG was highly expressed within the syncytial knots, we considered hCG to be a suitable specific marker to study the presence of syncytiotrophoblast aggregates in maternal lungs. hCG-positive multinucleate aggregates were observed in the lungs of six of the nine preeclamptic women. Following the observation that syncytial aggregates were present in the lungs of women with preeclampsia, we also stained control lung sections for hCG. Syncytial aggregates were observed in the lung samples of six of the 26 pregnant control subjects. Importantly, the women with preeclampsia had a significantly higher number of syncytial aggregates per 100 mm^2 lung tissue ($p<0{\cdot}05$, Mann-Whitney test, Figure 2). Syncytial aggregates were found in the pregnant control subjects whose gestational age was 10-40 weeks and in preeclamptic women with a gestational age of 32-39 weeks. We performed a separate analysis to exclude any potential effect of gestational age. Because the shortest gestational age in the preeclampsia group was 30 weeks, we performed an analysis in which we excluded the control subjects with a gestational age shorter than 30 weeks. Importantly, even though the gestational age of the resulting subset of controls was now similar to the preeclamptic women, the number of syncytial aggregates remained significantly higher in the preeclampsia group ($p < 0.05$; see Figure S1 in the online supplement). Aggregates were observed in subjects who died up to 13 days after delivery. The lung samples obtained from the additional control group of non-pregnant, non-hypertensive women contained no syncytial aggregates. The number of aggregates was not associated with gestational age, maternal age or the severity of preeclampsia.

SYNCYTIOTROPHOBLAST AGGREGATES IN THE MATERNAL LUNG RETAIN THE sFlt-1 PROTEIN

To test our hypothesis that syncytial aggregates retain sFlt-1 protein after transferring to the maternal compartment and becoming entrapped in the maternal lung, we stained the hCG-positive aggregates in the maternal lung samples for Flt-1 protein. Staining sequential sections for Flt-1 and hCG revealed that these proteins were co-localized within the aggregates (Figure 2). In the preeclampsia group, 56% of all hCG-positive aggregates were also positive for Flt-1; in contrast, in the control group, 26% of the syncytial aggregates were positive for Flt-1 protein ($p<0.05$; see Figure 3).

Y CHROMOSOME IN SITU HYBRIDIZATION STRONGLY SUPPORTS THE IDEA THAT MULTINUCLEATE AGGREGATES ARE OF FETAL ORIGIN

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

Discussion

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

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

principal source of sFlt-1. However, previous research has shown that approximately 25% of all sFlt-1 is derived from shed syncytial aggregates.[7] It has been estimated that near the end of pregnancy, approximately 3 grams of this placental material is shed into the maternal circulation (i.e. into the maternal lungs) daily.[15] Therefore, the cumulative quantity of these circulating aggregates—and their relative contribution to total sFlt-1 production—should not be underestimated.

The lungs of the preeclamptic women contained a significantly higher percentage of sFlt-1-positive syncytial aggregates than the control samples. This observation further supports the idea that preeclampsia is associated both with an increased number of circulating syncytial aggregates and with increased sFlt-1 expression within these aggregates. By releasing sFlt-1, these aggregates may contribute to the systemic endothelial dysfunction that is characteristic of preeclampsia. This finding is also consistent with the observation that preeclamptic placentas contain more syncytial aggregates that are heavily loaded with sFlt-1 than placentas obtained following uneventful pregnancies.

In addition, the presence of syncytial aggregates in maternal organs—particularly in the early stages of pregnancy—may play a key role in the development of immune tolerance. As early as gestational week 10, we observed syncytial aggregates in maternal lungs. Because preeclampsia rarely presents prior to 20 weeks of gestation,[11] we could not investigate the presence of syncytial aggregates in the lungs of preeclamptic women early in pregnancy. We did, however, observe syncytial aggregates in the lungs of preeclamptic women at gestational week 32 and later, and other groups have reported the presence of trophoblast fragments in maternal blood in earlier stages of preeclamptic pregnancy.[15] Altogether, circulating syncytial aggregates are present early in pregnancy, and we and others[16] have found a strong association between increased shedding of syncytial aggregates and preeclampsia. Thus, one may speculate that the release and transfer

of syncytial aggregates to the maternal compartment is an early event in the pathogenesis of preeclampsia. However, the relative contribution of sFlt-1 expression in these aggregates in early pregnancy is unclear.

The presence and persistence of fetal cells in maternal organs may also have both short-term and long-term implications for postpartum maternal health. Syncytial aggregates that remain in the maternal lungs may undergo further disaggregation, forming smaller microparticles. These sFlt-1-loaded microparticles may—via their release into the systemic maternal circulation—contribute to endothelial dysfunction in maternal organs other than the lungs. We found that even 13 days after delivery, hCG-positive syncytial aggregates can be detected within the maternal lungs. This finding supports the idea that placenta-derived syncytial aggregates may be involved in the post-partum complications that are associated with preeclampsia. Preeclampsia usually resolves rapidly after delivery, and its resolution is reflected by a parallel decrease in sFlt-1 levels.[17] However, in a subset of women, the symptoms and complications of preeclampsia can persist or present several days following delivery. Syncytial aggregates remain transcriptionally active up to 48 hours after delivery, and estimates suggest that during pregnancy, 25% of all circulating sFlt-1 is derived from circulating syncytial aggregates.[7] Therefore, we propose that these aggregates may play an important role in postpartum (pre)eclampsia.

It must be acknowledged that the placentas in our study were not obtained from the same women from whom we obtained the lung tissues. Therefore, the preeclampsia phenotype of the women whose lung tissues were investigated might have been more severe than the phenotype of the women who provided the placentas. As a consequence of this potential mismatch between phenotypes, we were unable to correlate placental sFlt-1 production to the portion of sFlt-1-loaded syncytial aggregates in the maternal lungs. To overcome this complication, an animal model could be used to study the association between placental sFlt-1 production and lung pathology.

Trophoblast cells are likely not the only fetal cell population that is present in the maternal lung. A previous study using mice suggested that fetal cells in the maternal lung are comprised of a mixture of cell types that includes trophoblasts, mesenchymal stem cells, and cells from the immune system.[18] We have now confirmed the presence of trophoblast cells in the human maternal lung. In the long run, the release of vital cells from the placenta may result in chimerism, as fetal cells can be retained in the maternal blood and organs for decades after delivery.[19, 20] Because retained fetal cells have stem cell-like properties,[21] it can be speculated that these cells provide a mechanism through which maternal health can be affected for decades after pregnancy.

PERSPECTIVES

In conclusion, we have demonstrated that multinucleate aggregates in the maternal lungs originate from the syncytiotrophoblast, that their presence is significantly associated with preeclampsia and that these aggregates retain the anti-angiogenic protein sFlt-1. Further studies are needed to determine the relevance and relative contribution of trophoblast cells—and other cell types—to maternal health. Likewise, understanding what drives the formation, detachment and transfer of syncytial knots to the maternal compartment—and why these knots produce sFlt-1—are important questions to be investigated. Nevertheless, this report highlights the importance of investigating further the role that syncytial aggregates play in preeclampsia and its complications.

NOVELTY AND SIGNIFICANCE

What is new?

- Within the placenta, syncytial knots are the principal site of expression of the anti-angiogenic factor sFlt-1.
- Placenta-derived syncytial aggregates that become lodged in the maternal lungs retain the anti-angiogenic factor sFlt-1.
- Preeclampsia is associated with significantly higher quantities of sFlt-1 loaded syncytial aggregates within the maternal lung.

What is relevant?

- Although the precise etiology of preeclampsia and its complications remains unknown, the condition is associated with excessive shedding of placental material into the maternal circulation.
- Placenta-derived syncytial aggregates within the maternal lungs contain sFlt-1 and may contribute to the systemic endothelial dysfunction that characterizes preeclampsia.
- Furthermore, retained fetal cells within the mother may have stem cell-like properties, thereby providing a mechanism through which maternal health can be affected for decades after pregnancy

Summary

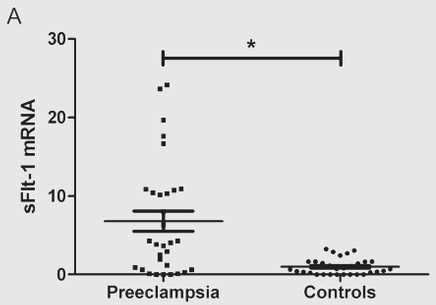
The current study confirms the important role of syncytial knots in placental sFlt-1 mRNA production. In addition, it demonstrates a significant association between preeclampsia and the presence of increased quantities of sFlt-1 containing syncytial aggregates in maternal lungs. These observations suggest that the transfer of syncytial aggregates into the maternal compartment likely contributes to the systemic endothelial dysfunction that characterizes preeclampsia.

Table 1

Characteristics	PE (n=9)	Controls (n=26)
Maternal age at death (years)	33.3 (4.6)	32.0 (5.2)
Death postpartum (%) †	100	42.3
GA at birth (w) *	35.2 (3.0)	38.5 (3.2)
Death Postpartum (h)	107.4 (157.2)	96.8 (180.0)
Death during pregnancy (%) †	0	57.5
GA at death (w)	-	22.3 (10.6)
Death-Autopsy time (h)	19.7 (13.8)	25.7 (14.7)
Gender offspring		
Male (%)	57.1	47.8
Female (%)	42.9	52.2
Parity	0.7 (1.1)	1.0 (1.2)

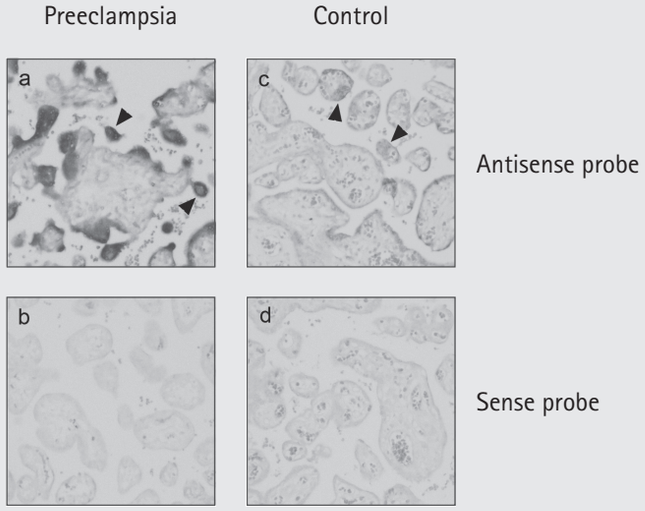
*Table 1: Data are given as mean ± SD. * p<0.05 † p<0.01, PE= preeclampsia*

Figure 1A



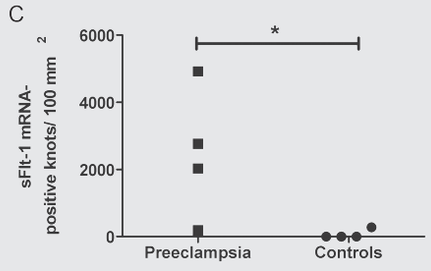
*Panel A shows the relative sFlt-1 mRNA levels in the placentas of women with preeclampsia and control subjects with mean+SEM (*p<0·001).*

Figure 1B



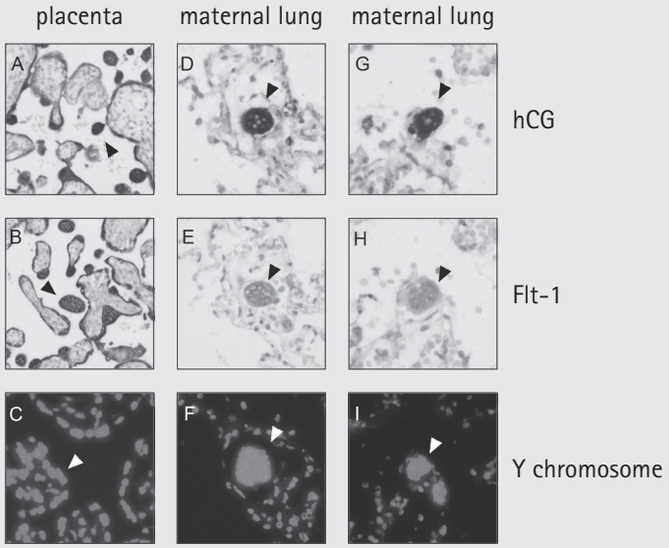
Panel B (full colour version inside cover) shows typical examples of sFlt-1 mRNA in situ hybridization in the placenta of a woman with preeclampsia (a and b) and a control subject (c and d). The term "syncytial knots" describes multinucleated structures that are loosely attached to the tips of placental villi in situ. Each column represents an individual placenta, and the various RNA probes are shown horizontally. The antisense probe (a and c) revealed that the syncytial knots (arrowheads) were the primary placental site of sFlt-1 mRNA production in both placentas. The sense probe (b and d) was used as a negative control.

Figure 1C



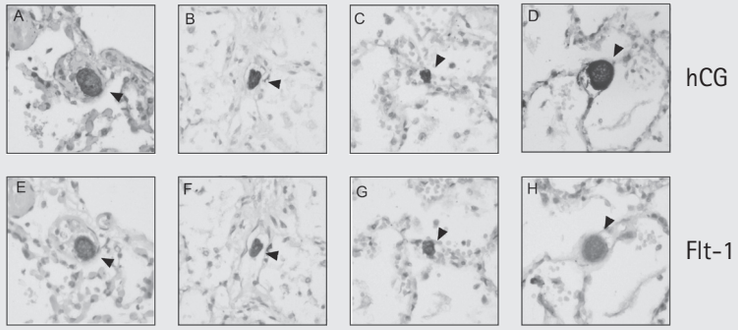
*Panel C shows the density of sFlt-1 mRNA-positive syncytial knots in the placentas of preeclamptic women and control subjects. *p<0·05.*

Figure 2A



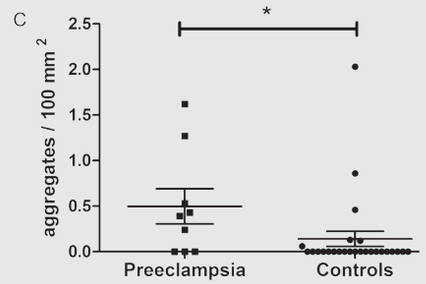
Panel A (full colour version inside cover) shows sections of placenta and sequential sections of maternal lungs that were stained for hCG or Flt-1 (using immunohistochemistry) or the Y chromosome (using in situ hybridization). To simplify the terminology, we use "syncytial knots" to describe multinucleated structures that are loosely attached to the tips of placental villi in situ and "syncytial aggregates" to describe detached multinuclear structures within the maternal lungs. The left column shows a placenta obtained from a preeclamptic patient, and the middle and right columns represent the lungs obtained from two women who were pregnant with a boy and died due to preeclampsia. The various stains are shown horizontally. Subpanels a-c show that within the preeclamptic placenta, the hCG (a) and Flt-1 (b) proteins are most abundantly present within the syncytial knots (arrowheads). In addition, Y chromosome in situ hybridization (c) shows the presence of the Y chromosome in the nucleus (visible as red puncta). Subpanels d and g show the presence of hCG-positive aggregates (arrowheads) in the lungs of two women who died due to preeclampsia. Subpanels e and h show the Flt-1 staining patterns in sections that were sequential to the sections shown in panels d and g, respectively. These images demonstrate that within the maternal lungs, hCG-positive aggregates also contain Flt-1 protein. The next sequential sections were used to perform Y chromosome in situ hybridization (subpanels f and i). These images show that the multinucleate aggregates contain Y chromosomes (arrowheads), indicating that it is very unlikely that these aggregates are not of fetal origin. Altogether, the figure demonstrates co-localization of hCG, Flt-1 and the Y chromosome in the multinucleate aggregates (arrowheads).

Figure 2B



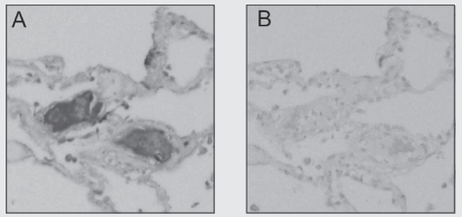
Panel B shows additional examples of multinucleate aggregates in maternal lungs, with co-localization of hCG (subpanels a-d) and Flt-1 (subpanels e-h). Each row shows matched sequential sections.

Figure 2C



*Panel C summarizes the number of hCG-positive syncytial aggregates within the maternal lungs of preeclamptic women (n=9) and control subjects (n=26). *p<0·05.*

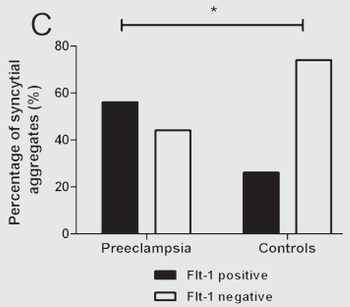
Figure 3AB



Panel A (full colour version inside cover) shows the presence of a hCG-positive aggregate in the lung of a control subject.

Panel B (full colour version inside cover) shows negative Flt-1 staining in a section that was sequential to the section shown in Panel A. These images demonstrate that within the lungs of control subjects, the minority of the hCG-positive aggregates also contain Flt-1 protein.

Figure 3C



*Panel C shows that within the preeclampsia group, 56% of all hCG-positive aggregates were also Flt-1 positive, whereas in the control group 26% of the syncytial aggregates showed positivity for Flt-1 protein. *p<0·05.*

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III Complement activation in the kidney during preeclampsia

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Abstract

A growing body of evidence suggests that complement dysregulation plays a role in the pathogenesis of preeclampsia. The kidney is one of the major organs affected in preeclampsia. Because the kidney is highly susceptible to complement deposits, we hypothesized that preeclampsia is associated with renal complement activation. We performed a nationwide search for renal autopsy material in the Netherlands using a computerized database (PALGA). Renal tissue was obtained from 11 women with preeclampsia, 25 pregnant controls, and 14 non-pregnant controls with hypertension. The samples were immunostained for C4d, C1q, MBL, properdin, C3d, C5b-9, IgA, IgG and IgM. Our findings in human samples were validated using a soluble fms-like tyrosine kinase 1 (sFlt-1) mouse model of preeclampsia. Preeclampsia was significantly associated with renal C4d —a stable marker of complement activation— and the classical pathway marker C1q. In addition, the prevalence of IgM was significantly higher in the kidneys of the preeclamptic women. No other complement markers studied differed between the groups. In the preeclampsia mouse model, the kidneys in the sFlt-1–injected mice had significantly more C4 deposits than the control mice. The strong association between preeclampsia and renal C4d, C1q, and IgM levels suggests that the classical complement pathway plays a role in the pathogenesis of renal injury in preeclampsia. Moreover, our finding that sFlt-1–injected mice develop excess C4 deposits indicates that angiogenic dysregulation may play an important role in complement activation within the kidney. We suggest that inhibiting complement activation may be beneficial for preventing the renal manifestations of preeclampsia.

Introduction

Preeclampsia is a severe multisystem pregnancy-related complication; worldwide, preeclampsia causes high maternal and perinatal morbidity and mortality rates.[1] Preeclampsia complicates 2-8% of pregnancies and is characterized by endothelial damage, resulting in maternal hypertension and proteinuria after gestational week 20.[2] The endothelial damage in preeclampsia is believed to arise from a dysregulation of proangiogenic and antiangiogenic factors, particularly the antiangiogenic factor soluble fms-like tyrosine kinase 1 (sFlt-1).[3]

Although the precise pathogenesis of preeclampsia is unknown, a growing body of evidence suggests that complement dysregulation plays a role in the development of preeclampsia.[4] In support of this notion, women with preeclampsia have complement dysregulation in the placenta, as well as elevated circulating levels of complement degradation products.[5, 6] In addition, individuals with mutations in genes that encode complement regulatory proteins are predisposed to developing preeclampsia.[7] Furthermore, treating preeclamptic mice with complement inhibitors can reverse proteinuria and histopathologic lesions.[8] Finally, Eculizumab, a terminal complement inhibitor, has been used successfully to reduce preeclampsia-associated conditions, thereby prolonging pregnancy in a patient with preeclampsia.[9]

In preeclampsia, the kidney is a major target organ that develops severe damage, as demonstrated by renal symptoms that include proteinuria and by abnormal renal histology.[10] These symptoms are believed to reflect endothelial damage due to increased sFlt-1 levels, which prevent vascular endothelial growth factor (VEGF) from maintaining the renal endothelium.[11] It has been shown that damage to the fenestrated glomerular endothelium can activate the complement system.[12-14] Interestingly, a case report showed glomerular complement deposits in a patient with preeclampsia, suggesting that the complement system may indeed play a key role

in the pathogenesis of renal damage in preeclampsia.[15] Furthermore, a recent study reported that patients with severe preeclampsia have a higher prevalence of urinary excretion of the terminal complement complex compared to control subjects.[16]

Because preeclampsia is characterized by complement dysregulation, the kidney plays an important role in preeclampsia, and the kidney is highly susceptible to complement deposits, we hypothesized that complement activation is involved in the renal manifestations of preeclampsia. If correct, this hypothesis would support the notion that inhibiting complement activation might be a viable option for treating the renal manifestations of preeclampsia. To test this hypothesis, we measured the presence of complement components in a unique cohort of renal autopsy tissue samples collected from preeclamptic patients and control patients; in addition, we studied complement components in a sFlt-1–induced mouse model of preeclampsia.

Methods

PATIENT SELECTION AND NATIONWIDE PALGA SEARCH FOR RENAL AUTOPSY TISSUE

To study the role of the complement system in the renal pathology of preeclampsia, we performed a nationwide search for renal autopsy tissues in the Netherlands using the Dutch Pathology Registry (PALGA), a histopathology and cytopathology network and registry that includes all pathology laboratories within the Netherlands.[17] The search parameters were as follows: “autopsy”, “women”, “age between 18 and 45 years”, and “since 1990”. We included all patients who were pregnant and who had a confirmed case of preeclampsia. In addition, two control groups were included in the study. The first control group consisted of pregnant women without a hypertensive disorder either prior to or during their pregnancy; this group was included to investigate the effect of pregnancy alone. The second control group consisted of non-pregnant young women with a medical history of chronic hypertension; this group was included to investigate the effect of hypertension alone. The search yielded paraffin-embedded kidney samples from 11 patients with preeclampsia,[18] 25 pregnant controls, and 14 non-pregnant chronic hypertensive controls. If available, clinical characteristics were obtained from autopsy-reports. The records of the National Maternal Mortality Committee of the Dutch Society of Obstetrics and Gynecology were used to confirm the cause of death of each pregnant case.[19] All tissue samples were coded and treated anonymously in accordance with Dutch national ethics guidelines (Code for Proper Secondary Use of Human Tissue, Dutch Federation of Medical Scientific Societies). This study was approved by the Medical Ethics Committee of the Leiden University Medical Center (P12.107).

sFlt-1 MOUSE MODEL OF PREECLAMPSIA

All animal experiments were performed at the Beth Israel Deaconess Medical Center in accordance with International Animal Care and Use Committee guidelines. We used the sFlt-1–induced mouse model of preeclampsia,[20] which overexpresses the sFlt-1 protein and develops high blood pressure, proteinuria, and endotheliosis. In brief, pregnant female CD1 mice (Charles River, Wilmington, MA) received a tail vein injection of 2×10^9 pfu of either an adenovirus encoding sFlt-1 (Ad-sFlt-1) or an equivalent dose of adenovirus empty vector CMV null (Vector Laboratories, Philadelphia) at gestational day 9.5. This model has been well characterized by a number of groups in both rats and mice and leads to hypertension, proteinuria and glomerular endothelial damage 7-10 days after adenoviral injection of sFlt-1.[3, 20-22] For our studies, mice were euthanized on gestational day 17.5 and one kidney from each mouse was frozen for immunofluorescence, and the other kidney was formalin-fixed and embedded in paraffin. Paraffin-embedded kidney sections were stained with Periodic Acid Schiff (PAS) or silver using standard protocols.

HISTOLOGY, IMMUNOHISTOCHEMISTRY, AND IMMUNOFLUORESCENCE

Sections of human kidney samples were stained with Periodic Acid Schiff (PAS) and silver using a standard protocol. To measure human renal complement activation, various complement system components were stained using immunohistochemistry. We used primary antibodies against the following proteins: C4d (Biomedica Gruppe, 1:50), a split product of C4 that binds covalently to the target tissue and can arise from the classical and mannose-binding lectin (MBL) pathways; C1q (DakoCytomation, 1:800), which reflects activation of the classical complement pathway; MBL (Sigma-Aldrich Biotechnology, 1:500), which reflects activation of the lectin pathway; properdin (kindly provided by the Department of Nephrology, Leiden University Medical Center, 1:200), which reflects activation of the alternative complement pathway; and C3d (Abcam, 1:800) and SC5b-9 (Quidel, 1:150), both of which are formed by activation of any of the three aforementioned pathways. To identify apoptotic cells, the samples were immunostained for caspase-3 (Cell Signaling Technology Inc., 1:300). Immunohistochemistry was performed after the sections were deparaffinized and treated for antigen retrieval. Staining was visualized using the appropriate HRP-labelled secondary antibodies with diaminobenzidine as the chromogen. Finally, the sections were counterstained with hematoxylin.

To examine the presence of immunoglobulin deposits in the human glomeruli, immunofluorescence was performed for IgA, IgG, and IgM separately. First, the sections were treated with protease XXIV (SigmaAldrich) at 37°C for one hour. The sections were then incubated for one hour with FITC-labeled rabbit anti-human IgA (DakoCytomation; 1:20), FITC-labeled goat anti-human IgG (Protos Immuno Research; 1:25), or FITC-labeled rabbit anti-human IgM (DakoCytomation; 1:20). To study classical complement activation in the mouse kidneys, frozen sections were stained for C4d using a rat monoclonal anti-C4 antibody (Cedarlane Laboratories, 1:200), which binds to murine C4, C4b, and C4d. To identify endothelial cells, adjacent sections were stained for CD31 using a rat monoclonal anti-CD31 antibody (BD Pharmingen, 1:100). A FITC-labelled secondary antibody was used to visualize the primary antibodies.

QUANTIFICATION OF IMMUNOHISTOCHEMISTRY AND IMMUNOFLUORESCENCE

The human kidney sections were scored histologically by an experienced renal pathologist who was blinded with respect to the subjects' clinical data. Each stained sample was evaluated and scored by two independent observers. Because the renal pathological manifestations of preeclampsia are present in the glomerulus, we scored the staining of the various markers in the glomerulus only, scoring ≥50 glomeruli per section. The immunostained complement components were scored semi-quantitatively as follows: 0 represents an absence of—or traces of—glomerular staining; 1 represents segmental glomerular staining; and 2 represents global staining of the glomeruli. If positive (i.e., a score of 1 or 2), the kidney sections were further classified as having either focal (10-50% of the glomeruli) or diffuse (>50% of the glomeruli) deposits. Caspase-3 staining was analyzed by counting the number of caspase-3–positive cells in 50 glomeruli and comparing the number of positive cells between the study groups. For immunofluorescence, the slides were analyzed for either the absence or presence of immune deposits in the glomeruli using both a fluorescence microscope (DM5500B, Leica Instruments) and a confocal laser-scanning microscope (LSM 700, Zeiss).

STATISTICAL ANALYSIS

Categorical variables were analyzed using the Chi-square test. Differences in quantitative parameters between groups were analyzed using a one-way ANOVA (for normally distributed data) or the non-parametric Kruskal-Wallis test (for non-normally

distributed data). Correlations between ordinal data and numerical data were calculated using a Spearman's or Pearson's coefficient, respectively. All analyses were performed using the SPSS statistical software package (version 20.0; IBM Corp.). Differences with $p<0.05$ were considered to be statistically significant.

Results

CLINICAL DATA

The clinical characteristics of the human subjects included in the study were previously described, and are shown in chapter 4, page 100.[23] The hypertensive control group was significantly older than the other study groups ($p<0.05$); no other significant differences were observed with respect to the remaining clinical characteristics.

HISTOLOGY

The majority (82%) of the women with preeclampsia had prominent glomerular lesions, including various degrees of endotheliosis, podocyte swelling, and double contours of the glomerular basement membrane (also known as tram tracking). As previously published, in this cohort, endotheliosis was present in 55% of the samples from the women with preeclampsia; in contrast, endotheliosis was less prevalent in the pregnant controls and hypertensive controls (12% and 15%, respectively; $p<0.05$ versus the patients with preeclampsia).[23] Tram tracking and podocyte swelling were present exclusively in the women with preeclampsia (in 36% and 18% of patients, respectively; $p<0.05$ versus the control groups).

IMMUNOHISTOCHEMISTRY

To study complement activation, we stained the kidney sections for several complement components. Figure 1 shows typical examples of immunostained adjacent kidney sections from a patient with preeclampsia ("PE"), a pregnant control ("PC"), and a hypertensive control ("HC"). The glomeruli in all 11 preeclamptic patients were positive for C4d; in contrast, only 15/25 (60%) pregnant controls and 3/14 (21%) non-pregnant hypertensive controls were positive for C4d. The C4d staining in the glomeruli was either segmental or global. Positive C4d staining was strongly associated with preeclampsia ($p<0.0001$). The presence of C4d correlated significantly ($p<0.05$) with endotheliosis and tram tracking. C1q was detected in 9/11 (82%) of the kidney sections obtained from women with preeclampsia; in contrast, C1q was detected in only 6/25 (24%) of the pregnant controls and 2/14 (14%) of the non-pregnant hypertensive controls. Positive C1q staining was significantly associated with preeclampsia ($p<0.01$), and C1q staining and C4d staining were positively correlated ($p<0.0001$). Detailed information regarding the staining patterns of C4d and C1q in the patient and control groups is given in Figure 1.

MBL was present in one preeclamptic patient and one pregnant control; in both samples, the staining pattern was segmental; no significant differences were found between cases and controls with respect to MBL staining. Properdin was not detected in any of kidney samples.

With respect to C3d, the staining pattern was usually segmental. In preeclamptic patients, 5/11 (45%) of the kidney sections were positive for C3d; in contrast, only 2/25 (8%) of the pregnant controls and 2/14 (14%) of the non-pregnant hypertensive controls were positive for C3d subjects. No significant difference was found between the patient and control groups, and no correlation was found between C4d and C3d staining (data not shown). The most abundant C5b-9 staining was detected in sclerotic glomeruli; C5b-9 was present only rarely in functioning glomeruli. However, a significant correlation ($p<0.05$) was found between C5b-9 and C3d staining (Figure S1 shows typical staining patterns of C3d and C5b-9 in the patient and control samples). In all of the aforementioned immunostained sections, all of the positive kidney sections had diffuse deposits of complement components. Finally, no significant

difference was found between the patient and control groups with respect to caspase-3 staining. Specifically, the samples from preeclamptic women had an average of 0.05 caspase-3–positive cells/glomerulus, and the samples from the pregnant controls and hypertensive controls averaged 0.02 and 0.12 caspase-3–positive cells/glomerulus, respectively.

IMMUNOFLUORESCENCE

IgA was not detected in any of the samples. In contrast, IgG deposits were detected at weak levels in a mesangial pattern. No significant difference was found between the three groups with respect to IgG positivity, with 27%, 8%, and 21% of the preeclamptic patients, pregnant controls, and non-pregnant hypertensive controls, respectively, testing positive for IgG. IgM (Figure 2) was detected in 36%, 4%, and 21% of the preeclamptic patients, pregnant controls, and non-pregnant hypertensive controls, respectively ($p<0.05$ between the groups) (Figure 2C). Typical examples of IgM-positive and IgM-negative sections are shown in Figure 2A and 2B, respectively. We also measured the prevalence of IgM staining based on whether the sections were C4d-positive or negative (Figure 2D). We found that 14% of the 21 C4d-negative kidney sections contained IgM deposits; 7% of the 14 kidney sections with segmental C4d staining contained IgM, and 27% of the 15 kidney sections with global C4d staining contained IgM. Although IgM deposits were more prevalent in the kidney sections with global C4d, this correlation was not significant. In contrast, the presence of IgM was correlated significantly with tram tracking ($p<0.001$).

RELATIONSHIP BETWEEN CLINICAL CHARACTERISTICS AND C4D

Among the 36 samples obtained from the preeclamptic patients and pregnant controls, 10 samples were negative for C4d, 11 samples were C4d-positive with segmental staining, and 15 samples were C4d-positive with global staining. Global C4d deposits were significantly correlated with increased gestational age ($p<0.05$), whereas C4d-negative staining was not correlated significantly with gestational age. Neither the level of proteinuria nor peak blood pressure was correlated with the pattern of C4d staining.

sFlt-1 MOUSE MODEL OF PREECLAMPSIA

Next, we used a sFlt-1 mouse model of preeclampsia[3, 20-22] to study whether sFlt-1–induced endothelial damage is associated with complement activation. As reported previously, injecting sFlt-1 into the tail vein caused a preeclampsia-like phenotype, with significantly elevated blood pressure, urinary albumin secretion, and endotheliosis (a characteristic renal lesion in preeclampsia), which was measured using open capillary volume.[3, 20] The percentage of C4-positive kidneys in the sFlt-1–injected mice was significantly higher than in the control mice (Figure 3). The sFlt-1–injected mice had significantly more C4-positive glomeruli ($p<0.05$; Figure 3C). Confocal immunofluorescence microscopy revealed that the C4 deposits were present on the endothelial cells. This finding was confirmed by studying adjacent sections, in which C4 and CD31 co-localized, indicating complement activation in endothelial cells.

Discussion

The mechanisms that underlie the renal pathology in preeclampsia are poorly understood. Here, we report that the kidney sections from all of the preeclamptic women in our study were positive for C4d deposits. In contrast, C4d deposits were significantly less prevalent in two control groups comprised of non-hypertensive pregnant women and non-pregnant women with chronic hypertension. Importantly, the significant correlation between C4d deposits and the classical complement pathway component C1q in glomeruli strongly suggests that the classical complement pathway was activated. The lack of significant correlation between C4d and MBL, and the absence of properdin staining, makes it extremely unlikely that complement activation is attributed to either the lectin or

alternative complement pathway. The hypothesis that angiogenic dysregulation plays an important role in triggering complement activation in the kidney is supported by our finding of excessive numbers of C4 deposits in the glomeruli of sFlt-1-injected mice, an established model of preeclampsia. Taken together, these findings suggest that preeclampsia is associated with activation of the classical complement pathway in the kidney.

We previously described a relationship between preeclampsia and classical complement pathway activation in the placenta.[5] Thus, both the current study and our previous study raise the question of what drives activation of the classical complement pathway in the setting of preeclampsia. In general, complement imbalance can arise from excessive activation and/or inadequate regulation of the complement system. Excessive complement activation can arise from angiogenic dysregulation, which is believed to cause the initial preeclampsia-related renal injury due to increased sFlt-1 levels preventing vascular endothelial growth factor (VEGF) from maintaining the renal endothelium.[3, 11] We hypothesized that the resulting endothelial damage in the kidney might drive excessive complement activation. In our study, the presence of the IgM isotype was significantly associated with preeclampsia. Although glomerular IgM deposits have been observed in a wide range of renal diseases, the role of these deposits has remained elusive, suggesting that these IgM deposits might not always be involved in the pathogenesis of these particular renal diseases. However, the presence of IgM deposits might have other explanations. First, they could reflect the binding of IgM antibodies to damaged endothelium. Natural IgM-antibodies play a major role in the clearance of damaged cells[24, 25], and they can bind to both hypoxic[26] and apoptotic cells[27, 28] through intracellular antigens that become externalized under these conditions. The binding of IgM antibodies to either hypoxic or apoptotic cells triggers the activation of the complement system.[26-28] Taken together, these studies strongly suggest that the initial endothelial damage —mediated via high

sFlt-1 levels in the kidneys of preeclamptic women—could trigger the binding of IgM antibodies, thereby activating the complement system. Our finding of classical complement pathway components in the glomeruli of women with preeclampsia, combined with excess deposits of C4 on glomerular endothelial cells in our sFlt-1–injected mice, highly support this hypothesis.

Secondly, the presence of IgM antibodies and the activation of the classical complement pathway in the kidneys of preeclamptic women could have resulted from auto-antibodies such as angiotensin II type 1 receptor agonistic antibodies (AT_1-AA),[29, 30] anti-phospholipid auto-antibodies,[31] and/or anti-laminin auto-antibodies.[32] In the context of preeclampsia, complement activation could result from these auto-antibodies binding to glomerular structures or by the deposition of circulating antibody–antigen immune complexes and their subsequent entrapment in renal tissue. In our study, although we observed glomerular immunoglobulins both in preeclamptic patients and in some controls, IgM was the only immunoglobulin isotype that was significantly more prevalent in the patients with preeclampsia. If immunoglobulin deposits had resulted from auto-antibodies, we would have expected to find increased IgG deposits in the kidneys of these women. Therefore, our observations suggest that it is unlikely that the glomerular complement deposits in the kidneys of the preeclamptic women were caused by auto-antibodies.

Inadequate regulation of the complement system may also have caused glomerular complement activation. In the kidney, several complement regulatory proteins are expressed at high levels,[33-35] suggesting the importance of renal complement regulation. In our study, we found no correlation between late complement cascade components and preeclampsia, suggesting that the complement cascade does not become activated—at least to an excessive degree—beyond the level of C3. Complement regulatory mechanisms may be responsible for this phenomenon. However, the association between preeclampsia and mutations in genes that encode complement

regulatory proteins suggests that inadequate complement regulation plays a role in preeclampsia.[7] Indeed, a putative mechanism for inadequate regulation is related to complement regulator factor H, which regulates both the alternative and classical complement pathways,[36] and mutations in factor H have been observed in relation to preeclampsia. Importantly, reduced levels of factor H have been related to angiogenic imbalance within the kidney.[37]

Regardless of which mechanism is responsible for renal complement activation in women with preeclampsia, understanding how complement activation contributes to the clinical manifestations of preeclampsia is essential. Given that complement activation is strongly associated with preeclampsia (and its characteristic angiogenic imbalance), inhibiting complement activation may be a promising therapeutic approach for targeting both the placental and renal manifestations of preeclampsia. Importantly, both proteinuria and the typical renal histological changes have been reversed in mouse models of preeclampsia that were treated using complement inhibitors.[8] In one patient with severe preeclampsia, the terminal complement inhibitor Eculizumab has been used successfully to reduce preeclamptic manifestations and to prolong pregnancy.[9]

PERSPECTIVES

Our study is the first to demonstrate extensive activation of the classical complement pathway in the kidneys of women with preeclampsia. The presence of excessive C4 deposits in our sFlt-1–induced preeclampsia mouse model strongly supports the notion that preeclampsia-related renal complement activation is initiated by endothelial damage. In summary, our results suggest that complement activation might contribute to renal injury in preeclampsia. Moreover, our findings provide evidence that inhibiting the complement system might significantly reduce both the renal and placental manifestations of preeclampsia.

NOVELTY AND SIGNIFICANCE

What is new?

- The kidney sections from all of the preeclamptic women in our study were positive for C4d deposits indicating that preeclampsia is associated with activation of the classical complement pathway in glomeruli.
- The hypothesis that angiogenic dysregulation plays an important role in triggering complement activation in the kidney is supported by our finding of excessive numbers of C4 deposits in the glomeruli of sFlt-1– injected mice, an established model of preeclampsia.

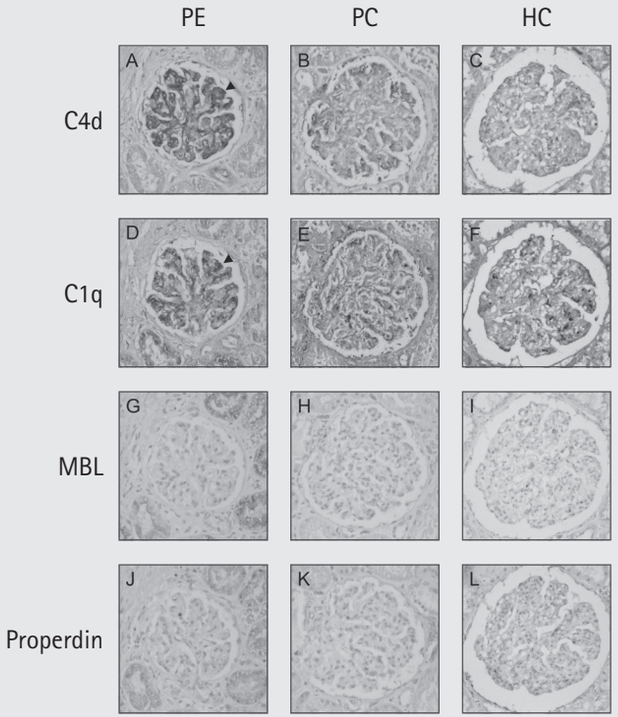
What is relevant?

- Our study suggests that initial endothelial damage —mediated via high sFlt-1 levels in the kidneys of preeclamptic women—could trigger the binding of IgM antibodies, thereby activating the complement system.
- Complement activation might contribute to renal injury in preeclampsia.
- Our findings provide evidence that inhibiting the complement system might significantly reduce the renal manifestations of preeclampsia.

Summary

The strong association between preeclampsia and renal C4d, C1q, and IgM levels suggests that the classical complement pathway plays a role in the pathogenesis of renal injury in preeclampsia. Moreover, our finding that sFlt-1–injected mice develop excess C4 deposits indicates that angiogenic dysregulation may play an important role in complement activation within the kidney. We suggest that inhibiting complement activation may be beneficial for preventing the renal manifestations of preeclampsia.

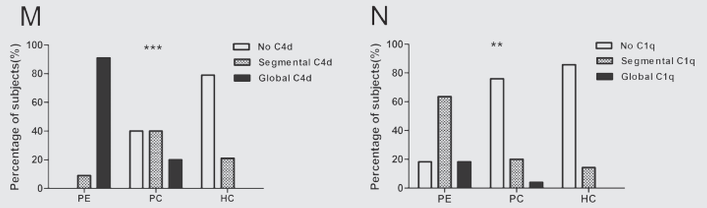
Figure 1



Immunohistochemistry of human kidney sections (full colour version inside cover)

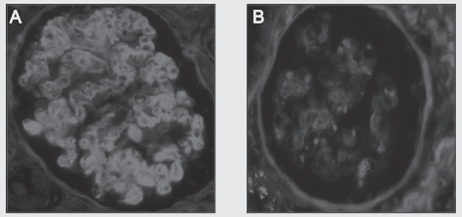
Adjacent sections of glomeruli were immunostained for C4d (A–C), C1q (D–F), mannose-binding lectin (G–I), or properdin (J–L). Each column contains adjacent sections and shows a single glomerulus. The left column shows a glomerulus from a patient with preeclampsia (PE), with global C4d staining. The middle column shows a glomerulus from a pregnant control (PC), with segmental C4d staining. The right column shows a C4d-negative glomerulus from a hypertensive control (HC). C1q staining was present in C4d-positive glomeruli (D) but also in C4d-negative glomeruli. In C4d-positive glomeruli, co-localization of C1q and C4d was observed (A and D). MBL was rarely observed (G-I) and properdin was never observed (J-L). Summary of the prevalence of each C4d (M) and C1q (N) staining pattern in the three groups. Kidneys sections from all preeclamptic patients were positive for C4d, with global staining in the majority of the kidney sections. In contrast, the majority of the pregnant and hypertensive controls showed a segmental or negative C4d staining pattern. Overall comparison revealed that C4d was significantly increased in preeclampsia (p<0.0001).

Figure 1



*Panel N shows the staining patterns for C1q. Overall comparison revealed that C1q was significantly increased in preeclampsia (p<0.01) (N). **p<0.01, ***p<0.0001.*

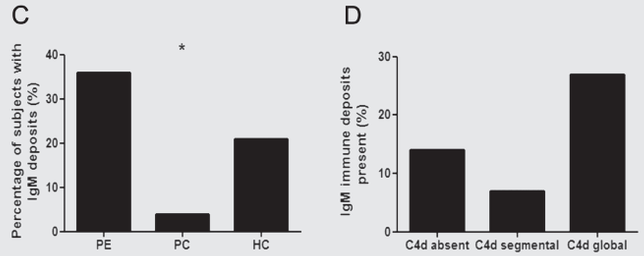
Figure 2AB



Immunofluorescence staining of IgM (full colour version inside cover)

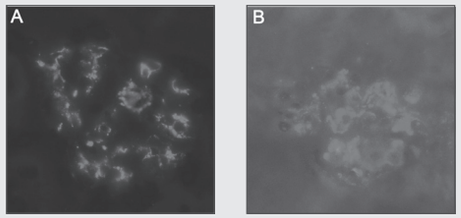
Representative images of an IgM-positive glomerulus (A) and an IgM-negative glomerulus (B). IgM deposits were significantly more prevalent in the kidney sections from the women with preeclampsia compared to the two control groups.

Figure 2CD



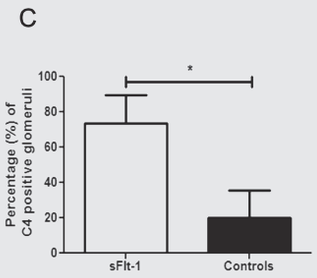
*Distribution of the percentage of IgM-positive sections based on C4d staining pattern (p>0.05) (C). *p<0.05. Distribution of IgM deposits according to C4d staining pattern Complement activation in the kidneys of sFlt-1–injected mice as a model of preeclampsia (D)*

Figure 3AB



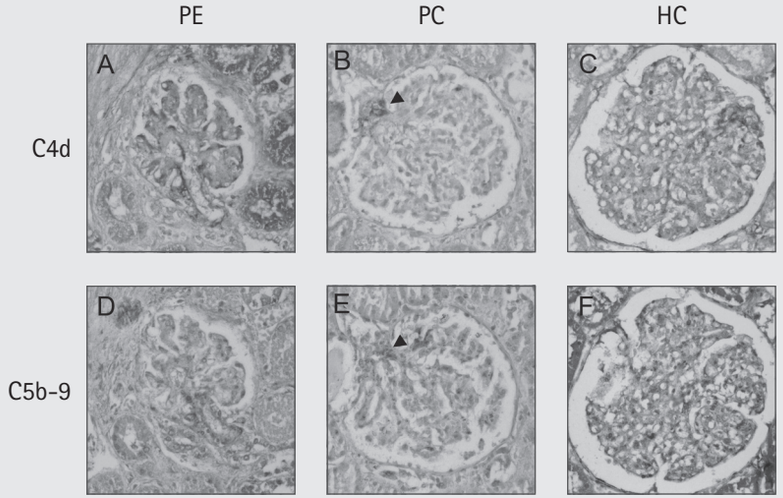
Representative images of C4 deposits in a glomerulus from a sFlt-1–injected (A) and control-injected (B) mouse.

Figure 3C



*Summary of the average (±SD) percentage of C4-positive glomeruli (C) in the kidneys of sFlt-1–injected mice (N=6 mice) and control mice (N=5 mice). *p<0.05.*

Figure S1



Immunohistochemical staining pattern of C3d and C5b-9 in human kidneys

Adjacent sections were immunostained for C3d (A–C) or C5b-9 (D–F). Each column represents an individual glomerulus. The left column shows a glomerulus from a patient with preeclampsia (PE), showing global staining. The middle column shows a glomerulus from a pregnant control (PC), with a segmental staining pattern (arrowhead). The right column shows a glomerulus from a hypertensive control (HC). C3d (A-C) deposits were observed in glomeruli from all study groups whereas C5b-9 (D-F) deposits were infrequently observed. However, C3d does co-localize with C5b-9 (arrowheads).

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IV Association of preeclampsia with podocyte turnover

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Abstract

BACKGROUND AND OBJECTIVES

Preeclampsia is a pregnancy-related complication that causes significant perinatal and maternal morbidity and mortality worldwide. Preeclampsia is characterized by hypertension and proteinuria, and increased shedding of podocytes into the urine is a common finding. This finding raises the question of whether preeclamptic nephropathy involves podocyte damage. We therefore studied podocyte-related changes in a unique sample of renal tissues obtained from women who died of preeclampsia.

DESIGN, SETTING, PARTICIPANTS AND MEASUREMENTS

Using a nationwide database of the Dutch Pathology Registry (PALGA), we identified a cohort of 11 women who died from preeclampsia. Three control groups were also identified and consisted of normotensive women who died during pregnancy (n=25), and non-pregnant controls either with (n=14) or without (n=13) chronic hypertension. Clinical data regarding the pregnant patients were obtained from the National Maternal Mortality Committee of the Dutch Society of Obstetrics and Gynaecology. Renal tissues were obtained. Glomerular lesions, including podocyte numbers, podocyte proliferation, and parietal cell activation were measured and compared between the four groups.

RESULTS

The preeclamptic patients had prominent characteristic glomerular lesions, including endothelial cell swelling, podocyte swelling, and double contours of the glomerular basement membrane. Importantly, we found that the number of podocytes per glomerulus did not differ significantly between the preeclamptic and control groups. However, preeclampsia was associated with a significant increase in intraglomerular cell proliferation (p=0.004) and activated parietal epithelial cells on a podocyte location (p=0.01).

CONCLUSIONS

Our findings suggest that the recently described mechanisms of podocyte replacement play a role in preeclampsia. Our results also suggest that the podocyte plays an important role in preeclamptic nephropathy and subsequent progressive renal damage, including focal and segmental glomerulosclerosis. These findings provide key new insights into the pathogenesis of preeclamptic nephropathy, and they open new possibilities for developing therapeutic modalities.

Introduction

Preeclampsia is a serious pregnancy-related complication that affects up to 8% of all pregnancies, thereby causing significant perinatal and maternal morbidity and mortality worldwide.[1] Preeclampsia is believed to arise–at least in part–from an imbalance between the pro- and antiangiogenic factors in the maternal circulation; the maternal kidney is particularly sensitive to this imbalance, as reflected by the occasional finding of severe proteinuria.[1, 2]

As far back as 1918, the glomerulus was recognized as the principal site of renal damage in preeclampsia.[3] Later, the glomerular podocyte became the focus of attention in relation to proteinuria. Because renal biopsies are rarely performed in pregnant preeclamptic patients, how the podocyte is affected by preeclampsia has remained largely unknown, although endotheliosis is generally considered a characteristic histopathological glomerular lesion in preeclampsia. Recently, Vikse[4] suggested that a previously undetected renal disease might become “overt” in the preeclamptic setting. Preeclampsia is a major risk factor for developing chronic kidney disease - in particular, focal and segmental glomerulosclerosis (FSGS), which is considered primarily a disease of podocytes - later in life. However, how preeclampsia increases the risk of renal disease is poorly understood.

Recently, our group and others reported significantly higher

numbers of podocytes in the urine (i.e. podocyturia) of women with preeclampsia compared to pregnant control subjects.[5, 6] This podocyturia remained in the preeclamptic patients up to one month after delivery, although their proteinuria resolved.[7] Structural changes in the podocyte, including abnormal expression of podocyte-related proteins, were recently reported in a limited number of patients with preeclampsia.[8, 9] These findings suggest that the podocyte plays a key role in preeclampsia. In particular, the increased shedding of podocytes into the urine of preeclamptic patients raises the question of whether the origin of preeclamptic renal disease involves podocyte damage. Moreover, this hypothesis is reminiscent of the podocytopathy that may underlie the subsequent renal deterioration that is seen in FSGS. To investigate the association between preeclampsia and glomerular lesions in general, and podocyte-related injury in particular, we collected a unique sample of renal autopsy tissues obtained from preeclamptic patients and control subjects.

Methods

PATIENT SELECTION AND NATIONWIDE PALGA SEARCH FOR RENAL TISSUE

Autopsy samples were obtained following a nationwide search of the Dutch Pathology Registry (PALGA), a histopathology and cytopathology network and registry that includes all pathology laboratories within the Netherlands.(10) We included pregnant patients who died from preeclampsia as defined by international guidelines established by the International Society for the Study of Hypertension in Pregnancy (ISSHP).[11] Three control groups were also obtained. One control group consisted of pregnant women without a hypertensive disorder either prior to, or during the pregnancy. The two other control groups consisted of non-pregnant young women either with, or without a medical history

of chronic hypertension. We obtained paraffin-embedded kidney samples taken from 11 preeclampsia patients, 25 normotensive pregnant controls, 14 chronic hypertensive non-pregnant controls, and 13 normotensive non-pregnant controls. The patients' clinical characteristics were obtained from their autopsy-reports. The cause of death in each pregnant case was confirmed by reviewing the records of the National Maternal Mortality Committee of the Dutch Society of Obstetrics and Gynecology.[12] A thorough review of the autopsy reports— including kidney weight—confirmed that the control subjects had no evidence of underlying renal disease. All tissues were coded and handled anonymously in accordance with the Dutch National Ethics Guidelines (Code for Proper Secondary Use of Human Tissue, Dutch Federation of Medical Scientific Societies). This study was approved by the ethics committee of the Leiden University Medical Center (P12.107).

HISTOLOGY, IMMUNOHISTOCHEMISTRY, AND IMMUNOFLUORESCENCE

Sections of renal tissue samples were stained with haematoxylin and eosin, periodic acid-Schiff (PAS), silver, and phosphotungstic acid-hematoxylin (PTAH) using standard methods. Immunohistochemistry was used to identify and count podocytes based on staining for WT-1, a podocyte-specific transcription factor.[13] To confirm the origin of swollen endothelial cells, we performed CD31 staining experiments. Because cell proliferation can affect the number of podocytes, we also performed immunohistochemical staining for Ki-67, a marker of cell proliferation.[14] The sections were deparaffinized, and after antigen retrieval, immunohistochemistry was performed. The sections were stained with antibodies against WT-1 (rabbit anti-human polyclonal antibody sc-192 lotnumber D2104, Santa Cruz Biotechnology, 1:250), or CD31 (Dako, 1:400) or Ki-67 (Thermo Fisher Scientific, 1:200), and staining was visualized using the appropriate secondary antibodies and diaminobenzidine

as the chromagen. Finally, the sections were counterstained with hematoxylin.

Double immunofluorescence stainings were performed. For the first double staining, we used an antibody against CD44 (a glycoprotein involved in cell adhesion, cell matrix interactions, and cell migration (Abcam, 1:200)), which is expressed by activated parietal epithelial cells[12] and an antibody against CD45 (a leukocyte common antigen, Ancell 1:800). Staining was visualized using goat anti-mouse Alexa 488 IgG2a and Alexa 546 Ig1. For a second double-staining experiment, we used antibodies against CD44 (Abcam, 1:200) and Ki-67 (Thermo Fisher Scientific, 1:200). Staining was visualized using the goat anti-mouse Alexa 488 IgG2 (1:200) and Alexa 546 Ig1 (1:200) secondary antibodies. We also performed a third double-staining experiment using antibodies against WT-1 (Santa Cruz Biotechnology, 1:250) and CD44 (Abcam, 1:200). WT-1 was visualized using the appropriate secondary antibody and diaminobenzidine as the chromagen, and CD44 was visualized using the FITC-labelled goat anti-mouse IgG antibody (1:200).

QUANTIFICATION OF HISTOLOGY

Sections were examined and scored by an experienced renal pathologist who was blinded with respect to the patients' clinical data. At least 50 glomeruli per section were examined, and the following parameters were scored: presence of endotheliosis, double contours of the glomerular basement membrane (tram tracking), swelling of podocytes, mesangial changes, glomerulitis, and focal and segmental glomerulosclerosis (FSGS). As described by previously Strevens et al[15], endotheliosis was scored semi-quantitatively as follows: 0 (no endotheliosis), 1 (<20% of the lumen was obliterated), 2 (20-80% of the lumen was obliterated), or 3 (>80% of the lumen was obliterated). Global sclerosis was recorded as a percentage of the total number of glomeruli scored. PTAH-confirmed microthrombi, interstitial fibrosis and tubular atrophy (IFTA), and acute tubular necrosis (ATN) were scored as either absent or present. To quantify vessel changes, the presence of hyalinosis and intimal fibrosis of arteries was scored. Finally, signs of ischemia, congestion, and edema were also evaluated.

MORPHOMETRY AND QUANTIFICATION OF IMMUNOHISTOCHEMISTRY AND IMMUNOFLUORESCENCE

Because differences between the groups with respect to glomerular surface areas can affect the absolute number of podocytes, PAS-stained slides were used to measure the surface areas of the glomerular tuft and Bowman's capsule of 30 randomly selected glomeruli (using ImageJ 1.47d software; National Institutes of Health, downloaded from http://rsb.info.nih.gov/ij). For the immunohistochemical and immunofluorescence stained sections, at least 30 glomeruli per section were analyzed by two observers who were blinded with respect to the patients' clinical data. The number of WT-1 positive nuclei per glomerular cross-section was counted using ImageJ 1.47d. Randomly selected regions of the outer renal cortex were analyzed. Because estimates of podocyte number vary widely between studies[16], we measured podocytes in a control group containing non-pregnant, non-hypertensive women as a measure of the number of podocytes per glomerular cross section in healthy adult women. Ki-67 staining was quantified by counting the number of Ki-67-positive cells within the glomeruli (both intraglomerular and lining Bowman's capsule). Finally, the presence of CD44-positive cells in the glomeruli was scored along the inner lining of Bowman's capsule—in an anatomic parietal epithelial cell location—and covering the glomerular basement membrane on a podocyte location (i.e., not counting endothelial cells) as previously described by Fatima *et al*[17]. The podocyte location was confirmed by co-localization between CD44 and WT-1. In addition, the number of cellular bridges (i.e., bridges between Bowman's capsule and the glomerular tuft) was scored in both CD44-stained and silver-stained samples. CD44-positive leukocytes, which were confirmed by co-staining with CD45, were excluded from the scoring analysis.

STATISTICAL ANALYSIS

Categorical variables were compared using the Chi-square test. Differences in quantitative parameters between groups were assessed using either the one-way ANOVA (for normally distributed data) or the non-parametric Kruskal-Wallis test (for non-normally distributed data). Correlations were calculated using either a Spearman's (for ordinal data) or Pearson's (for numerical data) coefficient. All analyses were performed using the SPSS statistical software package (version 20.0; Armonk, NY: IBM Corp). Differences with a *p*-value less than 0.05 were considered statistically significant.

Results

CLINICAL DATA

The clinical characteristics of the four groups are summarized in Table 1. The hypertensive non-pregnant control group was significantly (p=0.03) older than the other study groups. No other significant differences were observed with respect to the other clinical characteristics.

CHARACTERISTIC RENAL HISTOLOGY FINDINGS IN PREECLAMPSIA

The majority (81%) of the women with preeclampsia had prominent glomerular lesions, including various degrees of endotheliosis, swelling of podocytes, and tram tracking. Endotheliosis was present in 55% of the women with preeclampsia. Although endotheliosis is generally considered to be the principal feature of preeclamptic glomerular changes, it was also observed - albeit to a significantly lesser extent (p=0.003) - in both the pregnant control group (12%) and the hypertensive non-pregnant control group (15%). Both tram tracking (p<0.001) and podocyte swelling (p=0.02) were present in the preeclampsia group only. With the exception of one patient with preeclampsia, endotheliosis and tram tracking were not present simultaneously. The presence of podocyte changes was correlated significantly with the presence of endotheliosis (p=0.001). The presence of endotheliosis was not correlated with blood pressure, proteinuria, gestational age, or maternal age. No correlation was found between renal histopathological lesions and gestational age. The hypertensive non-pregnant control subjects had significantly more severe ischemic glomerular lesions than the preeclamptic patients; these and other lesions are summarized in Table 2. Supplemental Figures S1 and S2 show typical examples of renal histology from all four study groups.

MORPHOMETRIC ANALYSES

Glomerular surface areas did not differ significantly between the preeclampsia and control groups. However, the chronic hypertensive non-pregnant control subjects had significantly larger surface areas in the glomerular tuft and Bowman's capsule than the normotensive non-pregnant control subjects (Figure 1A and B). Similar results were obtained when we calculated glomerular volume using the Weibel-Gomez method.[18]

THE PREECLAMPSIA PATIENTS AND CONTROL GROUPS HAVE SIMILAR NUMBERS OF PODOCYTES

No significant difference in the number of podocytes was found between the preeclamptic group and the control groups (Figure 1C). Supplemental Figure S3A shows a typical example of WT-1 staining. WT-1 staining was not correlated with either the renal histopathological lesions or patient characteristics.

PREECLAMPSIA IS ASSOCIATED WITH INCREASED CELLULAR PROLIFERATION

The women with preeclampsia had significantly more intraglomerular Ki-67-positive cells compared to the hypertensive and normotensive non-pregnant control groups (p=0.004; Figure 2A). Furthermore, the women with preeclampsia had significantly more Ki-67-positive parietal epithelial cells than the pregnant controls and the hypertensive non-pregnant control subjects (p=0.02; Figure 2B). Supplemental Figure S3B shows a typical example of Ki-67 staining. Ki-67 and CD44 staining was co-localized (an example is shown in Supplemental Figure S4). Ki-67 staining was not correlated with either the renal histopathological lesions or patient characteristics.

PREECLAMPSIA IS ASSOCIATED WITH AN INCREASED NUMBER OF CD44 POSITIVE PARIETAL CELLS ON A PODOCYTE LOCATION

We scored the presence and location of CD44-positive/ CD45-negative cells within the glomeruli for the four study groups (Figure 3A-C). The podocyte location was confirmed by co-localization of CD44 and WT-1 staining (an example is shown in Figure S5A and B). The number of CD44-positive cells on a podocyte location was significantly higher in the women with preeclampsia than in all three control groups (Figure 3D). Moreover, the presence of CD44-positive cells was significantly associated with the presence of Ki-67-positive intraglomerular cells (p=0.03). Although a trend was observed between preeclampsia and CD44-positive cells on a parietal epithelial cell location, this association did not reach statistical significance (p=0.07; Figure 3E). The number of cellular bridges did not differ significantly between the study groups; however, the presence of cellular bridges was significantly associated with the presence of FSGS (p=0.02). An example of a cellular bridge is shown in Supplemental Figure S6.

Discussion

In our cohort of preeclamptic women, podocyte changes and tram tracking of the glomerular basement membrane were the most typical preeclampsia-associated lesions, occurring in 18% and 36% of patients, respectively; in contrast, none of the patients in our control groups had either of these lesions. Endotheliosis, a lesion that has been previously described as a characteristic of preeclampsia, was present in 55% of the preeclampsia patients, but it was also present – albeit at much lower percentages - in the pregnant controls and the hypertensive non-pregnant controls. We also provide the first report that although the number of glomerular podocytes is unaffected in preeclampsia (as determined by histological evaluation), preeclampsia is characterized by a higher number of activated parietal epithelial cells. A mechanism to explain our findings might come from elegant experiments by Appel et al., who used a rat model for lineage tracing and gene tagging and found that parietal epithelial cells migrate into the glomerular tuft.(19) Moreover, our findings strongly suggest that lost podocytes are replaced by progenitor cells of the parietal epithelium in the context of preeclampsia. Importantly, none of the patients had any indication of underlying renal disease, suggesting that these findings are likely attributable to preeclampsia.

Preeclampsia is characterized by an increase in the shedding of podocytes into the urine,[5-7] and this can occur before the onset of clinical manifestations.[20] Furthermore, the expression of podocyte-specific proteins such as nephrin and GLEPP-1 is significantly lower in kidney biopsies from patients with preeclampsia than from control subjects.[8, 9] Given the increased shedding of podocytes, our current findings that the number of glomerular podocytes remains stable suggests an increased turnover of podocytes in preeclampsia. Podocytes have traditionally been regarded as highly differentiated, non-dividing cells, which would imply that these cells cannot regenerate following podocyte injury and/ or loss. However, a

recent study reported that the parietal epithelial cells that line Bowman's capsule can replace injured and lost podocytes.[19] On the other hand, a recent mouse study reported that proteinuria inhibits the differentiation of parietal epithelial cells into podocytes by sequestering retinoic acid.[21]

Recently, it has been shown that parietal epithelial cells – but not podocytes – upregulate their de novo expression of CD44 (marker of cell migration) following podocyte injury and/ or loss.[22, 23] CD44 can also be expressed by endothelial cells.[24] However, in our study CD44 co-localized with WT-1, and CD44 positivity on a podocyte location was significantly higher in the preeclampsia patients than in the three control groups. Based on the aforementioned study describing parietal epithelial cell migration in a rat model [19], our findings suggest that during preeclampsia, activated parietal epithelial cells can migrate and replace lost podocytes. Additional data to support this mechanism included the increased cell proliferation and the co-localization of Ki-67 and CD44 staining that was observed in the glomeruli of the women with preeclampsia. Together with the observed co-localization between WT-1 and CD44, these findings confirm that the Ki-67-positive cells present on a podocyte location are indeed podocytes.

The replacement of lost podocytes by activated parietal epithelial cells is a compensatory mechanism that - if successful - is accompanied by remodeling the glomerular architecture.[25] However, under certain conditions, this replacement mechanism cannot compensate fully, thereby leading to renal damage that is histologically characterized by focal and segmental glomerulosclerosis. For example, in a mouse model of focal and segmental glomerulosclerosis,[23] an excessive proliferative response of parietal epithelial cells was involved in the progression of sclerotic lesions.[22, 23, 26] There is also evidence that in renal transplants, increased CD44 staining – an indicator of activated parietal epithelial cell - distinguishes early recurrent FSGS (which manifests with podocyte foot process effacement only) from

minimal change disease.[17] These authors speculated that CD44 expression in nephrotic patients without sclerosis has positive value in predicting progressive podocyte damage, including FSGS.[17] In our study, the patients with preeclampsia did not have significantly more FSGS in their kidneys; nevertheless, the significant correlation between FSGS and CD44-positive cellular bridges (connecting Bowman's capsule and the glomerular tuft) supports the previous notion that CD44 positive cells are involved in the formation of sclerotic lesions.[17, 23] The results reported by Fatima et al.[17] provide insight into the implications of our current findings, as women with preeclampsia have a higher risk of developing FSGS later in life[27]; moreover, we found that the preeclamptic women in our study had higher CD44 positivity on a podocyte location compared to the control groups. We therefore speculate that this increased CD44 positivity in preeclamptic kidneys is a sign of progressive podocytopathy and may have predictive value for FSGS in the long run. Our findings within the preeclamptic kidney during pregnancy might also explain the reported increased risk for women with preeclampsia to develop end-stage renal disease later in life.(4, 28) However, it bears mentioning that because we investigated autopsy material, we were unable to analyze any possible correlation between CD44 positivity during pregnancy and the development of sclerotic lesions later in life. The sequence of events and the aforementioned putative mechanisms that underlie podocyte replacement and subsequent podocytopathy during preeclampsia are illustrated in Figure 4.

An important lingering question is what early mechanism causes podocyte injury and loss during preeclampsia. In our study, preeclampsia was characterized by damage to all three layers of the glomerular filtration barrier. Consistent with a previous report, although endotheliosis was observed – albeit relatively rarely - in the pregnant controls and hypertensive non-pregnant controls, endotheliosis was most prevalent among the women with preeclampsia.[15] A dysregulation of—and the resulting imbalance

between—proangiogenic and antiangiogenic factors is believed to cause endotheliosis; in particular, increased levels of the antiangiogenic factor sFlt-1 (soluble Fms-like tyrosine kinase) can prevent vascular endothelial growth factor (VEGF) from maintaining the renal endothelium.[29] Because VEGF is essential for the interaction between endothelial cells and podocytes,[30] dysregulation of VEGF also affects the podocyte. The notion that dysregulation of these factors plays a role in the renal manifestations of preeclampsia is supported by studies showing that endotheliosis is a key feature of glomerular injury in a podocyte-specific VEGF-knockout mouse.[29] The same study also reported that similar lesions were observed in patients who were treated with the VEGF inhibitor bevacizumab.[29] Based on these observations, it is highly likely that angiogenic imbalance plays a causative role in the renal manifestations of preeclampsia. However, preeclampsia may arise from a variety of causative factors other than angiogenic imbalance alone.[31]

In conclusion, building on our previous report that preeclampsia is characterized by increased shedding of podocytes into the urine,[6] we now present the first report that the absolute number of glomerular podocytes is actually unchanged during preeclampsia. This finding might be explained by increased cell proliferation and/or significantly increased numbers of activated parietal epithelial cells, suggesting that these activated cells can migrate to a podocyte location. Our results indicate that podocytopathy plays a central role in preeclamptic nephropathy, thereby contributing – at least in part - to the increased risk of developing FSGS later in life. This notion is consistent with previous reports that podocytopathy plays a role in other forms of FSGS, and it lends credence to speculation that targeting the podocyte may have therapeutic value.[32, 33] Indeed, several studies showed that modulatig podocyte turnover by regulating activation of parietal epithelial cells[34] can be achieved by certain therapies such as ACE inhibitors,[35] and inhibitors of the Notch signalling pathway.[36] Whether the activation of parietal epithelial cells is a mechanism to compensate for ongoing podocyte injury and loss, or whether these cells contribute to glomerular injury - or both - remains to be investigated. Nevertheless, unraveling the mechanisms of podocyte damage and parietal epithelial cell recruitment in the setting of preeclampsia may lead to novel approaches for treating renal injury.

Acknowledgments

The authors would like to thank Floor Luken for her excellent technical support.

Characteristics	PE (n=11)	PC (n=25)	HC (n=14)	NPC (n=13)
Age (years)	32.5 (29.5-35.8)	31.0 (28.3-36.6)	41.8 (34.4-43.5) *	33.5 (24.6-40.0)
Gestational age (weeks)	35.7 (34.1-39.0)	33.4 (16.7-40.0)	NA	NA
Parity (mean (SD))	0.6 (1.0)	0.8 (1.0)	NA	NA
Proteinuria (g/ 24 hours)	0.36 (0.3-6.1)	NA	NA	NA
Blood pressure (mmHg)				
Systolic	160.0 (141.3-191.3) **	125.0 (113.5-137.5)	NA	NA
Diastolic	106.0 (87.5-120.0) ***	90.0 (70.0-90.0)	NA	NA
Antihypertensive therapy (n (%))	4 (36)	NA	4 (100) #	NA
Comorbidities (n (%))				
Sickelcell anemia	0 (0)	1 (4)	0 (0)	0 (0)
WPW syndrome	0 (0)	1 (4)	0 (0)	0 (0)
Asthma	0 (0)	2 (8)	0 (0)	0 (0)
Hyperhomocysteinemia	0 (0)	2 (8)	0 (0)	0 (0)
Hypertension	1 (9)	0 (0)	0 (0)	0 (0)
Epilepsia	1 (9)	0 (0)	0 (0)	0 (0)
Mammacarcinoma	0 (0)	0 (0)	1 (7)	0 (0)
Renal insufficiency	0 (0)	0 (0)	2 (14)	0 (0)
Depression	0 (0)	0 (0)	2 (14)	0 (0)
Obesity	0 (0)	0 (0)	3 (21)	1 (8)
Cause of death (n (%))				
Preeclampsia related	11 (100)	0 (0)	0 (0)	0 (0)
EUG	0 (0)	1 (4)	0 (0)	0 (0)
Thromboembolism	0 (0)	6 (24)	2 (14)	0 (0)
Amniotic fluid embolism	0 (0)	2 (8)	0 (0)	0 (0)
Arrhythmias	0 (0)	1 (4)	0 (0)	0 (0)
Cardiac arrest	0 (0)	1 (4)	2 (14)	0 (0)
Eci	0 (0)	2 (8)	1 (7)	0 (0)
Malignancy	0 (0)	1 (4)	1 (7)	0 (0)
Aortadissection	0 (0)	3 (12)	4 (29)	0 (0)
Infection	0 (0)	5 (20)	1 (7)	0 (0)
Cardiomyopathy	0 (0)	2 (8)	0 (0)	0 (0)
Pheochromocytoma	0 (0)	1 (4)	0 (0)	0 (0)
Cerebral bleeding	0 (0)	0 (0)	3 (21)	0 (0)
Suicide	0 (0)	0 (0)	0 (0)	5 (38)
High energy trauma	0 (0)	0 (0)	0 (0)	7 (54)
Drowning	0 (0)	0 (0)	0 (0)	1 (8)
Death-autopsy interval (hrs)	18.0 (6.0-32.3)	24.0 (20.5-24.0)	24.0 (12.0-48.0)	24.0 (24.0-60.0)

Table 1 (previous page): Patient characteristics

*Data are given as the median (IQR) unless otherwise specified. PE: preeclampsia, PC: normotensive pregnant controls, HC: hypertensive non-pregnant controls, NPC: normotensive non-pregnant controls. NA: Not applicable. SD: standard deviation. * p =0.03, ** p=0.001, *** p=0.006. # data on hypertensive therapy of the hypertensive controls was available from 4 patients.*

Histological parameters	PE (*n*=11)	PC (*n*=25)	HC (*n*=14)	NPC (*n*=13)	*p*-value
ATN (%)	0 (0)	4 (16)	3 (21)	3 (23)	0.41
Congestion (%)	0 (0)	0 (0)	0 (0)	3 (23)	0.007
Endotheliosis (%)	6 (55)	3 (12)	2 (14)	0 (0)	0.003
<20% of the lumen (%)	1 (17)	3 (100)	1 (50)	NA	
20-80% of the lumen (%)	3 (50)	0	1 (50)	NA	
>80% of the lumen (%)	2 (33)	0	0	NA	
FSGS (%)	1 (9)	2 (8)	5 (36)	0 (0)	0.03
Global sclerosis >1% (%)	1 (9)	0 (0)	5 (36)	1 (8)	0.04
Glomerulitis (%)	0 (0)	6 (24)	1 (7)	2 (15)	0.23
Hyalinosis (%)	1 (9)	4 (16)	7 (50)	10 (77)	<0.001
IFTA (%)	0 (0)	0 (0)	2 (14)	0 (0)	0.06
Intima fibrosis (%)	2 (18)	7 (28)	11 (79)	5 (39)	0.006
Ischemia (%)	0 (0)	1 (4)	3 (21)	1 (8)	0.17
Mesangium changes (%)	1 (9)	0 (0)	4 (29)	1 (8)	0.04
Microthrombi (%)	1 (9)	0 (0)	1 (7)	0 (0)	0.20
Edema (%)	1 (9)	0 (0)	0 (0)	1 (8)	0.34
Podocyte changes (%)	2 (18)	0 (0)	0 (0)	0 (0)	0.02
Tram tracking (%)	4 (36)	0 (0)	0 (0)	0 (0)	<0.001

Table 2: Renal histological parameters in the patients and control groups

Values are expressed as number of patients (%). ATN: acute tubular necrosis, FSGS: focal and segmental glomerulosclerosis. All FSGS cases were classified as the "Not otherwise specified" (NOS) variant. IFTA: interstitial fibrosis tubular atrophy. PE: preeclampsia, PC: normotensive pregnant controls, HC: hypertensive non-pregnant controls, NPC: normotensive non-pregnant controls.

Figure 1

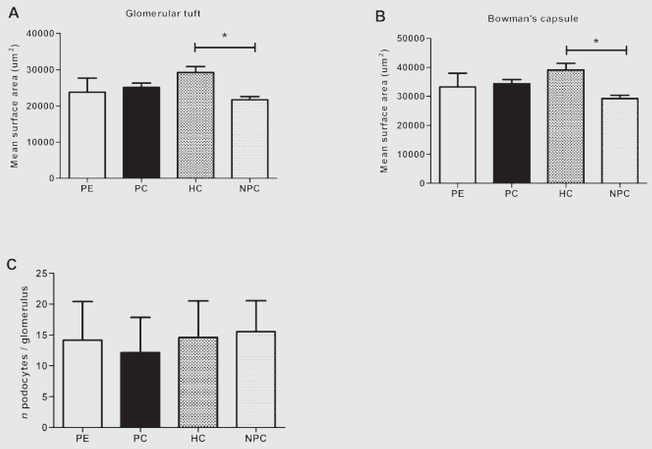


Figure 1: Morphometric analysis and podocyte number

*Glomerular surface areas were calculated for the glomerular tuft (A, * p=0.003) and Bowman's capsule (B, * p=0.002) in the preeclampsia patients and control groups. Panel C summarizes the number of WT-1 positive podocytes per glomerulus in the women with preeclampsia, and the control groups.*

Figure 2

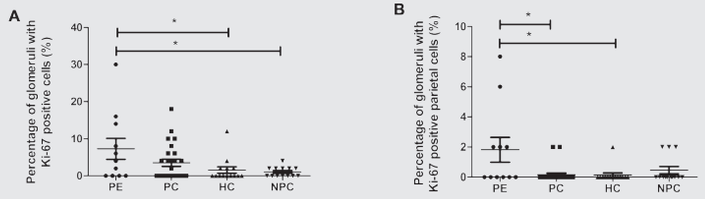
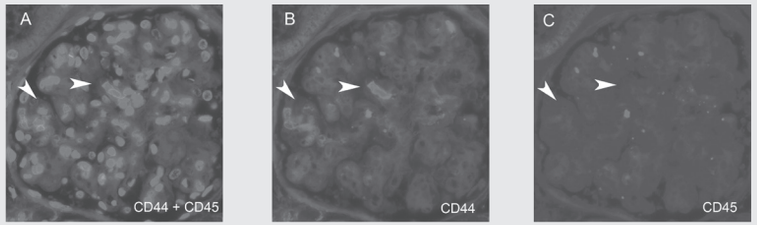


Figure 2: Ki-67 analysis

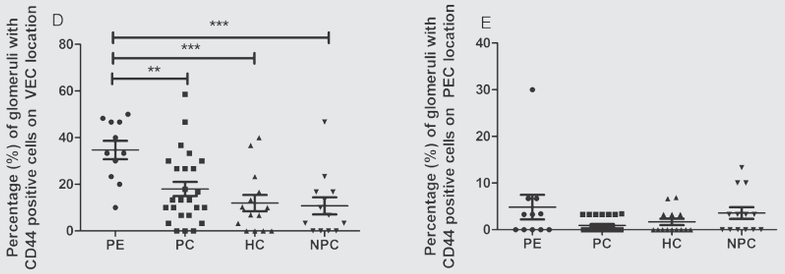
Panel A summarizes the percentage of glomeruli with Ki-67-positive cells (p=0.004), and panel B summarizes the percentage of glomeruli with Ki-67-positive parietal epithelial cells (* p=0.02). In A and B, each symbol represents an individual patient or control.*

Figure 3



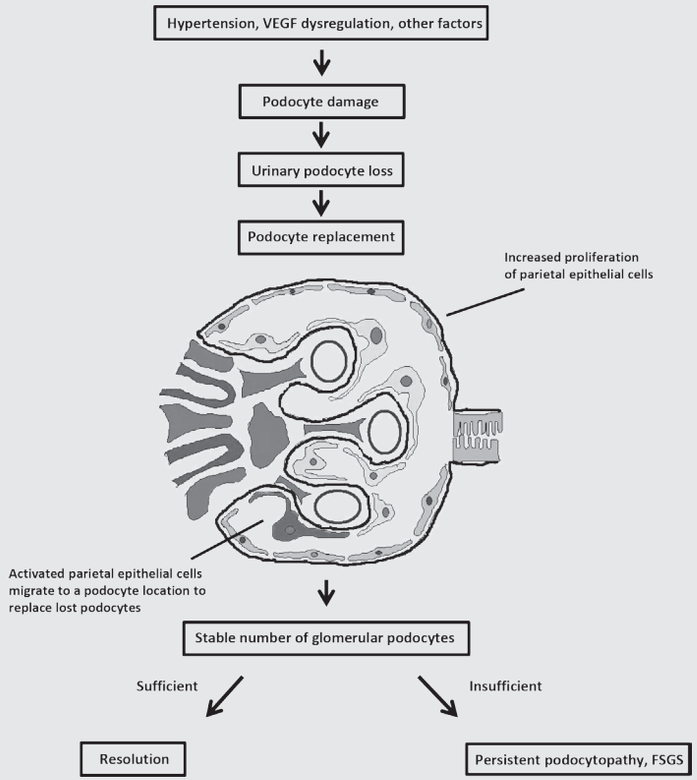
Double staining of CD44 and CD 45 (full colour version inside cover)

Sections were co-stained for CD44 (green) and CD45 (red), and the number of CD44-positive/ CD45-negative cells was scored within the glomeruli. Panel A shows double staining of CD44 positive/ CD45 negative cells on a podocyte location (arrowheads). The nuclei were counterstained with DAPI (blue). Note that the CD44-positive cells (B) are CD45-negative (C).



*Panel D summarizes the number of CD44-positive cells on a podocyte (visceral epithelial cell, VEC) location (** p =0.01, *** p =0.001). Panel E summarizes the number of CD44-positive cells on a parietal epithelial cell location (p=0.07). In D and E, each symbol represents an individual patient or control subject.*

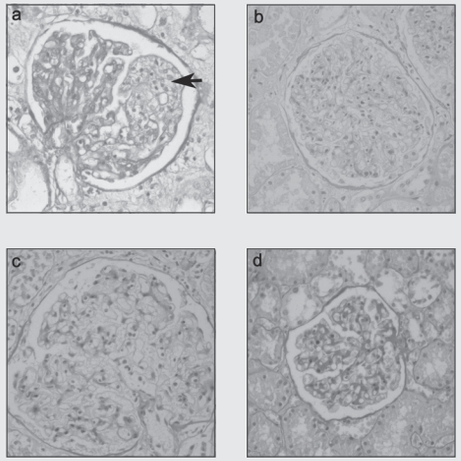
Figure 4



Schematic overview of the putative mechanism underlying podocyte replacement in preeclampsia

Hypertension, dysregulation of VEGF, and other factors cause damage and loss of podocytes during preeclampsia, resulting in increased shedding of podocytes into the urine. In preeclampsia, an increase in turnover of podocytes results from increased proliferation of parietal epithelial cells and higher numbers of activated parietal epithelial cells on a podocyte location. As a result of this increased turnover, the absolute number of glomerular podocytes is stable during preeclampsia. The replacement of lost podocytes by activated parietal epithelial cells is a compensatory mechanism, that – if sufficient – leads to the resolution of clinical symptoms, including proteinuria. However, if compensation is not sufficient, this mechanism can trigger persistent podocytopathy, with progressive proteinuria and renal function loss later in life, histologically characterized by focal and segmental glomerulosclerosis (FSGS).

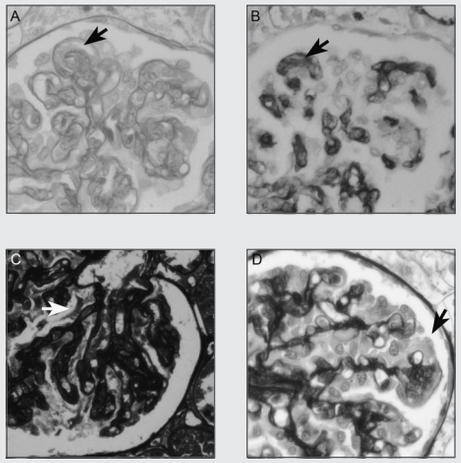
Figure S1



Typical examples of renal histology in preeclampsia and control subjects (full colour version inside cover)

PAS stain showing examples of the various glomerular lesions seen in patients and controls. In preeclamptic patients significantly more endotheliosis (arrow) was observed (a), while the majority of pregnant controls showed no glomerular pathology (b). In hypertensive controls a greater surface area of the glomerular tuft and Bowman's capsule was prominent (c), but non-pregnant controls showed generally normal glomeruli (d).

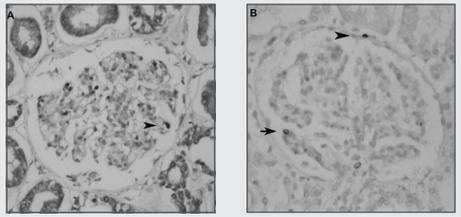
Figure S2



Renal histology in preeclampsia (full colour version inside cover)

This figure shows typical examples of histologic lesions in patients with preeclampsia. In preeclamptic patients significantly more endotheliosis (a, arrow). Note that endotheliosis (a, arrow) consists of endothelial cells, as shown by the positivity for the endothelial marker CD31 (b, arrow). In patient with preeclampsia significantly more tram tracking (c, arrow) was observed than in controls, and podocytes (arrow) were prominently present (d).

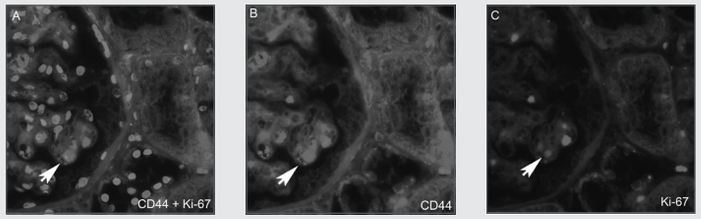
Figure S3



WT-1 and Ki-67 staining (full colour version inside cover)

Panel A shows a typical section with intraglomerular WT-1 positive cells (arrowhead) indicating podocytes. Panel B shows a typical section with Ki-67 positivity, a marker of cell proliferation, in the glomerular tuft (arrow), as well as in parietal epithelial cells (arrowhead).

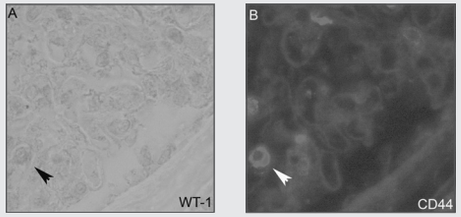
Figure S4



CD44 and Ki-67 staining (full colour version inside cover)

Sections were co-stained for CD44 (green) and Ki-67 (red). Panel A shows double staining of a CD44 positive/ Ki-67 positive cell on a podocyte location (arrow). The nuclei were counterstained with DAPI (blue). Note that the CD44-positive cells shown a membrane staining pattern (B) with Ki-67-positive nuclear staining pattern (C).

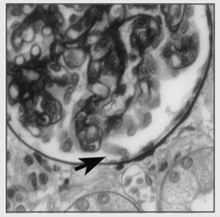
Figure S5



CD-44 positive cells on a podocyte location (full colour version inside cover)

Sections were co-stained for WT-1 (dark grey) and CD44 (green). Note the WT-1-positive cells indicating podocytes (A, an example is indicated by an arrow). In Panel B, a CD44-positive cell (arrow), is also WT-1 positive (A, arrow).

Figure S6



Cellular bridge (full colour version inside cover)

This figure shows an example of a cellular bridge (arrow) in a silver stain.

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V QPCR-based analysis of podocyturia is a feasible diagnostic tool in preeclampsia

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Abstract

Preeclampsia is a significant cause of maternal and fetal morbidity and mortality worldwide. A clinically useful screening test that can predict development of preeclampsia at an early stage is urgently needed. The detection of podocyturia by immunohistochemistry after cell culture has been noted as a reliable marker for preeclampsia. However, this method is laborious and carries the risk of cell-culture contamination. The aim of this study was to investigate the diagnostic value of qPCR as a rapid method to detect preeclampsia.

Clean-catch urine samples were collected from preeclamptic(n=35), healthy pregnant(n=34), and healthy non-pregnant(n=12) women. Furthermore, a control group of women with gestational hypertension(n=5) was included. qPCR analysis was performed for podocyte-specific markers. ROC-curve analyses were performed.

Significantly elevated mRNA levels of nephrin, podocin and VEGF were detected in preeclamptic women compared to healthy pregnant and healthy non-pregnant controls. In addition, significantly elevated levels of nephrin mRNA were detected in urine of preeclamptic women compared to women with gestational hypertension. A positive correlation ($\rho=0.82$, $p<0.0001$) was observed between nephrin and VEGF mRNA levels in preeclamptic women. ROC-curve analyses demonstrated a strong ability of this method to discriminate between the different study groups.

qPCR analysis of podocyte-specific molecules in urine samples is a rapid and reliable method to quantify podocyturia. We demonstrate that this method distinguishes preeclamptic patients from healthy controls and women with gestational hypertension. This method may be a tool for the detection of preeclampsia at an earlier stage, thereby preventing maternal and fetal morbidity and mortality.

Introduction

Preeclampsia affects 2-8% of all pregnancies and is an important cause of maternal and fetal morbidity and mortality worldwide. Preeclampsia is a pregnancy-specific, multisystemic disorder, with the kidney as one of the major target organs. Proteinuria and new-onset hypertension after 20 weeks of gestation are the defining diagnostic criteria for preeclampsia.[1]

Proteinuria arises when there is damage to the glomerular filtration barrier (GFB). The GFB consists of a basement membrane between a layer of fenestrated endothelium and a layer of glomerular visceral epithelial cells, known as podocytes. The histological hallmark of preeclampsia in the kidney is glomerular endotheliosis. Endotheliosis is characterized by the swelling of endothelial cells, enlarged glomerular volume with hypertrophy, and loss of glomerular endothelial fenestrae. Podocyte damage and foot process effacement are visible with electron microscopy.[2]

Although the exact pathophysiologic mechanisms leading to preeclampsia-related proteinuria remain unknown, it is evident that preeclampsia is associated with elevated serum levels of soluble Fms-like tyrosine kinase 1 (sFlt-1) and Endoglin (sEng) and reduced serum levels of Vascular Endothelial Growth Factor-A (VEGF-A).[3-5] VEGF-A regulates angiogenesis in all endothelial beds and is required in the early stages of vascular development. In the kidney, VEGF-A is expressed in tubular epithelial cells and in differentiated podocytes in the glomerulus. It appears that a paracrine/juxtacrine VEGF-signaling pathway is responsible for the cross-talk between endothelial cells and podocytes. It is thought that this signaling is crucial for the formation and maintenance of the glomerular filtration barrier.[6]

There is increasing evidence that podocytes play an important role in the renal manifestations of preeclampsia. It was demonstrated that a podocyte-specific conditional knock-out of VEGF-A in mice results in a loss of podocytes, the induction of

proteinuria, and glomerular abnormalities, such as thrombotic microangiopathy (TMA).[7] This mechanism also appears to be relevant in humans, as was clearly demonstrated by a study in which patients with malignancies were treated with anti-VEGF therapy and developed glomerular endotheliosis, proteinuria and TMA, similar to what is observed in women with preeclampsia.[7]

Based on experimental models, it was suggested that the appearance of podocytes in urine could be a specific marker for glomerular disease. In different *in vivo* models of podocyte damage with transient or continuing injury, evidence was found that urinary excretion of viable podocytes is confined to the phase of active, ongoing glomerular damage, whereas the detection of proteinuria cannot distinguish between ongoing damage and persistent glomerular defects in the barrier's function.[8] Garovic *et al.* showed for the first time that podocyturia as detected by immunohistochemistry on cultured podocytes is a highly sensitive and specific method for the detection of preeclampsia at disease onset.[9] Subsequently, Aita *et al.* showed that podocyturia was still present in patients with preeclampsia one month following delivery, while proteinuria disappeared.[10]

Although prospective studies are still awaited, the publication by Garovic *et al.* holds the promise of a novel, possibly even predictive biomarker for preeclampsia. The method described by Garovic *et al.* has important drawbacks. Cell culture and immunohistochemistry are laborious protocols and carry the risk of cell-culture contamination. We therefore investigated the potential of quantitative polymerase chain reaction (qPCR) to detect the mRNA level of podocyte-specific molecules in urine-samples of women with preeclampsia. This method has been shown to be rapid and accurate in other clinical settings.[11,12] We therefore hypothesized that this technique may be an alternative way to diagnose preeclampsia.

Methods

PATIENTS

A case-control study was conducted to investigate the potential of qPCR-based analysis of podocyturia as a biomarker for preeclampsia. Preeclamptic(n=35), healthy pregnant(n=34) and healthy non-pregnant(n=12) women were included. Furthermore, a control group of women with gestational hypertension(n=5) was included.

According to the ACOG-guideline[13] the definition of preeclampsia is a new-onset hypertension (defined as an arterial systolic blood pressure of >140mm Hg or arterial diastolic blood pressure >90 mmHg) and proteinuria (>300 mg in a 24-hour urine sample) after at least 20 weeks of gestation. Gestational hypertension was defined as new-onset hypertension, but without concurrent proteinuria. Hypertension and/or proteinuria had to be resolved within 12 weeks following delivery. Patients with known hypertension, renal disease or proteinuria before pregnancy were excluded. At the time of urine collection, all patients with preeclampsia were admitted to the Department of Obstetrics of the LUMC. To prevent contamination of the maternal urine with amniotic fluid or fetal urine, urine was collected from patients whose membranes were intact.

Healthy pregnant women were matched with the patients for parity, gestational age (±2 weeks) and age (±5 years). These women had no medical history of preeclampsia, other hypertensive disorders or renal disease. Healthy pregnant women were monitored at the obstetrical outpatient clinic of the LUMC.

Healthy non-pregnant women were residents of the Department of Obstetrics and Gynecology of the LUMC. These women were matched with the healthy pregnant women for parity.

Urine samples were collected following the guidelines of the medical ethical review commission of the LUMC and in accordance with the recent guideline of the Dutch Federation of Scientific Societies. All samples were coded and processed anonymously.

URINE COLLECTION AND CELL ISOLATION

Clean-catch urine samples were obtained and processed within two hours of collection. Urine-samples were transferred to tubes and centrifuged at 1500 rpm for 5 minutes. Centrifuged urine samples were stored at -20°C. Albumin and creatinine levels were measured from these urine samples. Pellets of centrifuged urine samples were washed with phosphate buffered saline (PBS) and centrifuged again at 1500 rpm for 5 minutes. The pellets were suspended in RNA-later and stored at -20°C until RNA isolation.

The RNA was isolated using the Trizol method. Briefly, the cell suspension in RNA-later was centrifuged at 13000 rpm for 2 minutes. Pellets were then dissolved with Trizol and RNA was isolated as previously described.[14]

QPCR

cDNA was generated with AMV reverse transcriptase (20 U/µl) (Roche), according to manufacturer's instructions. For the qPCR reaction iQ™ SYBR® Green supermix was used. Expression of the different podocyte-specific markers was measured using gene-specific primers. The primer sequences used are shown in Table S1. We measured expression of genes that code for proteins that localize to the slit diaphragm of podocyte foot processes (nephrin and podocin). These markers are known to be podocyte-specific. GAPDH was used as a positive control. The levels of VEGF mRNA were also measured. In the kidney, VEGF is expressed in podocytes and in the proximal tubular epithelium. To exclude the possible presence of proximal tubular epithelial cells, mRNA levels of megalin were also measured. mRNA levels detected by qPCR were corrected for creatinine concentration and calculated per ml urine. To investigate whether the podocyte-specific mRNA levels correlated with the number of viable podocytes, mRNA was isolated from six serially diluted samples of immortalized podocytes (kindly provided by M. Saleem).[15]

CELL CULTURE

From a subgroup of patients 5 ml of each urine sample was used to culture the cells on 8-chamber culture slides as described previously.[9,16] The following day the medium was removed, and the slides were fixed with methanol. All eight chambers were incubated with an anti-podocin antibody (kindly provided by Dr. C. Antignac of Hopital Necker, Paris, France) at a dilution of 1:500.[17] The slides were scored blindly for the presence of podocytes. Nucleated, positive-stained cells were considered to be podocytes. Podocyturia is expressed as the ratio of the number of podocytes to the creatinine content.

STATISTICAL METHODS

Descriptive statistics are reported as the means ± SD. Patient characteristics were compared using an unpaired T-test. The mRNA levels of VEGF and GAPDH in the different groups were compared using nonparametric tests (Kruskal-Wallis and Mann-Whitney U-test). All measurements were carried out in duplicate. The mean measurement was used for the statistical analysis. Because there were non-detects for nephrin and podocin (Table S2) censored boxplots were drawn and p-values were calculated using R-package NADA (Nondetects And Data Analysis for environmental data) software, as described previously.[18] Correlations between the expression of the different markers, cultured podocytes and clinical characteristics were calculated using a Spearman's coefficient. Using the receiver operating characteristic (ROC) curve, the area under the curve (AUC), sensitivity and specificity of three markers (VEGF, nephrin and podocin) treated separately and in combination were calculated. All significance tests were two-tailed and conducted at the 0.05 significance level using Graphpad Prism version 5.

Results

PATIENT CHARACTERISTICS

Patient characteristics of the different patient groups are shown in Table 1. As expected, women with preeclampsia had a significantly higher body mass index, elevated systolic and diastolic blood pressures, elevated levels of proteinuria, earlier deliveries and infants with lower birth weights compared to healthy pregnant controls. Women with gestational hypertension had significantly elevated systolic and diastolic blood pressures compared to healthy pregnant controls.

mRNA LEVELS

qPCR was performed for nephrin, VEGF, podocin, GAPDH and megalin. mRNA levels for these proteins were examined in urine-samples of preeclamptic, non-preeclamptic pregnant and non-pregnant control women. In all samples, expression of GAPDH was detectable and there were no differences in the mRNA levels of this gene between the groups.

Significantly elevated levels of podocin ($p<0.001$), nephrin (p=0.01) and VEGF ($p<0.001$) were detected in the urine samples of preeclamptic women compared to healthy pregnant controls (see Figure 1). The mRNA levels of podocin ($p<0.00001$), nephrin ($p<0.001$) and VEGF (p=0.01) were also found to be significantly elevated in samples from preeclamptic women compared to healthy non-pregnant controls. Furthermore, the mRNA levels of podocin ($p<0.01$) and VEGF (p=0.03) were significantly higher in the group of healthy pregnant women than in the healthy non-pregnant controls. For nephrin, the difference between these two groups was not significant. Non-detects (*i.e.* mRNA levels were under the detection limit of the qPCR) were observed for podocin and nephrin (Table S2). No megalin mRNA was detectable in any of the analyzed urine samples (data not shown). Furthermore, an additional control group containing women with gestational hypertension was analyzed using qPCR. Significantly higher levels of nephrin mRNA were detected in the urine from the preeclamptic women than from the women with gestational hypertension ($p<0.05$). In addition, the levels of VEGF and podocin mRNA were increased 9.5-fold and 4.0-fold respectively, in the urine from preeclamptic patients, although these differences did not reach significance (data not shown).

To investigate whether the mRNA levels of podocyte-specific markers correlated with the number of viable podocytes, mRNA was isolated from a serial dilution of a known quantity of immortalized podocytes (kindly provided by M. Saleem). The Spearman's correlation coefficient revealed that the number of podocytes was significantly correlated with nephrin mRNA levels (R=0.98, $p<0.01$) as shown in Figure 2.

To determine whether relative expression levels were different between the various podocyte markers, mRNA levels were correlated with one another. Using Spearman's correlation coefficient, a correlation of 0.82 ($p<0.0001$) between nephrin and VEGF was observed in the preeclamptic women and in the pregnant controls (see Figure 3). Likewise, a Spearman's correlation coefficient was measured to test whether the severity of proteinuria correlated with the mRNA levels of podocyte-specific markers. The albumin/creatinine ratio did not correlate with the mRNA levels of either the combined or separate podocyte-specific markers in the patients with preeclampsia or in the controls.

CELL CULTURE

To determine whether the mRNA levels of podocyte-specific markers were correlated with the number of viable podocytes in patient samples, cells were cultured from urine collected from a subgroup of patients, and each patient's nephrin mRNA level was plotted against the patient's corresponding podocyte count. A Spearman's correlation coefficient revealed that the number of podocytes correlated significantly (R=0.72, $p<0.05$) with the levels of nephrin mRNA.

TEST CHARACTERISTICS: ROC CURVE ANALYSIS

A Receiver Operating Characteristic (ROC) curve analysis was performed. The area under the curve (AUC), sensitivity and specificity for the three markers (VEGF, nephrin and podocin) separately (see Table 2) and combined (see Table 2 and Figure 4) were calculated to discriminate between the different groups. The three markers combined had a higher overall value for the different calculated characteristics than when the markers were treated separately. For the group of preeclamptic women compared to healthy pregnant controls, the AUC for the three markers combined was 0.82, with sensitivity and specificity values of 68.6 and 88.2, respectively. The test characteristics for the group of women with preeclampsia compared to healthy non-pregnant women, again combining the three markers, were as follows: the AUC was 0.99, the sensitivity 94.3 and the specificity 91.7. All of these results are summarized in Table 2.

Discussion

To our knowledge, this is the first study to describe the use of qPCR to quantify podocyturia in the context of preeclampsia. In the present study, significantly elevated levels of nephrin, podocin and VEGF mRNA were detected in the urine of preeclamptic women as compared to levels in healthy pregnant controls and healthy non-pregnant controls. Significantly higher levels of nephrin mRNA were detected in urine from preeclamptic women than in urine from women with gestational hypertension.

In addition, we observed significantly elevated mRNA levels of VEGF and podocin in the urine of healthy pregnant controls as compared to healthy non-pregnant controls. Furthermore, the AUC, sensitivity and specificity for the three markers (nephrin, podocin and VEGF) treated separately and in combination indicate that this method is capable of discriminating between preeclamptic and non-preeclamptic women. Taken together, qPCR-based analysis of podocyturia is a highly promising, non-invasive method for detecting preeclampsia.

Despite decades of research into preeclampsia, predicting which women are at increased risk of developing this condition remains problematic. Many studies have attempted to identify a predictive biomarker. Numerous molecules in serum of patients with preeclampsia, such as sFlt-1, VEGF, sEng, Placental Growth Factor, P-Selectin, and Placental Protein 13, have been proposed as candidates for early detection of preeclampsia. However, no single molecule has proven to be reliable as a predictive tool.[19]

Our observation that mRNA levels of podocin, nephrin and VEGF are significantly elevated in the urine of preeclamptic women compared to those in non-preeclamptic patients is in agreement with the results of Garovic *et al.*[9] In this case-control study, the authors report an impressive correlation between podocyturia detected by immunologic staining of podocyte-specific markers and the presence of preeclampsia. Podocyturia was a highly sensitive and specific (both 100%) predictor and the correlation with the condition was stronger than for any of the known angiogenic factors. Although our sensitivity and specificity values are not 100%, our confirmation of the correlation between podocyturia and preeclampsia as described by Garovic *et al.* is an important step for the use of podocyturia as a potential early biomarker for preeclampsia.

There are also several interesting differences between our data and the results of Garovic *et al.* that bear mentioning. In normotensive women, and in women with either hypertension or proteinuria but without preeclampsia Garovic *et al.* did not observe podocyturia as measured using a urinary podocin-staining protocol. In contrast with these results, our qPCR-based analysis revealed the expression of podocyte-specific molecules in the urine of both pregnant and non-pregnant healthy women. This finding indicates that in normal pregnancy - and even in non-pregnant states - there is loss of podocytes in the urine that can be detected using qPCR.

Interestingly, these results are in agreement with the most recent study by Garovic *et al*., which illustrated that podocyturia, shown by the identification of a podocyte-specific peptide using mass spectrometry, was measurable in women with a normal pregnancy and was further increased in preeclampsia.[16]

Furthermore, we found that the mRNA levels of the podocyte-specific markers were lower in women with gestational hypertension than in preeclamptic patients, with nephrin being the most prominently reduced ($p<0.05$). These results suggest that measuring the expression of podocyte-specific markers can distinguish between gestational hypertension and preeclampsia.

The ultimate goal of qPCR analysis of podocyturia is the detection of pre-clinical stages of preeclampsia prior to the occurrence of proteinuria, thereby identifying women at risk of developing the disease. In this stage, it is likely that the number of podocytes in the urine is relatively low. Therefore, a sensitive test is needed to detect small differences in the quantity of podocytes. In this context, qPCR analysis is a promising method.

One of the challenges in the current study was to ensure both the quality and quantity of the RNA isolated from urinary cells. To minimize the possibility that differences in mRNA levels were caused by variations in the processing methods, every urine sample was handled by following to a strict protocol. Because the RIN (RNA Integrity number) showed that the RNA in most samples was partially degraded, we used random primers to synthesize the cDNA. In addition, specific PCR primers were chosen to generate small amplification fragments. Although some samples were negative (non-detects) for nephrin and/or podocin (Table S2), the housekeeping gene GAPDH was detected in every sample, and its expression levels were similar among all study groups. This result suggests that the quality of RNA isolated from urinary cells was sufficient for amplification. Therefore, the possibility of non-detects being attributable to RNA degradation seems negligible.

The method used by Garovic *et al*. has several disadvantages

compared to the method described in the present study. Cell culturing followed by immunostaining of urinary podocytes carries the risk of contamination during cell culture. Moreover, this method is relatively time-consuming (a test result can be produced in two days at the earliest), whereas the detection of podocyturia using qPCR can be performed within four hours.

An important issue is the choice of the markers used in the experiments. Podocin and nephrin are proteins that are expressed in the slit-diaphragm of the podocyte foot process, and can therefore be used as podocyte-specific markers.[20,21] VEGF was chosen as an additional marker because its expression is known to be high in podocytes.[22] As VEGF is also expressed in the proximal tubular epithelium, we examined the expression of megalin, which is expressed on tubular epithelial cells only. Because we could not detect megalin mRNA in our samples, the observed VEGF mRNA was likely derived from podocytes.

We found a significant correlation ($p<0.0001$) between the mRNA levels of VEGF and nephrin, indicating that the mRNA levels are a representative marker for the number of podocytes in the urine. Although preeclampsia has been associated with reduced VEGF signaling, the increased VEGF mRNA levels measured in our study seem to reflect the high abundance of podocytes in the urine of women with preeclampsia. Previous work performed by Baelde *et al.* demonstrated a strong correlation between the mRNA levels of nephrin and podocin and the number of podocytes as measured by WT-1 staining (a podocyte-specific stain) in kidney biopsies.[23]

Importantly, it is difficult to discriminate between viable and apoptotic podocytes using qPCR analysis of urinary sediments. However, our finding that the number of cultured, viable podocytes correlates strongly with the expression levels of podocyte-specific markers suggests that measuring the expression of these markers provides a reliable estimate of the number of viable podocytes. However, based on this experiment, although we cannot exclude the possibility that qPCR may also detect mRNA from apoptotic cells,

our results nevertheless show that mRNA levels increase with an increase in the number of viable podocytes.

An interesting observation in the present study was the significant difference in the mRNA levels of VEGF and podocin in healthy pregnant controls compared to levels in healthy non-pregnant women. This finding suggests a physiologic shedding of podocytes during normal pregnancy. Previous studies show that preeclampsia is associated with a maternal systemic inflammatory response (MSIR).[24,25] Interestingly, a milder MSIR is also seen during normal pregnancy. It is proposed that this MSIR only leads to the clinical symptoms of preeclampsia when one or more maternal systems decompensate. According to this model, the increased podocyturia seen in healthy pregnant controls may be the result of endothelial dysfunction due to the physiologic MSIR, whereas the further increase of podocyturia seen in preeclamptic women resembles the dysregulation and decompensation of one or more maternal systems.

The present study has two primary limitations. First, we measured mRNA levels in the urine of women with preeclampsia, gestational hypertension and healthy pregnant controls. Previous studies have reported significant higher levels of podocyte-specific mRNA detected using qPCR in patients with Diarrhea+ Hemolytic Uremic Syndrome (D+ HUS)[11], IgA nephropathy[26] and diabetic nephropathy[26,27] relative to healthy controls. Therefore, it is difficult to rely solely on podocyturia to differentiate between preeclampsia and other underlying renal diseases.

Secondly, because we used a case-control study design, it is impossible to calculate the predictive value of our qPCR analyses. A previous study suggested that podocyturia might be more sensitive than proteinuria as a marker of glomerular disease severity.[8] Interestingly, in the present study, the albumin/creatinine ratio was not correlated with the mRNA levels of podocyte-specific markers in patients with preeclampsia. Aita *et al.* reported that podocyturia can be present in patients with preeclampsia even after delivery, whereas proteinuria has disappeared by this stage.[10] In addition, several other studies have suggested the potential use of podocyturia for detecting ongoing glomerular damage.[28,29]

In conclusion, a prospective study is needed to assess whether podocyturia at an early stage is a more reliable marker for preeclampsia than conventional biomarkers, such as proteinuria.

PERSPECTIVES

We have shown for the first time that qPCR-based analysis of podocyte-specific molecules in urine samples is a rapid and sensitive method for quantifying podocyturia in patients with preeclampsia. Our current results are promising and qPCR-detected podocyturia may be a useful diagnostic tool to detect preeclampsia during the early stages of pregnancy. A prospective study should assess whether this test can predict which women are predisposed to preeclampsia. If so, this would be an important, much-needed step toward improving the diagnosis of preeclampsia and possibly toward therapies for the prevention of maternal and fetal morbidity and mortality worldwide.

NOVELTY AND SIGNIFICANCE

What is New?

qPCR-based analysis of podocyte-specific molecules in urine samples for quantifying podocyturia in patients with preeclampsia.

What is Relevant?

- Preeclampsia is a common pregnancy-specific hypertensive disorder with serious maternal and fetal morbidity and mortality.
- A clinically useful screening test that can predict the development of preeclampsia at an early stage is urgently needed to prevent fetal and maternal morbidity and mortality worldwide.

Summary

We have shown that qPCR-based analysis of podocyte-specific molecules in urine samples is a rapid and sensitive method for quantifying podocyturia in patients with preeclampsia.

Acknowledgments

We like to thank Clara Kolster-Bijdevaate and Marjolein Verhart for all their help with patient inclusion and collection of urine samples.

Characteristics	PE (n=35)	PC (n=34)	GH (n=5)	NPC (n=12)
Maternal age (years)	31.80 ± 5.49	31.49 ± 5.83	30.00 ± 2.34	32.17 ± 5.98
Parity	0.8 ± 0.93	0.77 ± 0.84	0.40 ± 0.55	0.75 ± 0.87
Body Mass Index	29.15 ± 9.62*	23.31 ± 3.21	26.47 ± 2.78	-
Blood pressure				
Systolic (mmHg)	145.3 ± 24.9*	114.86 ± 9.66	142.00 ± 9.75 *	-
Diastolic (mmHg)	105.6 ± 19*	69.0 ± 6.59	95.00 ± 6.12 *	-
Albumin/creatininee ratio	123.6*	0.6	Negative	-
Gestational age (weeks)				
at time of urine collection	31.46 ± 3.35	31.22 ± 3.73	36.17 ± 2.45	-
at time of delivery	32.7 ± 3.3*	39.8 ± 1.34	37.54 ± 0.80	-
Birth weight infants (g)	1716.5 ± 839.5*	3530.8 ± 518.0	2631.20 ± 673.93	-

Table 1: Patient characteristics

*Data are given as the mean ± SD. * Denotes a statistically significant difference (p<0,01) compared to the healthy controls. PE: preeclampsia, PC: pregnant control, GH: gestational hypertension, NPC: non-pregnant control.*

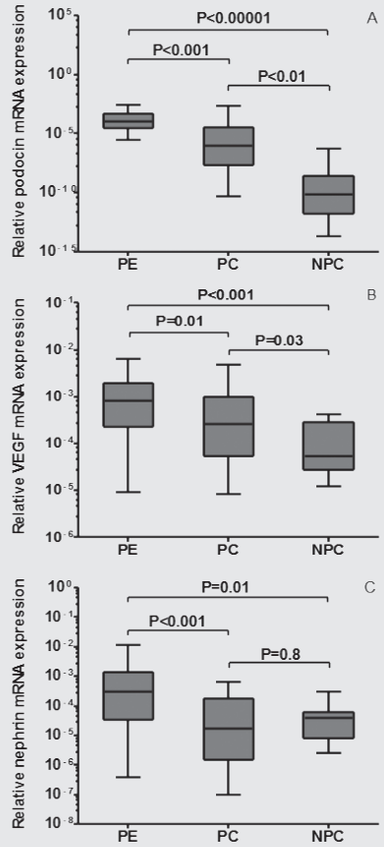
Table 2

Markers	AUC	Sensitivity	Specificity
1. PE vs. PC			
Podocin	0.69	37.1	88.2
VEGF	0.68	48.6	73.5
Nephrin	0.76	51.4	79.4
Combined	**0.82**	**68.6**	**88.2**
2. PE vs. NPC			
Podocin	0.85	74.3	100
VEGF	0.87	82.9	66.7
Nephrin	0.77	100	0
Combined	**0.99**	**94.3**	**91.7**
3. PC vs. NPC			
Podocin	0.76	100	0.0
VEGF	0.71	100	0.0
Nephrin	0.50	100	0.0
Combined	**0.85**	**85.3**	**58.3**

Test characteristics

In this table, the Area Under the Curve (AUC), sensitivity and specificity for the three markers (VEGF, nephrin and podocin) treated separately and in combination are shown. The test characteristics are calculated for the different groups. PE: preeclampsia, PC: pregnant control, NPC: non-pregnant control.

Figure 1

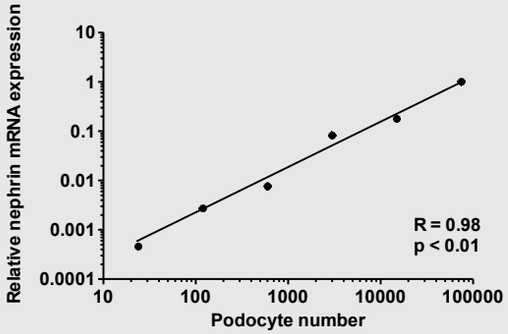


Boxplots for VEGF, nephrin and podocin

This figure shows the boxplots for podocin (A), VEGF (B) and nephrin (C) mRNA levels. For podocin and nephrin, censored boxplots are shown. A regular boxplot was drawn for VEGF. The detection limits for podocin and nephrin were $4.95 * 10^{-8}$ and $3.83 * 10^{-7}$, respectively.

For podocin and VEGF, the calculated differences between the different groups were found to be statistically significant. Differences in nephrin mRNA levels were found to be statistically significant between the examined groups, except for levels between the healthy pregnant controls and the healthy non-pregnant controls. A logarithmic scale was used for all panels. PE: preeclamptic patients, PC: pregnant controls, NPC: non-pregnant controls.

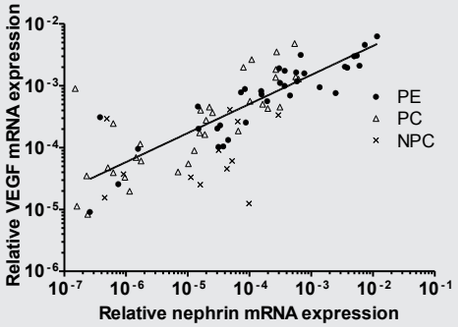
Figure 2



Correlation between number of immortalized podocytes and nephrin mRNA level

In this figure, the statistically significant correlation between the number of immortalized podocytes in six samples and nephrin mRNA level is shown. An R-value of 0.98 (p<0.01) was calculated using a Spearman's coefficient.

Figure 3



Correlation between nephrin and VEGF mRNA expression levels

In this figure, the statistically significant correlation between nephrin and VEGF mRNA levels is shown. An R-value of 0.82 (p<0.0001) was calculated using a Spearman's coefficient. Logarithmic scales are used for both axes. PE: preeclamptic patients, PC: pregnant controls, NPC: non-pregnant controls.

Figure 4

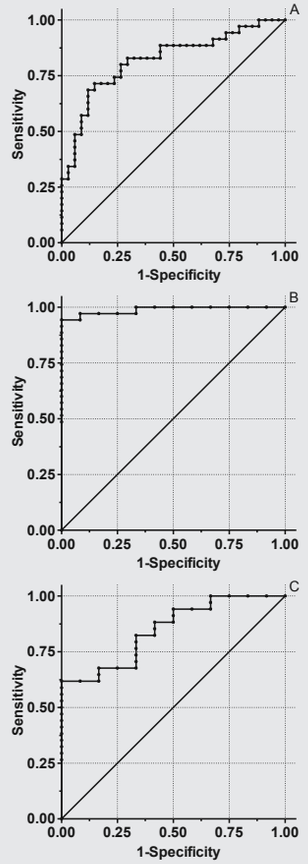


Figure 4: ROC curves

In this figure, the ROC curves for the three combined markers (VEGF, nephrin and podocin) are shown. In panel A, the ROC curve is shown for the preeclamptic group compared to the healthy pregnant controls. The calculated AUC was 0.82. Panel B shows the ROC curve for the preeclamptic group compared to the healthy non-pregnant women. Here, the AUC was 0.99. Panel C shows the ROC curve for the healthy pregnant women compared to the healthy non-pregnant women. The AUC for this analysis was 0.85.

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VI Using first-trimester urinary metabolomics profiling to identify markers of preeclampsia

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Submitted

Abstract

INTRODUCTION

Preeclampsia is a severe pregnancy complication with high maternal and fetal morbidity and mortality rates. The pathophysiology of preeclampsia is poorly understood, and no viable screening test exists. We therefore evaluated whether urinary metabolomics can be used to identify predictive biomarkers of preeclampsia in the first trimester.

METHODS

Urine samples were obtained from pregnant women between 11 weeks 0 days and 13 weeks 6 days of gestation. We performed a nested case-control study that included 73 women who later developed preeclampsia and 138 control subjects who were matched for Body Mass Index and gestational period at the time of sample collection. Urine samples were analyzed using Proton Nuclear Magnetic Resonance (^{1}H-NMR), and the concentrations of 36 metabolites were compared between the two groups. Because ethnicity appeared to be a strong confounding factor, a stratified analysis was performed for the two largest ethnic subgroups, Caucasians and Blacks.

RESULTS

In the first trimester, the Caucasian patients who later developed preeclampsia had significantly different concentrations of 18 metabolites compared to the control Caucasian subjects. In contrast, the Black patients who later developed preeclampsia had significantly different concentrations of three metabolites compared to the control Black subjects.

DISCUSSION

This is the first study to use urinary metabolomics in an attempt to identify markers for predicting preeclampsia early in pregnancy. Our finding of differential metabolite concentrations in urine samples obtained before the onset of preeclampsia may facilitate early disease prediction and provide insight into the pathogenesis of preeclampsia.

Introduction

Preeclampsia is a severe pregnancy-related condition characterized by hypertension and proteinuria after gestational week 20.[1] Preeclampsia is associated with high maternal morbidity and mortality rates,[2, 3] and fetal outcome is often compromised by a combination of intra-uterine growth restriction and preterm delivery.[1] Although the pathophysiology of preeclampsia is poorly understood, evidence suggests that the disease results from a complex interaction between poor placental perfusion—as a consequence of ineffective remodeling of the spiral arteries in early pregnancy—and a maternal response to placenta-derived components causing systemic endothelial dysfunction.[1, 4]

Currently available models for predicting preeclampsia depend upon a combination of maternal factors (including demographics, medical history, and obstetric history) and clinical tests (including booking blood pressure and uterine artery Doppler velocimetry).[5, 6] However, the development of high-end analytical technologies in the past two decades has opened new possibilities for using exploratory, hypothesis-free approaches to screen for new markers of preeclampsia. Metabolomics—and in particular metabolomics of bodily fluids—is one such promising approach.[7] Metabolomics is a discipline that arose from the explosive development of analytical sciences and a re-discovery of the holistic nature of biology.[7] The most promising aspect with respect to the medical sciences is the ability to provide a methodological framework for exploratory

studies, thereby generating new ideas and facilitating the discovery of novel associations between disease pathogenesis and the metabolic composition of bio-fluids.[7]

In the setting of preeclampsia, metabolomics analysis has been performed using plasma samples.[8-11] Recently, a pilot study measured urinary metabolomics in patients with preeclampsia at gestational week 36.[12] Changes in the plasma metabolome are reflected in the urine; importantly, urine samples are relatively easy to obtain and are usually free of proteins and lipids.[13] Moreover, urinary metabolomics has been used successfully to study other diseases.[14, 15] Here, we present the first study that uses urinary metabolomics to identify first-trimester biomarkers for predicting preeclampsia.

Methods

PATIENTS AND URINE COLLECTION

This nested case-control study is part of a larger prospective study currently being conducted by the Fetal Medicine Foundation in, London, UK to predict important fetal and obstetric disorders in the first trimester. The long-term objective of the project is to develop and evaluate existing and newly identified biomarkers that can be used to predict preeclampsia. The details of the larger study have been described previously.[8, 9] Pregnant women residing in the London area were prospectively screened from March 2006 through September 2009. Each participant provided written informed consent, and the study was approved by the King's College Hospital Research Ethics Committee. In brief, pregnant women at 11 weeks 0 days through 13 weeks 6 days gestation were recruited. Maternal characteristics and medical history were documented, and first-trimester ultrasound was performed, including crown-rump length (CRL) and uterine artery Doppler pulsatility index, was performed. Maternal urine samples were obtained and stored at -80°C for subsequent laboratory analysis. The study cohort consisted of 80 single-fetus pregnancies that subsequently developed preeclampsia and a matched group of 160 control subjects who did not develop preeclampsia. Each preeclamptic patient was matched for both Body Mass Index (BMI) and maternal age with two control subjects who delivered a healthy, full-term neonate with appropriate birth weight; in addition, the control subjects did not develop a hypertensive disorder of pregnancy, and they each provided a urine sample within three days of assessment of their matched preeclampsia case. Preeclampsia was defined based on the International Society for the Study of Hypertension in Pregnancy.[16] No HELLP syndrome or gestational hypertension cases were included.

NUCLEAR MAGNETIC RESONANCE (NMR) SAMPLE PREPARATION

Samples were thawed, centrifuged at 3,000´*g* for 5 min at 4°C to remove any precipitates, then transferred into deep 96-well plates. For sample preparation, 540 µl urine was mixed with 60 µl of pH 7.4 buffer (1.5 M K_2HPO_4 in D_2O) containing 4 mM sodium 3-trimethylsilyl-tetra-deuteriopropionate (TSP) and 2 mM NaN_3. The samples were mixed and transferred to NMR tubes using two Gilson 215 liquid handlers controlled by a Bruker Sample Track LIMS system (Bruker BioSpin, Karlsruhe, Germany).

NMR SPECTROSCOPY

^{1}H NMR data were collected at 27°C using a Bruker 600 MHz AVANCE II spectrometer equipped with a 5 mm TCI cryogenic probe head and a z-gradient system. After automatic tuning and matching of the probe head, 90° pulses were automatically calibrated for each individual sample using a homonuclear-gated nutation experiment[17] on the locked and shimmed samples. One-dimensional (1D) ^{1}H-NMR spectra were recorded using the first increment of a NOESY pulse sequence with presaturation (γB_1 = 50 Hz) during a relaxation delay of 4 seconds, with a mixing time of 10 msec to ensure efficient

water suppression.[18] Sixteen scans of 65,536 points covering 12,335 Hz were recorded and zero-filled to 65,536 complex points. Prior to Fourier Transformation (FT), an exponential window function was applied with a line-broadening factor of 1.0 Hz. The spectra were automatically phased, baseline corrected, and referenced to the internal standard (TSP ; δ 0.0). 2D *J* resolved (*JRES*)[19] spectra with presaturation (γB_1 = 50 Hz) were acquired with 12,288 data points over a spectral width of 10,000 Hz (F2 dimension), performing two scans for each of the 40 increments. Pre-FT data were weighted in both dimensions using a sine-bell function, and the spectra were tilted by 45° to provide orthogonal chemical shift (F2) and coupling constant (F1) axes; the spectra were subsequently symmetrized about the F1 axis. The chemical shift scale was referenced to an internal standard (TSP; δ 0.0). 2D-NMR spectra (^{1}H-^{1}H COSY, ^{1}H-^{1}H TOCSY and ^{1}H-^{13}C HSQC-DEPT135) were also recorded for selected samples and were used for spectra annotation.

DATA ANALYSIS

The metabolites were annotated using reference spectra from the Bruker Bioref database and an in-house reference data set. The identities of the metabolites were confirmed using Statistical Total Correlation SpectroscopY (STOCSY) method.[20] The identified metabolites were quantified using deconvolution and subsequent integration of their resonance via in-house automation routine. Absolute concentrations were calculated using TSP as an internal reference. Metabolite concentrations were normalized to creatinine and log-transformed. Principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) were performed to identify trends in the data using SIMCA software. The Mann-Whitney U test was used to compare the metabolite concentrations between the study groups. Differences with a *p*-value <0.05 were considered statistically significant.

Results

CLINICAL CHARACTERISTICS

The clinical characteristics of the preeclamptic patients and control subjects are summarized in Table 1. After excluding some samples because of insufficient sample volume or analytical failure, ^{1}H-NMR spectra of the urine samples from 73 patients with preeclampsia and 138 control subjects were included in the final analysis. The two study groups differed significantly with respect to ethnicity and birth weight.

MULTIVARIATE ANALYSIS

To investigate the primary sources of variance within our data, we subjected the entire data set to principal component analysis (PCA). The score plot of the PCA model (29 components explaining 66% of the variability; see Figure 1A) shows that the differences between the preeclampsia patients and control subjects are not reflected by the first two principal components (which account for approximately 18% of the total variance). Moreover, an attempt to build a regression model with diagnosis as class identity—preeclampsia versus non-preeclampsia—resulted in a poor, unstable model (data not shown). One possible explanation for this observation is that a strong confounding factor may have influenced the data. Because ethnic background differed significantly between the two groups, ethnicity may be one such confounding factor. To test this possibility, we re-plotted the data in Figure 1A according to each subject's ethnic background (Figure 1B). The two largest ethnic subgroups in our cohort—Caucasians (i.e., people of European descent) and Blacks (i.e., people of African descent)—influenced the data significantly. Because the remaining ethnic subgroups—South Asian, East Asian, and Mixed ethnicity—comprised only 10% of the entire cohort, we performed the subsequent analyses using the Caucasian (*n*=119) and Black (*n*=69)

subgroups only. A PCA model based on this subset of the data (25 components accounting for 66% of the variance) shows that the differences between these two major ethnic groups are represented in the first two principal components (Figure 2A). Thereafter, using a supervised analysis, we built a two class PLS-DA regression model using ethnicity as class identity (Figure 2B). This approach yielded a robust model with a cumulative explained variance (RY2) of 0.828 and predictive fraction (Q2Y) of 0.498.

Given the abovementioned results, ethnicity is likely the confounding factor that obscured the differences in urinary metabolic profiles between our preeclampsia patients and control subjects. Therefore, we examined the differences between the Caucasian preeclampsia patients and Caucasian control subjects, and between the Black preeclampsia patients and Black control subjects. However, multivariate modeling of these data did not yield a statistically sound model (data not shown).

Next, we explored whether ethnicity was an equally strong confounder among the control subjects and/or among the patients with preeclampsia. In the control group, a two class PLS-DA regression model with ethnicity as a class identity yielded a strong, statistically valid model. However, we did not obtain a valid model when we applied the same approach to the preeclampsia group (Supplemental Figure S1).

UNIVARIATE ANALYSIS

Because our supervised multivariate modeling was inconclusive, we next performed a targeted analysis. We selected a set of 36 physiologically relevant metabolites with consistent resonances in each preeclampsia patient and control subject. The identified metabolites were then quantified using spectral deconvolution and analyzed using the nonparametric Mann-Whitney U test with one-tailed exact *p*-value. Because ethnicity appeared to be a strong confounder, we compared the metabolite concentrations between the patients and control subjects within the Caucasian and Black subgroups. Table 2 compares the concentrations of 36 individual metabolite concentrations between the Caucasian preeclamptic patients and control subjects, and between the Black preeclamptic patients and control subjects. Remarkably, within the Caucasian subgroup, 18 of the 36 metabolites differed significantly between the preeclamptic patients and the control subjects. Within the Black subgroup, three metabolites differed significantly between the preeclamptic patients and control subjects. Two of these metabolites—2-hydroxyisobutyric acid and trigonelline—differed significantly in the Caucasian subgroup as well; the third significant metabolite—formate—did not differ significantly between the Caucasian preeclamptic patients and controls.

Discussion

Preeclampsia is a multifactorial syndrome, and understanding its pathophysiology requires a broad, bias-free view provided by modern "omics" technologies such as metabolomics. Here, we performed an exploratory metabolomics analysis in an attempt to identify a first-trimester urinary metabolomics signature among women who develop preeclampsia later in their pregnancy. We found that ethnic background was a confounding factor, prompting us to analyze the two primary ethnic groups separately. Within the Caucasian subgroup, 18 of the 36 metabolites differed significantly between the women who later developed preeclampsia and the control subjects. Within the Black subgroup, three metabolites differed significantly between the women who later developed preeclampsia and the control subjects.

Measuring the urinary metabolomics profile can be useful for assessing specific conditions; however, the eventual predictive signatures of those profiles are closely correlated with various factors, including the patient's lifestyle, diet, and ethnicity.[21, 22] Indeed, in our cohort, ethnicity appeared to be a strong confounder, as shown by the two-class PLS-DA model in which ethnic origin

was used as a class identity (Figure 2B). A straightforward multivariate case-control based model provided poor results, and it is unknown why we were unable to identify strong metabolic correlates when we used a case-control modeling strategy on each ethnic subgroup separately; however other confounding factors should certainly be considered. Moreover, given the complex physiological and metabolic changes that occur during pregnancy, in combination with the abovementioned confounding factors, a prospective follow-up study throughout pregnancy may yield more insight into pregnancy-related physiology.

Here, using a more targeted analysis and simple nonparametric univariate tests, we found that the metabolites that are associated with preeclampsia differed between the two major ethnic subgroups. Because this is the first study to investigate preeclampsia-related urinary metabolomics in the first trimester of pregnancy, we can only compare our results with studies that investigated metabolomics differences in plasma and with a recent pilot study of the urinary metabolome at gestational week 36.[12] Because the studies that measured plasma included either ethnically diverse groups[8, 9, 11] or one ethnic subgroup[10] of patients with preeclampsia and controls subjects, it is certainly conceivable that ethnicity might have influenced the outcome of these studies.

In our study, we observed a striking number of metabolites that differed significantly between the ethnic subgroups. However, these results should be interpreted with caution, as the disproportionate number of preeclamptic patients and control subjects in the Caucasian subgroup may have influenced the results. In contrast, the number of patients and control subjects in the Black subgroup was more balanced, and only a relatively small number of metabolites differed significantly between the preeclampsia patients and control subjects. Two of the metabolites that differed in the Black subgroup—2-hydroxyisobutyric acid and trigonelline—overlapped with the Caucasian subgroup. Thus, the pathophysiology of preeclampsia might differ—at least partially—among different ethnic groups.

A thorough discussion of the functional significance of the metabolites that differed significantly in our cohort is beyond the scope of this article. However, in both ethnic subgroups, the concentrations of two metabolites—2-hydroxyisobutyric acid and trigonelline—were significantly lower in the patients with preeclampsia than in their respective control subjects. The first metabolite, 2-hydroxyisobutyric acid, is a relatively well-studied compound, and increasing concentrations of this metabolite are associated with conditions that involve energy metabolism deficiency.[23] Remarkably, in our study we observed significantly lower concentrations of 2-hydroxyisobutyric acid in the patients with preeclampsia. In a recent pilot study, Austdal *et al.* reported that samples collected at gestational week 35-36 contained similar concentrations of 2-hydroxyisobutyric acid between preeclamptic patients and control subjects.[12] The second metabolite, trigonelline, also differed significantly between preeclamptic patients and control subjects in both ethnic subgroups. Although trigonelline excreted through the urine is usually dietary in origin,[24] trigonelline can be produced endogenously via methylation of nicotinic acid and involving S-adenosyl-methionine. To the best of our knowledge, experimental evidence for endogenous trigonelline synthesis exists for rodents only, and the endogenous synthesis of this metabolite is largely unexplored in humans.[25]

Low concentrations of trigonelline are associated with impaired lung function,[26] helminth infection,[27] and diabetes mellitus.[28] In particular, studies regarding diabetes mellitus lend credence to the notion that trigonelline is functionally significant in hypertension.[29] Administering trigonelline to rats with type 2 diabetes normalized several hypertension-related enzymes, including angiotensin converting enzyme (ACE).[29] Austdal *et al.* also reported that trigonelline levels are lower in patients with preeclampsia at 35-36 weeks of gestation.[12] This finding is consistent with our observation that trigonelline levels were significantly lower in the women who later developed preeclampsia than in the control subjects, regardless

of their ethnicity. Whether low levels of trigonelline are associated with the development of hypertension, or whether preeclampsia (and its associated hypertension) induces low levels of trigonelline—or both—remains to be investigated.

In conclusion, this study is the first urinary metabolomics screen in pregnant women who later developed preeclampsia. Our finding that the levels of specific metabolites differ in first-trimester urine samples between patients who develop preeclampsia and control subjects may provide a viable method for predicting preeclampsia early, even before symptoms develop, and may provide key insight into the disease's pathogenesis. Future studies using a larger cohort of patients are needed to expand these findings—particularly with respect to the role of ethnicity. Ideally, these studies should combine ^{1}H-NMR with other metabolomics techniques such as mass spectrometry. A comprehensive metabolomics analysis will likely increase our understanding of the pathophysiology of preeclampsia, which in turn will facilitate the development of preventive and therapeutic strategies.

Table 1

Characteristics	PE (n=73)	Controls (n=138)
Maternal age, years (SD)	32.3 (5.9)	32.5 (5.7)
Nulliparity, *n* (%)	40 (54.8)	61 (44.2)
Smoking, *n* (%)	2 (2.7)	7 (5.1)
Ethnicity, *n* (%) *		
Caucasian	24 (32.9)	95 (68.8)
Black	35 (47.9)	34 (24.6)
South Asian	7 (9.6)	2 (1.4)
East Asian	1 (1.4)	1 (0.7)
Mixed	6 (8.2)	6 (4.3)
BMI (SD)	28.6 (5.2)	28.0 (5.1)
PE early, *n* (%)	15 (20.5)	NA
Live birth, *n* (%)	71 (97.3)	138 (100)
Birth weight, g (%)	2575.8 (945.9)	3541.9 (363.5)*

Patient characteristics

*BMI: Body Mass Index; PE: preeclampsia. PE early: preeclampsia requiring delivery before gestational week 34. *p<0.001.*

Metabolite	Caucasian subjects (n=119)			Black subjects (n=69)		
	PE, n=24	Control, n=95	p-value	PE, n=35	Controls, n=34	p-value
1-Methylnicotinamide	9.6 (13.4)	17.8 (13.8)	0.160	18.0 (36.9)	17.4 (13.9)	0.877
2-Hydroxyisobutyric acid	23.1 (37.9)	53.7 (23.8)	0.002	41.3 (35.4)	51.2 (39.3)	0.037
Acetate	169.5 (118.5)	198.4 (131.4)	0.022	217.9 (140.8)	238.1 (130.9)	0.366
Acetoacetate	36.7 (93.8)	70.3 (96.2)	0.071	60.0 (128.3)	57.3 (83.7)	0.658
Acetone	6.6 (5.9)	11.5 (7.7)	0.017	12.0 (12.1)	11.6 (67.5)	0.995
Alanine	190.1 (375.3)	381.9 (311.4)	0.025	403.9 (435.0)	467.5 (275.4)	0.204
Carnitine	617.6 (1593.7)	1047.8 (1250.5)	0.119	651.7 (1094.6)	901.0 (886.4)	0.333
Citrate	1778.5 (2134.5)	3832.4 (2742.0)	0.003	3520.3 (3803.8)	3846.2 (2334.0)	0.734
Creatine	572.6 (827.8)	567.1 (836.1)	0.496	309.1 (865.3)	676.1 (632.1)	0.173
Creatinine	4714.0 (5518.2)	8165.4 (5360.8)	0.019	8364.9 (7433.7)	8237.3 (5662.4)	0.503
Dimethylamine	128.1 (162.7)	212.6 (126.3)	0.032	211.3 (380.2)	268.4 (180.3)	0.241
Dimethylglycine	41.0 (42.2)	63.6 (53.6)	0.014	60.1 (53.1)	59.21 (51.56)	0.269
Formate	132.4 (136.7)	171.1 (123.7)	0.174	139.1 (135.6)	212.8 (99.6)	0.030
Fumarate	0.8 (1.1)	4.1 (4.5)	0.001	4.2 (6.9)	3.4 (6.0)	0.807
Glucose	86.75 (207.4)	239.7 (192.2)	0.015	261.4 (228.5)	215.3 (208.5)	0.368
Glycine	1604.8 (2034.8)	2269.9 (1562.0)	0.105	2266.1 (3147.1)	2370.1 (1987.8)	0.571
Guanidinoacetate	515.7 (683.6)	856.5 (466.9)	0.141	770.6 (733.2)	823.7 (575.7)	0.734
Hippurate	690.4 (867.4)	1102.6 (829.9)	0.094	1133.6 (801.2)	1300.9 (950.5)	0.139
3-Hydroxybutyrate	28.5 (37.4)	31.1 (53.0)	0.952	33.5 (40.0)	22.6 (37.8)	0.584
3-Hydroxyisovaleriate	135.7 (214.6)	210.3 (174.1)	0.100	189.6 (197.7)	239.0 (229.8)	0.102
Isobutyrate	14.6 (22.7)	35.9 (31.4)	0.002	38.0 (35.9)	44.4 (43.6)	0.338
Lactate	262.7 (465.2)	602.6 (456.8)	0.006	578.8 (409.9)	533.8 (314.4)	0.620
Methylguanidine	213.2 (205.9)	321.0 (186.7)	0.031	324.9 (286.4)	329.8 (283.0)	0.676
Phenylacetylglycine	266.7 (391.3)	511.6 (330.4)	0.034	412.7 (446.4)	555.1 (519.1)	0.159
Propylene glycol	31.9 (50.2)	43.0 (48.4)	0.194	41.3 (38.7)	28.1 (36.3)	0.813
Pyruvate	29.9 (48.4)	51.9 (44.3)	0.135	37.8 (47.8)	47.1 (47.4)	0.146
Scyllo-inositol	36.4 (54.4)	80.7 (52.0)	<0.001	85.2 (60.8)	75.1 (59.3)	0.461
Succinate	47.5 (54.1)	102.5 (59.1)	<0.001	77.2 (106.6)	91.8 (77.8)	0.536
Sucrose	17.5 (31.9)	24.0 (37.4)	0.153	8.7 (32.8)	10.3 (35.9)	0.898
Trigonelline	7.8 (18.8)	26.7 (41.6)	<0.001	30.1 (46.4)	80.5 (71.4)	0.009
TMAO	253.9 (382.3)	391.5 (280.6)	0.130	316.5 (353.5)	402.6 (255.3)	0.610
Tyrosine	63.6 (69.6)	64.6 (50.7)	0.595	71.7 (62.6)	77.9 (67.6)	0.516
Valine	18.3 (18.9)	28.2 (15.5)	0.037	27.0 (20.9)	31.6 (29.1)	0.284
Pseudouridine	158.8 (190.1)	256.7 (155.6)	0.062	282.8 (262.0)	285.6 (210.3)	0.541
Glyoxylate	1.5 (1.1)	1.2 (1.3)	0.723	0.9 (2.5)	1.4 (2.5)	0.842
Hypoxanthine	9.4 (16.9)	20.9 (20.8)	0.055	13.2 (38.4)	15.1 (18.9)	0.945

Table 2 (previous page): Concentrations of metabolites in the urine of the indicated groups measured using nuclear magnetic resonance

Values represent the concentrations of metabolites in micromolar. Data are shown as median (standard deviation). PE: preeclampsia.

Figure 1

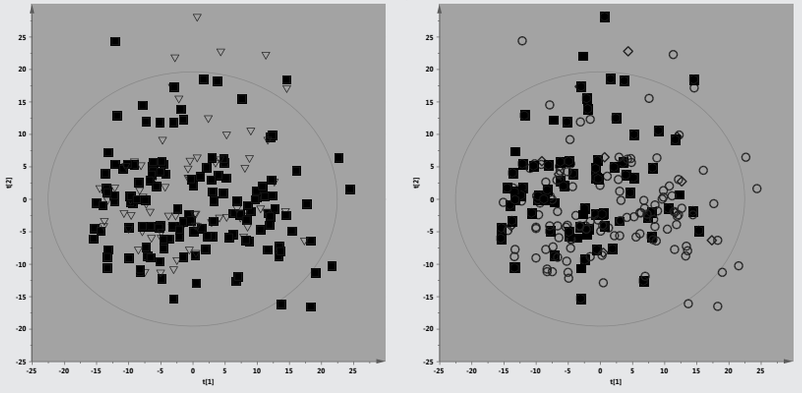

Figure 1A *Figure 1B*

Principal components analysis plot

Figure 1A shows a principal components analysis plot of the patients with preeclampsia (in open triangles) and control subjects (in solid squares). The x-axis represents the first principal component (the most significant vector), and the y-axis represents the second principal component (the second most significant vector). No separation was observed between the patients with preeclampsia and the control subjects based on 29 components that explained approximately 70% of the variance. Figure 1B shows the same principal components analysis plot, with the subjects coded according to their ethnic background. The open circles represent Caucasians, the solid hexagons represent Blacks, the open diamonds represent Asians (both South and East Asian), and the grey triangles represent mixed ethnicity.

Figure 2

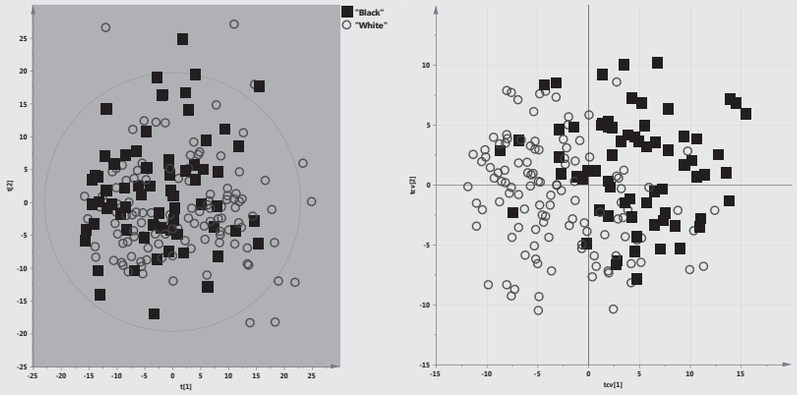

Figure 2A *Figure 2B*

Principal component analysis plot based on ethnicity

Principal component analysis plot based on the two largest ethnic groups in the cohort, Caucasians (shown in open circles) and Blacks (shown in solid hexagons). 25 components explain approximately 66% of the variance (Figure 2A). Figure 2B shows a partial least squares discriminant analysis (PLS-DA) model (cross-validated score plot). R2X = 0.202, R2Y = 0.828, Q2 = 0.498; p=4.59058e-24.

Figure S1

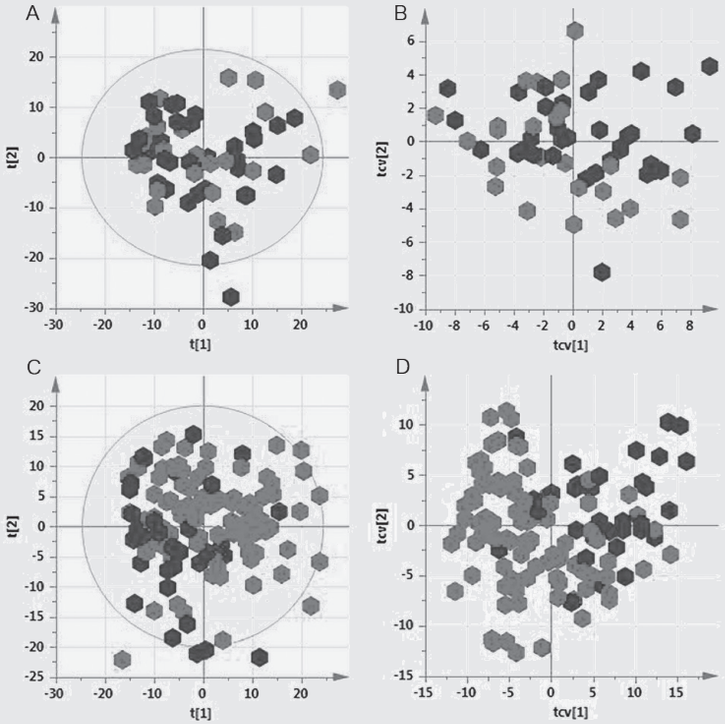

Figure S1: Influence of ethnic background

Principal Component Analysis (PCA; panels A and C) and PLS-DA (panels B and D) models with ethnic origin as class identity. The dark grey symbols represent Blacks, and the light grey symbols represent Caucasians. For the patients with preeclampsia, the PCA (A) resulted in a non-significant PLS-DA model (B; R2X = 0.102, R2Y = 807, Q2 = 0.009, p=0.683). For the control subjects, the PCA (C) resulted in a significant PLS-DA model (D; R2X = 0.202, R2Y = 0.771, Q2 0.404, p=2.99147e-12).

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VII Summary and general discussion

Summary

Preeclampsia is a complication of pregnancy which can suddenly change from a relatively mild phenotype into a life-threatening situation. One of the organs that is always involved during preeclampsia is the kidney. The placenta plays an important role in the renal pathophysiology of preeclampsia. The placenta produces excessive amounts of anti-angiogenic factors which are associated with systemic endothelial dysfunction. Although the underlying mechanisms of renal injury during preeclampsia remain unclear, women with preeclampsia have an increased risk of developing renal disease later in life. This observation suggests that preeclampsia 'marks' the mother—putatively in combination with pre-existent conditions—which might contribute to serious sequel throughout her life.

The widespread endothelial dysfunction in preeclampsia is believed to be due to increased serum levels of anti-angiogenic factors—in particular sFlt-1—produced by the placenta.[1] The studies described in CHAPTER 2 were focused on the mechanisms of sFlt-1 production within the placenta, and the systemic spreading of this anti-angiogenic factor. During both preeclampsia and uncomplicated pregnancy, the placenta is the principal source of sFlt-1. The outermost layer of the placenta, the syncytiotrophoblast, forms 'knots' that contain the anti-angiogenic protein sFlt-1, particularly during preeclampsia. Previous research has shown that these knots—called syncytial aggregates after detachment from the placenta—account for approximately 25% of all sFlt-1 protein in the maternal circulation.[2] In this thesis, the spread of these syncytial aggregates was further investigated. We showed that—within the placenta—syncytial knots are indeed the primary production site of sFlt-1, which is significantly higher in placentas from patients with preeclampsia. These knots detach from the placenta, and the systemic spread of the syncytial aggregates was confirmed by the presence of hCG-positive multinucleate aggregates in the lungs of pregnant women. We showed that preeclampsia is associated with higher numbers of syncytial aggregates in the maternal lung. In lung samples obtained from women who were carrying a male fetus co-localization of hCG and the Y-chromosome was observed, which strongly supports the idea that these aggregates are of fetal origin. Importantly, we observed that women with preeclampsia show increased sFlt-1 expression within these aggregates, as compared to the non-preeclamptic pregnant control subjects. It may be speculated that these aggregates influence the development of local immune tolerance in maternal organs early in pregnancy. Later in pregnancy, the sFlt-1 produced by these aggregates may cause endothelial injury. The presence and persistence of fetal cells in the maternal circulation and organs may also have both short- and long-term consequences for postpartum maternal health. On short-term, the sFlt-1-loaded syncytial aggregates may undergo further disaggregation, forming smaller particles. Via the release of these smaller particles into the maternal circulation, it is not unlikely that these sFlt-1-loaded smaller particles contribute to endothelial dysfunction in maternal organs other than the lungs. On long-term, the fetal cells may result in chimerism, as they can be retained in the maternal blood and organs for decades after delivery.[3] These cells—having stem cell-like properties—might affect maternal health for years after pregnancy, but it is currently unknown whether they cause disease or might have beneficial effects, or both.[4, 5]
Within the kidney, endothelial dysfunction can be caused by an imbalance between anti- and pro-angiogenic factors. In particular, vascular endothelial growth factor-A (VEGF-A)-signaling—necessary for the maintenance of the glomerular filtration barrier—seems to be affected during preeclampsia. Putative mechanisms playing a role in renal injury during preeclampsia were investigated in the studies described in CHAPTERS 3 and 4. Previous studies have shown that injury to the fenestrated endothelium within the glomerulus can lead to complement activation.[6] Combining this observation

with the notion that preeclampsia is characterized by complement dysregulation—shown in placentas[7-9] and serum[10]—we explored complement activation in the preeclamptic kidney in the studies described in chapter 3.

In the studies described in chapter 3 we investigated renal autopsy tissues from women who died due to preeclampsia, as well as control subjects, for the presence of complement components. Preeclampsia was significantly associated with the presence of C4d—a stable marker of complement activation—and markers of classical pathway activation. To explore whether complement activation during preeclampsia in the kidney is caused by antibody-mediated injury, we performed immunofluorescence stainings on the renal autopsy tissues mentioned, for the presence of immunoglobulins. Immune deposits of the IgM-subtype were significantly more frequently observed in kidneys of women with preeclampsia, whereas IgG deposits were sporadically observed in all study groups, and IgA was never observed. It is not possible to investigate the causative role of sFlt-1 in the activation of the complement system in the kidney in vivo in humans. Therefore, a sFlt-1-induced-mouse model of preeclampsia was used. Mice injected with sFlt-1 show increased deposition of C4 in the kidneys as compared to control mice. These findings suggest that angiogenic imbalance may play an important role in activation of the complement system in the kidney during preeclampsia. The observations described in this chapter raise the question: what causes the presence of the IgM-subtype in glomeruli of patients with preeclampsia? Although glomerular IgM deposition is observed in a variety of renal diseases and its role remains elusive, the presence of this subtype might have several explanations. Firstly, the presence of the IgM-subtype could reflect the binding of IgM antibodies to endothelium injured by sFlt-1. Natural IgM-antibodies play a major role in the clearance of damaged cells,[11, 12] and they can bind to both hypoxic[13] and apoptotic cells[14, 15] through intracellular antigens

that become externalized under these conditions. Secondly, IgM is a large molecule, and due to its polymeric nature, it easily binds non-specifically to endothelium.[16] Finally, although we did not observe other immunoglobulin subtypes, it may be hypothesized that the presence of IgM might be caused by auto-antibodies.[17] Altogether, the studies in this chapter show that preeclampsia is strongly associated with activation of the classical pathway of the complement system in the kidney. These findings suggest that complement activation might amplify local inflammation, and could contribute to the renal injury in preeclampsia. Our findings further support the concept of complement inhibition as a therapeutic tool, targeting both the renal and placental manifestations of preeclampsia.

The extent of renal injury during preeclampsia was investigated in the studies described in chapter 4. Preeclampsia is characterized by loss of podocytes into the urine. Therefore, this chapter was devoted to quantification of the numbers of podocytes within the glomeruli of patients with preeclampsia and of control subjects, using the same renal autopsy tissues as in chapter 3. The numbers of glomerular podocytes did not differ significantly between the preeclamptic and control groups. Podocyte injury and loss are often associated with increased parietal epithelial cell activation. Therefore, we evaluated parietal epithelial cell activation in the setting of preeclampsia. Women with preeclampsia showed significantly higher numbers of activated parietal epithelial cells on a podocyte location. It remains to be investigated whether activation and proliferation of parietal epithelial cells compensate for ongoing podocyte injury and loss, or whether these cells contribute to glomerular injury, or both. The findings described in this chapter may provide new insights into the renal complications of women with preeclampsia later in life. Parietal epithelial cell activation is associated with focal and segmental glomerulosclerosis (FSGS),[18] and women with preeclampsia have a higher risk of

developing FSGS later in life.[19] As further discussed below, we hypothesize that the higher numbers of activated parietal epithelial cells in the preeclamptic kidney as described in this chapter, may play an important role in the pathogenesis of the development of FSGS later in life.

It is evident from the studies described in chapters 3 and 4, that the kidney—and in particular, the glomerulus—is targeted during preeclampsia. An important cause of this, as mentioned earlier, is believed to be the angiogenic imbalance, in which an excess of particularly sFlt-1 prevents VEGF-A from adequately maintaining the renal endothelium. As a VEGF-A-signaling pathway is responsible for cross-talk between endothelial cells and podocytes, it is believed that both endothelial cells and podocytes are injured in situations of angiogenic imbalance. Injured podocytes may detach from the glomerular basement membrane. After detachment from the glomerular basement membrane, podocytes are shed into the urine. Podocyturia during preeclampsia was further investigated in the studies described in CHAPTER 5. In this chapter, we explored the potential of using quantitative polymerase chain reaction (qPCR) as a means to detect the mRNA levels of podocyte-specific molecules in urine samples of women with preeclampsia and of control subjects. Women with preeclampsia showed significantly elevated urinary mRNA levels of nephrin, podocin, and VEGF as compared to normotensive pregnant control subjects, and non-pregnant control subjects. In addition, significantly elevated levels of nephrin mRNA were detected in urine of women with preeclampsia as compared to that of women with gestational hypertension. We found mRNA encoding for podocyte-specific molecules in urine of non-pregnant women, normotensive pregnant women, and women with gestational hypertension, which indicates that during all these conditions, there is a (physiological) loss of podocytes in urine. It may be speculated that normal pregnancy, gestational hypertension, and preeclampsia are a continuous spectrum of (mild) endothelial

dysfunction, contributing to podocyturia. The results from the studies described in this chapter demonstrate that qPCR is a highly promising, non-invasive method for quantifying podocyturia in patients with preeclampsia, but it still needs to be assessed whether measuring podocyturia is a more reliable marker than conventional biomarkers are. Importantly, a very recent study showed that podocyturia precedes proteinuria and the clinical features of preeclampsia,[20] which is discussed in detail on page 168 of this chapter.

To identify possible first-trimester biomarkers in preeclampsia, the work described in CHAPTER 6 focused on the presence of metabolites in early-pregnancy urine samples, and their potential role in predicting preeclampsia. The set of metabolites that reflects the organism under a particular set of conditions, is called the *metabolome*.[21] The metabolome is the product of environmental and genetic conditions, and provides a new logical framework to elucidate disease etiology. In the studies described in chapter 6, first trimester urine samples were analyzed using nuclear magnetic resonance spectroscopy. The concentrations of 36 metabolites were compared in a nested case-control study of 73 women who developed preeclampsia later during pregnancy and 138 pregnant control subjects derived from a prospective cohort study consisting of 33.602 singleton pregnancies.[22] Although ethnicity appeared to be a strong confounding factor, we identified a first trimester urinary metabolomics signature within the two largest ethnical subgroups—Caucasians and Blacks—for patients developing preeclampsia later in pregnancy. The significantly different urinary metabolomics signature in the first trimester of pregnancy in women destined to develop preeclampsia, potentially holds the promise of a screening test, and offers insight into the pathogenesis of preeclampsia.

General Discussion

ON RENAL PATHOPHYSIOLOGY IN PREECLAMPSIA

Because the kidney is always involved in preeclampsia, the causes and consequences of kidney injury, both on the short- and long term, will be discussed below.

CAUSES AND CONSEQUENCES OF ENDOTHELIAL INJURY

As mentioned earlier, endothelial injury is considered to play an essential role in the pathogenesis of preeclampsia. A significant part of this injury is thought to arise by the excessive amounts of the anti-angiogenic factor sFlt-1, which is produced by the placenta during pregnancy. However, not all pregnant women with high sFlt-1 serum concentrations develop preeclampsia. The overlap of sFlt-1 concentrations between women with preeclampsia and women with uncomplicated pregnancies illustrates this phenomenon.[1] Therefore, the rise in sFlt-1 can not solely be held accountable for the endothelial injury observed during preeclampsia. It remains debatable why some patients are so sensitive to high concentrations of sFlt-1, whereas other patients abide the high concentrations of sFlt-1 very well. sFlt-1 binds to VEGF-A, which leads to low concentrations of bio-active VEGF-A.[23, 24] Thereby, the physiological functions of VEGF-A are impaired, including proliferation, angiogenesis, lymphogenesis, inducing endothelial cell permeability, and vasodilation.[23, 24] Perhaps, pre-existent endothelial injury increase the vulnerability for a rise in sFlt-1. It is known that conditions associated with endothelial injury predispose to preeclampsia, such as diabetes mellitus, obesity, and hypertension.[25] It may be that women with such pre-existent endothelial injury are more vulnerable to the stress imposed on the endothelium during pregnancy.[26-28] Thus, the rise in sFlt-1 which occurs during preeclamptic pregnancy might aggravate pre-existent endothelial injury. Furthermore, other anti-angiogenic factors, such as soluble Endoglin,[29] have been described to play an important role in the endothelial injury during preeclampsia. These observations support the hypothesis that endothelial injury during preeclampsia is a multifactorial condition. Taken together, there is an overlap between the concentrations of anti-angiogenic factors in healthy pregnant women and women with preeclampsia. This phenomenon could be explained by the assumption that healthy pregnant women are not as sensitive to high concentrations of anti-angiogenic factors, or might not have pre-existent endothelial injury, or both. Rising levels of anti-angiogenic factors alone may not be sufficient to cause systemic endothelial injury.

Preeclampsia is associated with certain genetic variants.[30] These genetic variants concern genes associated with the coagulation, fibrinolysis, immunological, and renin-angiotensin-aldosterone systems. In addition, these genetic variants also seem to concern genes involved in cardiovascular disease and its risk factors, such as diabetes mellitus, obesity, and hypertension.[30] Illustratively, women with preeclampsia have an 8-fold higher risk of death from cardiovascular causes later in life than women who have had an uncomplicated pregnancy.[31] However, not all women with preeclampsia do develop cardiovascular disease later in life.[32, 33] The question that remains is, whether preeclampsia is the cause of remaining endothelial injury, causing cardiovascular disease later in life, or that the genetic make-up and other risk factors of these women result in pre-existent endothelial injury and predispose to both preeclampsia and cardiovascular disease.

The different genetic predispositions associated with preeclampsia could also be the reason for distinct metabolic profiles in women with preeclampsia, compared to pregnant control subjects. In a recent study, a metabolomics signature in serum was described to be specific for preeclampsia.[34] In the results described in chapter 6, we observed that the levels of specific metabolites differ in first-

trimester urine samples between patients who develop preeclampsia and control subjects, and we found that ethnic background was a confounding factor. These findings may provide a viable method for predicting preeclampsia early, even before symptoms develop, and may provide key insight into the disease's pathogenesis. It may be, that the factors playing a role in early pregnancy could also contribute to the pre-existent endothelial injury, and subsequent vulnerability for sFlt-1 in preeclampsia.

Within the murine preeclamptic kidney, endothelial injury through high sFlt-1 levels manifests as endotheliosis (swelling of endothelial cells).[35] However, there is increasing evidence that complement activation also contributes to the kidney injury in preeclampsia. The results described in chapter 3 are the first observations to suggest a putative role that injured endothelium itself might play in initiating activation of the complement system during preeclampsia. In particular, the presence of complement deposits in a sFlt-1-induced-mouse model of preeclampsia, strongly supports the hypothesis that sFlt-1 induced endothelial injury is—at least in part—responsible for activation of the complement system. Activation of the complement system can be the result of binding of auto-antibodies.[36, 37] In the kidney specifically, complement activation could result from binding of auto-antibodies to glomerular structures, or by glomerular deposition of circulating immune complexes.[6] In preeclampsia, immune complex deposition and subsequent complement activation could, for instance, result from binding of angiotensin II type 1 receptor agonistic antibodies (AT1-AA). These antibodies are frequently observed in women with preeclampsia,[17] and in a murine model the presence of these antibodies resulted in glomerular complement activation.[38] However, not all women with preeclampsia have circulating antibodies against the angiotensin II type I receptor. In the work described in chapter 3 of this thesis, glomerular complement activation was observed in all studied women with preeclampsia. Although the number of patients studied was relatively small, this finding suggests that

other mechanisms than binding of auto-antibodies might also be responsible for activation of the complement system. Indeed, another putative cause for complement activation is the interplay between the complement system and dysregulation of pro- and anti-angiogenic factors resulting in angiogenic imbalance. Within the kidney there is evidence that complement activation is a consequence of angiogenic imbalance. The high concentrations of circulating sFlt-1 bind to VEGF-A, which leads to low concentrations of bio-active VEGF-A.[23, 24] In vivo cell experiments showed that on the podocytes' surface, low concentrations of VEGF-A lead to reduced concentrations of the important complement regulator, factor H.[39] Dysfunction of factor H is also associated with atypical Hemolytic Uremic Syndrome (aHUS).[40] Since aHUS and preeclampsia show overlapping histopathologic renal lesions—such as double contours of the glomerular basement membrane and signs of thrombotic microangiopathy—the underlying pathogenic mechanisms of these conditions may share the same pathways. In aHUS, the presence of mutations in factor H is associated with the deposition of complement products on the surface of platelets, resulting in a prothrombotic state.[41] The other way around, complement may be activated on the membrane of activated platelets.[42] Thus, these findings may suggest that complement dysregulation during preeclampsia, in particular regarding factor H, is linked to conditions associated with high incidence of thrombosis, which indeed seems to be the case.[43] This multifaceted vicious cycle may aggravate the kidney injury during preeclampsia. In following, there are several questions that need to be answered. Is activation of the complement system a cause or a consequence of endothelial injury? Is activation of the complement system within glomeruli of patients with preeclampsia a result of sFlt-1 induced endothelial injury? Or is the complement system activated through autoantibodies directed against the angiotensin II type I receptor, thereby causing endothelial injury? And does this endothelial injury then cause activation of the complement system, and thereby activation of the coagulation system?

PODOCYTURIA: PREDICTION AND A GLIMPSE INTO THE FUTURE

Endothelial injury is thought to give rise to the clinical manifestations of preeclampsia, including hypertension and kidney injury.[35] Clinically, this kidney injury results in worsening of kidney function and loss of proteins in the urine: proteinuria. Within the relatively short period of preeclamptic pregnancy, the glomerular filtration barrier is severely injured, which leads to leakage of proteins. Interestingly, Yu et al.[44] demonstrated that in rat models of puromycin aminonucleoside-induced nephrosis, mesangioproliferative nephropathy, and hypertensive nephropathy, proteinuria is present during both active and chronic phases of glomerular injury, whereas podocyturia is confined to active, ongoing glomerular damage only. Based on these findings, podocyturia seems to be a good marker of ongoing glomerular damage.

In the work described in chapter 5 of this thesis, increased podocyte specific mRNA levels were observed in urine of women with preeclampsia, as compared to pregnant control subjects. This rapid and sensitive method seems useful in quantifying podocyturia. The question that remains is: is podocyturia a diagnostic tool to detect preeclampsia during the early stages of pregnancy, i.e. before the onset of clinical symptoms? Recently, a very promising paper was published, in which the authors describe that podocyturia precedes both proteinuria and the clinical features of preeclampsia.[20] In all preeclamptic patients, podocyturia was detected in the second trimester, whereas podocyturia was never observed in normotensive controls, nor in patients who developed gestational hypertension. Podocyturia in the second trimester had a significantly greater sensitivity and specificity (both 100%, providing perfect likelihood ratios) for the subsequent diagnosis of preeclampsia than any single angiogenic factor, such as sFlt-1 or s-Eng, or a combination of these factors. These findings implicate promising possibilities of using podocyturia for accurate identification of pregnant women

at risk for preeclampsia. Importantly, these findings also add to our understanding of the pathophysiologic mechanisms leading to preeclampsia-related proteinuria. Podocyturia was observed in the second trimester before the onset of proteinuria.[20] This may indicate that glomerular loss of podocytes may lead to a disruption of the glomerular filtration barrier and, thereby, to proteinuria. However, it has also been described that proteinuria impairs podocyte regeneration,[45] and it may be speculated that proteinuria therefore leads to podocyturia. Another possibility is that proteinuria and podocyturia are both caused by one upstream mechanism. It could also be the case that proteinuria and podocyturia happen at the same time, but are the result of different underlying mechanisms. Despite the significant loss of podocytes in the urine, the work described in this thesis showed that the number of glomerular podocytes is unaffected in preeclampsia. The higher number of activated glomerular parietal epithelial cells present in kidneys from women with preeclampsia may suggest that lost podocytes are replaced by progenitor cells of the parietal epithelium.

The replacement of lost podocytes by activated parietal epithelial cells could be a favourable phenomenon. In a Munich Wistar Frömter (MWF) rat model of spontaneous glomerular injury, this compensatory mechanism has been described to contribute to remodeling of the glomerular architecture, if successful.[46] Perhaps this mechanism could be responsible for the fact that the glomerular lesions in preeclampsia usually disappear within a few weeks after delivery.[47, 48] However, instead of a repair response by parietal epithelial cells, murine studies have illustrated the important role of parietal epithelial cells in the development of FSGS, by showing that an excessive proliferative response of parietal epithelial cells is involved in the progression of FSGS.[49] In human renal transplants, activated parietal epithelial cells are present in significantly higher numbers in early recurrent FSGS than in minimal change disease.[18] Women with preeclampsia have a higher risk of developing

FSGS later in life[19], but the pathogenesis of this phenomenon is poorly understood. Based on the aforementioned studies, one may hypothesize that an excessive proliferative response of parietal epithelial cells may lead to kidney injury, in particular FSGS.

We found higher numbers of activated parietal epithelial cells in kidneys from women with preeclampsia than in the control groups. Taking the abovementioned studies into consideration, it could be hypothesized that the higher numbers of parietal epithelial cells in the preeclamptic kidney could be a sign of repair, or a sign of progressive podocytopathy. In case the higher numbers of parietal epithelial cells are a sign of progressive podocytopathy, this podocytopathy may be associated with the risk of developing FSGS on the long run. In addition, the replacement of lost podocytes by activated parietal epithelial cells might also be responsible for the increased risk (relative risk of approximately 4) for women with preeclampsia to develop end-stage renal disease.[50] This increased risk could be a direct effect of preeclampsia. However, preeclampsia itself is also associated with an increased risk of cardiovascular disease, and cardiovascular disease is in turn associated with kidney disease.[51] Therefore, it is challenging to distinguish the precise role of preeclampsia in the development of kidney disease. However, the risk of kidney disease and micro-albuminuria one decade after preeclampsia seems to be greater than the risk of cardiovascular disease,[52, 53] which underscores the hypothesis that preeclampsia might induce a primary renal insult.

PATIENTS' PERSPECTIVES

No single factor can be held responsible for the development of kidney disease during and after preeclampsia. A variety of risk factors and mechanisms probably underlies this phenomenon. Regardless of which factors contribute, preeclampsia is an important risk factor for subsequent chronic kidney disease. This notion calls for early detection and intervention. To start with, early prediction of preeclampsia—for example by using podocyturia or urinary metabolomics analysis—could provide an opportunity for close surveillance and preventive strategies, such as early delivery. It lends credence to speculation that targeting factors that contribute to the pathogenesis of kidney disease in preeclampsia may have therapeutic value. For instance, murine models have shown reversal of proteinuria and of histologic changes typical of preeclampsia when treated with complement inhibitors.[54] In humans, Eculizumab—a terminal complement inhibitor—has recently been administered in a woman with preeclampsia. This resulted in abrogation of preeclampsia manifestations and prolongation of pregnancy.[55] Further research is warranted to validate these findings in a larger clinical study, especially regarding the potential side effects of the treatment. In addition, several studies showed that targeting the excessive proliferative response of parietal epithelial cells[56] can be achieved by therapies, such as ACE-inhibitors[57] and inhibitors of the Notch-signaling pathway.[58] Similarly to Eculizumab, ACE-inhibitors, and Notch-signaling pathway inhibitors should be further studied in randomised controlled trials, in particular regarding the side effects for the mother and the fetus. Furthermore, long-term follow-up is recommended after delivery, especially with respect to hypertension, obesity, and insulin resistance.[51] Whether monitoring of these risk factors and usage of the abovementioned therapeutic options will improve the renal condition during and after preeclampsia and might decrease the risk of kidney disease later in life, should be subject of future studies.

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Nederlandse samenvatting

Pre-eclampsie—in de volksmond zwangerschapsvergiftiging genoemd—is een ernstige zwangerschapscomplicatie, waarvan het beloop plotseling kan veranderen van een milde aandoening tot een levensbedreigende situatie. Pre-eclampsie wordt gekenmerkt door een hoge bloeddruk en eiwitverlies in de urine tijdens de zwangerschap. Het eiwitverlies in de urine treedt op doordat de nier één van de organen is die beschadigd raakt tijdens pre-eclampsie. Het is nog niet geheel duidelijk welke mechanismen deze schade precies veroorzaken. Bovendien lijkt de schade tijdens de zwangerschap tevens consequenties te hebben voor de gezondheid van de moeder in haar latere leven. Vrouwen die een zwangerschap met pre-eclampsie hebben doorgemaakt hebben namelijk een licht verhoogd risico op het ontwikkelen van nierziekten. Dit lijkt erop te duiden dat pre-eclampsie een soort ‘ voetafdruk’ achterlaat in het lichaam van de moeder, hetgeen kan leiden tot chronische schade op de langere termijn. Gedacht wordt dat de oorzaak van pre-eclampsie een verkeerde aanleg en werking van de placenta is. De placenta produceert, met name tijdens pre-eclampsie, grote hoeveelheden anti-angiogenetische factoren. Deze anti-angiogenetische factoren remmen angiogenetische factoren. Angiogenetische factoren zijn belangrijk voor de aanmaak en groei van bloedvaten. Als anti-angiogentische factoren deze functies remmen, kan dat leiden tot schade aan het endotheel (de binnenbekleding van de bloedvaten) in het hele lichaam. Dit proefschrift beschrijft de schade—en de onderliggende mechanismen van deze schade—in de nieren tijdens pre-eclampsie en onderzoekt eveneens de mogelijkheden om pre-eclampsie te voorspellen door gebruik te maken van specifieke moleculen in urine.

HOOFDSTUK 2 gaat over één van de belangrijke anti-angiogenetische factoren, sFlt-1. In dit hoofdstuk zijn de experimenten beschreven waarbij werd onderzocht waar deze factor wordt geproduceerd in de placenta en hoe deze factor in het lichaam van de moeder terechtkomt. Hoewel sFlt-1 ook tijdens

normale zwangerschappen door de placenta gemaakt wordt, zijn de hoeveelheden sFlt-1 in het bloed van de moeder tijdens pre-eclampsie veel hoger. In de placenta wordt sFlt-1 vooral gemaakt door de buitenste laag cellen, de zogenoemde syncytiotrofoblast. Met name tijdens pre-eclampsie vormt deze laag fragmenten, die grote hoeveelheden van sFlt-1 bevatten. De fragmenten laten los en komen in de bloedsomloop van de moeder terecht. In dit hoofdstuk hebben wij de fragmenten daadwerkelijk teruggevonden in de longen van de moeder . In de longen van vrouwen met pre-eclampsie werden veel meer placenta fragmenten gevonden dan in longen van vrouwen die geen pre-eclampsie tijdens de zwangerschap hadden gehad. Het is goed mogelijk dat het circuleren van deze fragmenten van de placenta ervoor kan zorgen dat vrouwen met pre-eclampsie complicaties ontwikkelen na de bevalling. Het is zelfs mogelijk dat deze fragmenten van de placenta de gezondheid van de moeder nog jaren na de zwangerschap kunnen beïnvloeden.

Zoals in het voorgaande is vermeld, is de nier een orgaan dat beschadigd raakt tijdens pre-eclampsie. Gedacht wordt dat de endotheelschade in de nier voor een groot deel veroorzaakt wordt door het effect van de anti-angiogenetische factoren. In de nier bevinden zich veel bloedvaatjes in de filterlichaampjes—glomeruli genaamd—waar het bloed wordt gefilterd tot urine. Voor de filtratiebarrière in de glomeruli is de angiogenetische factor, vasculaire endotheel groei factor-A (VEGF-A), erg belangrijk. Deze angiogenetische factor zorgt voor communicatie tussen de verschillende lagen cellen die samen de filtratiebarrière vormen om zo deze barrière goed te kunnen onderhouden. Tijdens pre-eclampsie zijn er in het bloed veel hogere concentraties van sFlt-1 dan tijdens een gezonde zwangerschap. sFlt-1 bindt aan VEGF-A waardoor er veel minder ongebonden VEGF-A overblijft om te worden gebruikt voor de communicatie tussen de verschillende lagen van de filtratiebarrière. De mogelijke mechanismen die een

rol spelen bij het ontstaan van de nierschade bij pre-eclampsie werden onderzocht in de studies beschreven in HOOFDSTUK 3 en HOOFDSTUK 4. Eerdere studies hebben laten zien dat schade van het endotheel in de nier kan leiden tot activatie van het complementsysteem. Het complementsysteem is een onderdeel van het immuunsysteem en speelt een belangrijke rol bij de afweer. Aangezien recente studies hebben laten zien dat tijdens pre-eclampsie het complementsysteem geactiveerd is in bijvoorbeeld de placenta en het bloed van de moeder, werd de activatie van het complementsysteem in nieren van vrouwen met pre-eclampsie onderzocht in hoofdstuk 3.

In de studies beschreven in dit hoofdstuk werd de activatie van het complementsysteem onderzocht in de nieren van vrouwen die waren overleden aan pre-eclampsie en in de nieren van vrouwen die tijdens de zwangerschap waren overleden, maar zonder pre-eclampsie. De laatste categorie vrouwen fungeerde als controlegroep. In de nieren van vrouwen met pre-eclampsie werd altijd complementactivatie gevonden, terwijl dit in een veel lager percentage bij de controlegroepen werd gezien. Het complementsysteem kan op verschillende manieren geactiveerd worden. Deze verschillende manieren worden onderverdeeld in drie verschillende ‘ routes’ die alle kunnen leiden tot activatie van het complementsysteem. Dit hoofdstuk beschrijft de onderzoeken die zijn verricht om uit te zoeken welke route van het complementsysteem geactiveerd is in de nieren van vrouwen met pre-eclampsie. De onderzoeken in hoofdstuk 3 wijzen erop dat de zogenoemde klassieke route geactiveerd wordt, maar hoe deze route precies geactiveerd raakt, was nog niet geheel duidelijk. Omdat het onmogelijk is dit mechanisme in de mens te onderzoeken werden in dit hoofdstuk ook muizen onderzocht. Muizen die werden geïnjecteerd met sFlt-1 lieten meer complementactivatie in de nier zien dan muizen zonder sFlt-1. Samengevat, laten de onderzoeken in dit hoofdstuk zien dat pre-eclampsie gekenmerkt wordt door

activatie van de klassieke route van het complementsysteem in de nieren. Deze bevinding suggereert dat complementactivatie zou kunnen leiden tot een lokale ontstekingsreactie en zo zou kunnen bijdragen aan de schade in de nieren bij pre-eclampsie. Bovendien zou naar aanleiding van deze resultaten gespeculeerd kunnen worden dat remming van complementactivatie een mogelijke therapeutische optie zou kunnen zijn om de schade tijdens pre-eclampsie in de nier te beperken.

De nierschade tijdens pre-eclampsie werd onderzocht in de studies beschreven in hoofdstuk 4. In dit hoofdstuk werden karakteristieke afwijkingen gevonden in de nieren van vrouwen met pre-eclampsie, namelijk zwelling van endotheelcellen (endotheliose genaamd), verdubbeling van de glomerulaire basaalmembraan en zwelling van podocyten. Podocyten zijn speciale cellen die deel uit maken van de filtratiebarrière. Zij hebben voet-achtige uitlopers, vandaar de naam podocyte, aangezien ‘ podo’ in het Latijn voet betekent. Een eerdere studie liet zien dat vrouwen met pre-eclampsie podocyten verliezen in de urine. Daarom beschrijft dit hoofdstuk of ook het aantal podocyten in de nieren van vrouwen met pre-eclampsie veranderd is. In andere studies is bovendien ook aangetoond dat verlies van podocyten kan leiden tot de aanmaak van ‘nieuwe podocyten’ vanuit andere cellen in de glomerulus. Deze aanmaak van ‘nieuwe podocyten’ werd ook onderzocht in dit hoofdstuk met behulp van speciale technieken. De resultaten in het hoofdstuk laten zien dat het aantal podocyten in de nieren van vrouwen met pre-eclampsie niet verschilde met de controlegroepen. Wel werden er meer ‘nieuwe podocyten’ gevonden in de nieren van vrouwen met pre-eclampsie. Het is dus goed mogelijk dat door het verlies van podocyten in de urine, andere cellen uit de glomerulus de verloren podocyten vervangen en zo het verlies compenseren. Aan de andere kant zouden deze ‘nieuwe podocyten’ ook juist voor schade kunnen zorgen. Hoewel toekomstig onderzoek zou moeten uitwijzen welk van deze twee mogelijkheden—of beide—van toepassing zijn bij

pre-eclampsie, zouden de bevindingen in dit hoofdstuk wel een nieuw licht kunnen werpen op de ontstaansmechanismen van de nierschade later in het leven bij vrouwen met pre-eclampsie.

In de hoofdstukken 3 en 4 is duidelijk geworden dat de nier—en met name de glomerulus—beschadigd raakt tijdens pre-eclampsie via verschillende mechanismen. Met name door de anti-angiogenetische factoren kunnen zowel de glomerulaire endotheelcellen als podocyten beschadigd raken. Podocyten die ernstig beschadigd zijn kunnen loslaten en op die manier in de urine terechtkomen. De toename van verlies van podocyten in de urine tijdens pre-eclampsie, ook wel podocyturie genoemd, werd onderzocht in de studies beschreven in HOOFDSTUK 5. Eerdere studies onderzochten de aanwezigheid van podocyten in urine door gebruik te maken van immunohistochemische kleuringen op gekweekte podocyten. Aangezien deze methode arbeidsintensief is en veel tijd kost, werd in hoofdstuk 5 onderzocht of het meten van podocyt-specifieke moleculen—met behulp van een quantitatieve polymerase chain reaction (qPCR)—in urine van vrouwen met pre-eclampsie en controle vrouwen een betere methode zou zijn. In de urine van vrouwen met pre-eclampsie werden veel hogere waarden van podocyt-specifieke moleculen gevonden dan in urine van gezonde zwangere controle vrouwen en niet-zwangere controle vrouwen. Aangezien ook podocyt-specifieke moleculen werden gevonden in de controle groepen, lijkt verlies van podocyten in urine fysiologisch te zijn. Het is dus goed mogelijk dat zowel een normale zwangerschap, als een zwangerschap met hoge bloeddruk en een zwangerschap met pre-eclampsie een continue spectrum vormen en zo in meer of mindere mate gepaard gaan met verlies van podocyten in de urine. Een recente studie laat zien dat podocyturie voorafging aan de klinische verschijnselen van pre-eclampsie, wat suggereert dat het meten van podocyturie zou kunnen voorspellen welke vrouwen een verhoogde kans hebben op het ontstaan van pre-eclampsie later tijdens de zwangerschap.

HOOFDSTUK 6 gaat over de mogelijkheden om pre-eclampsie al vroeg in de zwangerschap te voorspellen. In dit hoofdstuk werden urine-monsters, van vrouwen die elf tot dertien weken zwanger waren, onderzocht op de aanwezigheid van bepaalde ‘metabolieten’ om zo pre-eclampsie te kunnen voorspellen. Metabolieten zijn de tussen- of eindproducten die ontstaan nadat een stof stofwisseling heeft ondergaan. De samenstelling van verschillende metabolieten in het lichaam wordt het ‘metaboloom’ genoemd. Het metaboloom is een resultaat van omgevings- en genetische factoren en kan zo inzicht bieden in het ontstaan van bepaalde aandoeningen. In hoofdstuk 6 werden urine-monsters geanalyseerd met behulp van nuclear magnetic resonance en werd de concentratie van 36 metabolieten vergeleken tussen zwangere vrouwen die later pre-eclampsie ontwikkelden en zwangere vrouwen die een ongecompliceerde zwangerschap doormaakten. De etnische afkomst van de vrouwen bleek de resultaten sterk te beïnvloeden. Binnen de grootste etnische subgroepen—Blanken en Afrikanen—kon een specifiek metabolietensamenstelling in de urine worden gevonden bij vrouwen die later in de zwangerschap pre-eclampsie ontwikkelden. De samenstelling van deze metabolieten in eerste trimester urine van vrouwen die later pre-eclampsie ontwikkelen zou in de toekomst wellicht gebruikt kunnen worden om pre-eclampsie te kunnen voorspellen en zou bovendien inzicht kunnen geven in het ontstaan van pre-eclampsie.

De bevindingen beschreven in dit proefschrift laten zien dat pre-eclampsie een ernstige zwangerschapscomplicatie kan zijn, die de nodige effecten op de nier heeft.

Toekomstig onderzoek moet uitwijzen of het behandelen met middelen die bijvoorbeeld complementactivatie tegengaan, een bijdrage zou kunnen leveren aan de gezondheid van vrouwen met pre-eclampsie en hun kinderen tijdens hun zwangerschap en in de navolgende jaren. Tevens hebben de studies in dit proefschrift de

mogelijkheden verkend om pre-eclampsie te voorspellen met behulp van verschillende markers in de urine. Ondanks de veelbelovende resultaten zouden deze onderzoeken eerst op uitgebreide schaal getest moeten worden voordat het kan worden toegepast in de dagelijkse praktijk. Het combineren van verschillende factoren in één model om pre-eclampsie te voorspellen, bijvoorbeeld de metabolieten in de urine, de leeftijd van de moeder, de Body Mass Index en boekingsbloeddruk, lijkt op dit moment de beste resultaten te geven. Vrouwen die hierbij een verhoogd risico laten zien op het ontwikkelen van pre-eclampsie, kunnen dan vaker worden gezien door een verloskundige of gynaecoloog tijdens de zwangerschap, om indien nodig, zo snel mogelijk actie te kunnen ondernemen. De beschadigingen van de nier tijdens de zwangerschap zouden mogelijk kunnen bijdragen aan het verhoogde risico dat vrouwen met pre-eclampsie hebben op nierziekten en hart- en vaatziekten. Het monitoren van de bloeddruk, glucosewaarden en overgewicht na de bevalling bij vrouwen die een zwangerschap met pre-eclampsie doormaakten zou een eerste stap in de goede richting kunnen zijn om zoveel mogelijk schade te voorkomen.

Curriculum Vitae

Marlies Penning werd op 24 februari 1986 geboren in Schiedam. Het grootste deel van haar jeugd bracht zij door in Duiven, samen met haar ouders en zusje Iris. Na het doorlopen van het vwo op het Candea College in Duiven werd zij toegelaten tot de studie Geneeskunde aan het Leids Universitair Medisch Centrum (LUMC) in 2004. Haar studie Geneeskunde werd een jaar onderbroken om plaats te nemen in het bestuur van de Medische Faculteit der Leidse Studenten (M.F.L.S.) in de functie van Lid Intern. Het daarop volgende jaar was zij voorzitter van de Studentenraad van het LUMC. Als student en co-assistent verbleef zij tijdens haar studie in Ghana, Jordanië en Malawi. Tijdens deze buitenlandse stages raakte zij geboeid door andere culturen. Gedurende haar wetenschapsstage op de afdeling Pathologie en de afdeling Verloskunde van het LUMC werd de basis gelegd voor haar wetenschappelijke carrière, resulterend in het huidige proefschrift. Zij ontving een beurs vanuit het LUMC in het kader van het MD/PhD traject waarmee zij, na het behalen van haar artsexamen in 2011, vanaf januari 2012 gedurende twee jaar voltijd onderzoek kon verrichten. Tijdens haar onderzoek werd zij begeleid door dr Hans Baelde, dr Kitty Bloemenkamp en prof. dr Jan Anthonie Bruijn. In januari 2014 startte Marlies als ANIOS op de afdeling Gynaecologie en Verloskunde (opleider dr J. C. M. van Huisseling) van het Groene Hart Ziekenhuis in Gouda. Per april 2014 is zij in ditzelfde ziekenhuis gestart met de opleiding tot arts Internationale Gezondheidszorg en Tropengeneeskunde (opleider drs M. Lagro). Zij hoopt in 2016 deze opleiding af te ronden en samen met haar vriend Maarten van der Deijl—die eveneens in opleiding tot tropenarts is—enkele jaren in het buitenland te gaan werken.

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