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THE ROLE OF ANGIOGENIC AND IMMUNOLOGICAL FACTORS IN PREECLAMPSIA

PhD thesis

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List of Abbreviations

ADAMTS13	von Willebrand factor cleaving protease
APLS	antiphospholipid syndrome
ART	assisted reproduction techniques
BMI	body-mass index
CSF-1	colony-stimulating factor-1
DAB	3,3'-diaminodbenzidine
IFN-γ	interferon-γ
ISSHP	International Society for the Study of Hypertension in Pregnancy
EDTA	ethylene diamine tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FIGO	International Federation of Gynecology and Obstetrics
FRETS-VWF73	fluorigenic substrate
GM-CSF	granulocyte-macrophage colony-stimulating factor
HRP	horse radish peroxidase
LBW	low birthweight
LIF	leukemia inhibitory factor
MIP-1α	macrophage Inflammatory Protein-1 alpha
NK cells	natural killer cells
OPD	ortho-phenylenediamine
PlGF	placental growth factor
PRES	posterior reversible encephalopathy syndrome
PVDF	polyvinylidene difluoride
SDS	sodium dodecyl sulphate
SGA	small for gestational age
SLE	systemic lupus erythematosus
STBM	syncytiotrophoblast-derived microparticles
TBS-T	tris-buffered saline containing 0.05% tween 20
TGF-ß1	transforming growth factor-β1
VEGF-A	vascular endothelial growth factor-A
VEGF-C	vascular endothelial growth factor-C
VWF	von Willebrand factor

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VWF:Ag	von Willebrand factor antigen

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1. Introduction

1.1. Preeclampsia: Significance, Definition, Epidemiology

The preeclampsia is still a major obstetrical issue, leading to maternal and fetal complications, affecting the maternal and fetal morbidity and even the mortality (1, 2). The definition of the preeclampsia has been changing over the last decades. The preeclampsia is one of the hypertensive disorders in pregnancy. This condition is a multisystem progressive pregnancy specific disorder, characterised by new onset of hypertension and proteinuria or the new onset of hypertension with a significant end-organ dysfunction with or without proteinuria typically presenting after 20 weeks of gestation or postpartum (3). Preeclampsia is estimated to occur in 2 to 5 percent of all pregnancies in the United States, Canada and Western Europe, (4, 5) and is one of the leading causes of maternal morbidity. Annually, preeclampsia is responsible for over 70,000 maternal deaths and 500,000 fetal deaths worldwide (3). The incidence varies in geographical regions. The lowest incidence as low as 0.4% reported from Vietnam (6). While the condition is especially common in women indigenous to, or with ancestry from, sub-Saharan Africa (7).

Many authors distinguish between the early onset and the late onset preeclampsia. The reason is the differences in both the neonatal and maternal complications of the disease, which occur more often before the 34 weeks of pregnancy and are more severe than after the 34th weeks of pregnancy (8). Some of the risks factors for the preeclampsia like obesity, diabetes, advanced maternal age, chronic hypertension are becoming more often among the pregnant woman occurring growing incidence of the preeclampsia in the western countries (9). Preeclampsia is a significant contributor both fetal and maternal health (10).

Maternal complications:

- Placental abruption
- Disseminated coagulopathy
- HELLP syndrome
- Pulmonary oedema/aspiration
- Acute renal failure

- Liver failure or haemorrhage
- Eclampsia
- PRES
- Stroke
- Death
- Long-term cardiovascular morbidity

Fetal complications

- Preterm delivery
- Fetal growth restriction
- Hypoxia-neurologic injury
- Perinatal death
- Long-term cardiovascular morbidity associated with low birthweight (fetal origin of adult disease)

Preeclampsia plays a substantial role in maternal deaths. It has been reported that in low-to-middle income countries, preeclampsia and similar hypertensive pregnancy disorders are the cause of death for 14% of mothers (11).

The most severe maternal complications affect the central nervous system, kidney, liver, lungs, and the haemostasis. The definitive therapy of the preeclampsia is terminating the pregnancy. This results in iatrogenic preterm delivery. This iatrogenic preterm delivery and the disease-characteristic intrauterine growth restriction are responsible for most of the neonatal complications. 15% of all preterm deliveriesare iatrogenic due to the treatment of preeclampsia and fetal growth restriction (11). The neonate is usually admitted to the intensive care unit, and in severe cases the maternal treatment could be necessary as well, causing tremendous medical expenses worldwide.

Long-term consequences of the preeclampsia could affect not only the mother but her child as well. We know the following long-term maternal complications: chronic hypertension, coronary artery diseases, cardiovascular mortality, venus thromboembolism, renal insufficiency, type II, diabetes mellitus, hypothyreosis, cognitive and psychological disturbances. The newborn could be affected from hypertension in its adult age, insulin resistance, diabetes mellitus, neurological diseases,

mental illness. In addition to preeclampsia, the preterm delivery and the intrauterine growth restriction are contributing to these diseases.

There are several clinical risk factors to preeclampsia, which should be evaluated by a detailed history of the pregnant woman during the antenatal care, usually at first clinic session (13, 14, 15). Previous preeclampsia, chronic hypertension, antiphospholipid syndrome, pregestational diabetes, use of assisted reproductive technologies and obesity (pre-pregnancy BMI >30 kg/m^2) show the strongest association with preeclampsia.

1.2. The pathogenesis of preeclampsia

The first recorded mention of preeclampsia dates back to around 400 BC when Hippocrates described the symptoms of headache, heaviness, and convulsions during pregnancy as bad (16). However, the term “preeclampsia” was first used by François Boissier de Sauvages in the 18th century (16). Since then, many pioneers have contributed to the understanding of preeclampsia, but despite extensive research, the pathophysiology of this relevant disease is still not entirely understood.

A series of epidemiologic data outlines the crucial role of immunologic factors in the development of preeclampsia (17). The incidence of the disorder is higher among primiparas than among multiparas. The protective effect of multiparity vanishes if the pregnancy is conceived from a new partner. (18). The incidence of preeclampsia is also higher when pregnancy is achieved through oocyte, sperm, or embryo donation (19). Maternal-fetal immunologic maladaptation is the main factor contributing to the development of preeclampsia, according to these observations (17, 20).

1.2.1. The two-stage model of preeclampsia

The two-stage model of preeclampsia is a widely accepted theory that explains the pathogenesis of preeclampsia as a sequential two-step occurrence. Abnormalities in the development of placental vasculature early in pregnancy may result in relative placental underperfusion/hypoxia/ischemia, which then leads to release of antiangiogenic factors into the maternal circulation that alter maternal systemic endothelial function and cause hypertension and other manifestations of the disease (hematologic, neurologic, cardiac, pulmonary, renal, and hepatic dysfunction). The first stage is the pathological development of the placenta due to deficient trophoblastic invasion, which occurs

during the first half of pregnancy. These conditions lead to the second stage, which is the development of maternal symptoms during the second half of pregnancy (21). The first stage is also referred to as the preclinical phase, while the second is known as the clinical phase. Immunological procedures are involved in both stages, although the contributing antigens and effectors vary between the stages forming different "immunological interfaces" (20).

1.2.2. The immunology of the first stage

The first immunological interface occurs between maternal immunocompetent cells, including uterine NK cells, macrophages, T-lymphocytes, and dendritic cells, and extravillous trophoblastic cells.

The interaction between uterine natural killer (NK) cells and the cytotrophoblast plays a crucial role in regulating trophoblastic invasion. When activated by extravillous trophoblastic cells, NK cells produce various cytokines such as interferon (IFN)-γ, granulocyte-macrophage colony-stimulating factor (GM-CSF), macrophage inflammatory protein-1α (MIP-1α), colony-stimulating factor-1 (CSF-1), leukemia inhibitory factor (LIF), and angiogenic factors including VEGF-A, VEGF-C, PlGF, angiopoietin-1, angiopoietin-2, and transforming growth factor β1. These factors collectively control trophoblastic invasion and remodeling (22-25).

1.2.3. The immunology of the second stage

The second interface is formed by the circulating maternal immune cells, such as T-lymphocytes, monocytes, NK cells, and dendritic cells, and the syncytiotrophoblasts (26).

The uteroplacental malperfusion resulting from defective trophoblastic invasion and inadequate remodeling leads to placental hypoxia and ischemia. (27). Obstructive lesions are observed in the spiral arteries of the placenta in preeclamptic patients. These lesions are frequently accompanied by thrombosis of the spiral arteries, further impeding placental perfusion (28).

The placenta in pre-eclampsia experiences both oxidative stress and endoplasmic reticulum stress, both strongly linked to inflammation. These stressors release soluble factors into the maternal circulation, contributing to the manifestation of maternal

symptoms associated with preeclampsia (20). The maternal preeclampsia is associated with a systemic inflammatory response, marked by the activation of leukocytes and endothelial activation (29).

The systemic endothelial activation and dysfunction lead to vasoconstriction, hypertonus, and increased vessel permeability, resulting in edemas. Glomerular endotheliosis accompanied by proteinuria is observed in the kidneys, while microthrombi form in blood vessels due to the activation of the clotting mechanism and platelets (30). A widespread inflammatory response within blood vessels coincides with systemic oxidative stress and triggers an acute phase reaction. Levels of positive acute phase proteins increase, while negative acute phase proteins decrease, and these changes are also linked to alterations in metabolism (20, 31-34).

The components of the maternal systemic inflammatory response in preeclampsia (34):

• Clotting system activation (35)
• Complement activation (36-38)
• Endothelial cell activation
• Elevated plasma/serum concentrations of pro-inflammatory cytokines (TNF-α, interleukin-6 and -8, (IL-6, IL-8)
• Leukocyte activation / Leukocytosis
• Platelet activation

1.2.4. Altered expression of placental angiogenic and anti-angiogenic factors

Several key angiogenic factors are associated with placental angiogenesis, and their aberrant expression and release into the maternal circulation is thought to contribute to the pathophysiology of preeclampsia (39).

Soluble fms-like tyrosine kinase-1 (sFlt-1) is the naturally occurring form of the Flt-1 receptor in the circulation. The Flt-1 receptor serves as the endothelial receptor for both VEGF-A and PlGF. sFlt-1 binds with high affinity to and antagonizes the circulating angiogenic VEGF-A and PlGF (40). Increased placental expression of sFlt-1 has been observed in preeclampsia, resulting in elevated concentrations of sFlt-1 in the circulation. The elevated serum sFlt-1 concentration is associated with reduced levels of free VEGF-A and PlGF in maternal circulation. Additionally, sFlt-1 inhibited the VEGF-A and PlGF-induced relaxation of rat renal arterioles. Furthermore,

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administration of an sFlt-1 adenovirus vector to pregnant rats led to hypertension, proteinuria, and glomerular endotheliosis, characteristic of preeclampsia (41). On the other hand, treatment with recombinant VEGF-A or PlGF alleviated symptoms induced by sFlt-1 overexpression in experimental animals (42-43). The increase in circulating sFlt-1 levels and the decrease in free PlGF levels precede the clinical manifestations of preeclampsia by weeks (44).

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2. Objectives

1. Our objective was the examination of plasma von Willebrand factor cleaving protease (ADAMTS13) activity, von Willebrand factor (VWF) antigen levels, and von Willebrand factor multimer structure in preeclampsia.

2. Analysis of systemic activation of the complement system via the classical, lectin, and alternative pathways in preeclampsia.

3. Methods

3.1. Study patients

Our study on ADAMTS13 utilized a case-control design. It included 67 preeclamptic patients, 70 healthy pregnant women with uncomplicated pregnancies, and 59 healthy non-pregnant women. Similarly, our investigation into the activation of the complement system followed a case-control approach, involving 60 preeclamptic patients, 60 healthy pregnant women, and 59 healthy non-pregnant women. Participants were recruited from the 1st Department of Obstetrics and Gynecology and the Department of Obstetrics and Gynecology of the Kútvölgyi Clinical Center at Semmelweis University in Budapest, Hungary. All participants were Caucasian and lived in the same geographic area in Hungary. Exclusion criteria included multifetal pregnancies, chronic hypertension, diabetes mellitus, autoimmune diseases, angiopathy, renal disorders, maternal or fetal infections, and fetal congenital anomalies. All participants were fasting, and no pregnant women were in active labor or had ruptured membranes. The healthy non-pregnant women were in the early follicular phase of their menstrual cycle (days 3 to 5) and were not using hormonal contraception.

Preeclampsia was defined by elevated blood pressure (≥140 mmHg systolic or ≥90 mmHg diastolic on two or more occasions at least 6 hours apart) after 20 weeks of gestation in women with previously normal blood pressure, along with proteinuria (≥0.3 g/24 hours). Blood pressure normalized by 12 weeks postpartum in all preeclamptic patients. Severe preeclampsia was identified by either a blood pressure of ≥160 mmHg systolic or ≥110 mmHg diastolic, or proteinuria of ≥5 g/24 hours. Women with eclampsia or HELLP syndrome were excluded from the study. Early onset preeclampsia was defined as occurring before 34 weeks of gestation (between 20 and 33 completed weeks). Fetal growth restriction was diagnosed if the fetal birth weight was below the 10th percentile for gestational age and gender, based on Hungarian birth weight percentiles (45).

The study protocol received approval from the Regional, Institutional Committee of Medical Ethics at Semmelweis University, and written informed consent was obtained from each participant. The study was conducted in accordance with the Declaration of Helsinki.

3.2. Sampling, preparation, and storage of biological specimens

Blood samples were obtained from an antecubital vein and collected into plain and EDTA-anticoagulated tubes. The samples were then centrifuged at room temperature with a relative centrifugal force of 3000×g for 10 minutes. The resulting serum and plasma aliquots were stored at −80°C until analysis.

3.3. Determination of plasma ADAMTS13 activity

The fluorogenic substrate FRETS-VWF73 was obtained from Peptides International (Louisville, KY, USA) and used to measure ADAMTS13 enzyme activity following the supplier's protocol with slight modifications (46). In brief, citrated plasma samples were diluted 1:20 in assay buffer (5 mM Bis-Tris, 25 mM CaCl2, 0.005% Tween 20, pH 6.0) and combined with 5 µM FRETS-VWF73 substrate solution (20 µl each) in white 384-well plates. Fluorescence readings were taken at 37°C every 2 minutes for 1 hour using a Chameleon microplate reader (Hidex, Turku, Finland) equipped with a 340 nm excitation and a 460 nm emission filter. The reaction rate was determined by performing linear regression analysis of the fluorescence over time. A standard curve was created using a two-fold dilution series of normal human plasma (pooled from citrated plasma samples of 10 healthy blood donors), with 100% ADAMTS13 activity set to the reaction rate observed in the 1:20 diluted sample. The intra-assay coefficient of variation (CV) was below 5%, and the inter-assay CV ranged from 6% to 9% (measured at 60% and 100% activity levels). Additionally, ADAMTS13 enzyme activity was measured in six plasma samples using the collagen-binding assay, as previously described (47). Pooled citrated plasma from 10 healthy blood donors was used as a reference. The results from the FRETS-VWF73 assay were consistent with those from the collagen-binding method (Spearman $R=0.82$; $p<0.05$).

3.4. Determination of plasma VWF antigen (VWF:Ag) levels

VWF antigen (VWF:Ag) levels in citrated plasma were quantified using an enzyme-linked immunosorbent assay (ELISA) with commercially available antibodies from Dakopatts (Glostrup, Denmark). Microtiter plates (Immunoplate Maxisorb, Nunc, Roskilde, Denmark) were coated with polyclonal rabbit anti-human VWF antibody, diluted 1:800 in sodium bicarbonate buffer at pH 9.6, and incubated overnight at 4°C. The plates were then washed with Tris-buffered saline containing 0.05% Tween 20 (TBS-T). Plasma samples, diluted 1:500, or standard control plasma, with a dilution range from 1:250 to 1:8,000, were added to the plates and incubated for 2 hours at room temperature. After washing again with TBS-T, HRP-conjugated polyclonal rabbit anti-human VWF antibody was added and incubated for 1 hour. This was followed by the addition of ortho-phenylenediamine (OPD) as the substrate. The optical density was read at 492 nm. Results were expressed as percentages relative to a standard composed of pooled plasma from 10 healthy blood donors.

3.5. Analysis of the multimer structure of von Willebrand factor

We examined the multimeric pattern of VWF in EDTA plasma samples using sodium dodecyl sulfate (SDS)-agarose gel electrophoresis. In short, low-resolution (0.8%) agarose gels (Seakem HGT, Lonza, Basel, Switzerland) were prepared on glass plates using Tris-Borate buffers as recommended by the agarose manufacturer. Electrophoresis was carried out at a constant current of 20 mA per gel for 6 hours on a plate cooled to 18°C. Proteins were then transferred to PVDF membranes (Immobilon-P, Millipore Corp., Bedford, MA, USA) using a tank electro-blotting method. To improve the transfer of the largest VWF molecules, the gels were treated with 1 mM β-mercaptoethanol (mercaptolysis) for 10 minutes before blotting (48). VWF was detected by immunostaining with HRP-labeled polyclonal rabbit anti-human VWF antibody (DakoCytomation, Glostrup, Denmark) and DAB substrate. Quantitative multimer analysis was performed as described previously (49). Membranes were digitized using a GS-800 (Bio-Rad Laboratories, Richmond, CA, USA) calibrated densitometer and analyzed with QuantityOne software (Bio-Rad Laboratories). The percentage of large multimers, defined as oligomers larger than the 20-mer (band 10), was calculated using

reflective density versus relative front data and was used to describe VWF multimer distribution.

3.6. Assessment of the complement system

Levels of C3a, Bb, C4d, and SC5b9 were measured using Quidel ELISA kits (San Diego, California, USA) following the manufacturer's instructions (Cat. No.: A015, A027, A008, A029, respectively). Radial immunodiffusion was performed to quantify C3, C4, C9, and C1-inhibitor levels using antibodies from DiaSorin (DiaSorin, Inc., Stillwater, Minnesota, USA). C9 values are expressed as a percentage of normal pooled human serum, while C3, C4, and C1-inhibitor assays were calibrated using a serum protein calibrator.

Factor H and C4b-binding protein antigens were measured with homemade sandwich ELISA assays using monoclonal–polyclonal antibody pairs. For C4b-binding protein, sheep polyclonal PC026 (The Binding Sites, Birmingham, UK) and mouse monoclonal A215 (Quidel) were used; for factor H, sheep polyclonal PC030 (The Binding Sites) and mouse monoclonal A229 (Quidel) were used. The factor H assay was calibrated against pooled human serum and expressed as a percentage, while the C4b-binding protein assay was calibrated against purified C4b-binding protein (Hyphen BioMed, Neuville sur Oise, France).

3.7. Determination of MBL–MASP2 Activity

The activity of MBL–MASP2 was determined using the C4 fixation method (50, 51) with minor additional changes. In brief, microtiter plates (Nunc Maxisorp F96, Roskilde, Denmark) were coated with 1 mg/ml mannan from Saccharomyces cerevisiae (Sigma–Aldrich M7504, St. Louis, USA) in 0.1M Na2CO3, pH 9.6 (coating buffer) for 1 hour at 37°C. The mannan solution was pre-treated with Bio-Bead SM-2 adsorbents (Bio-RAD 152-8920, Berkeley, CA, USA) to remove detergent traces detectable in some batches. The wells were then washed three times with 20 mM HEPES, 140 mM NaCl, 5 mM EDTA, 0.1% Tween-20, pH 7.4 (blocking buffer) and blocked with 300 µl of blocking buffer for 2 hours at 4°C.

After washing with 20 mM HEPES, 140 mM NaCl, 5 mM CaCl2, 0.1% Tween-20, pH 7.4 (washing buffer), the mannan-coated wells were incubated with 50 µl of human serum samples diluted 1:1 in 40 mM HEPES, 2 M NaCl, 10 mM CaCl2, pH 7.4 (serum dilution buffer) for 1 hour at 4°C to capture MBL–MASPs. The wells were then washed twice with 20 mM HEPES, 1 M NaCl, 5 mM CaCl2, 0.1% Tween-20, pH 7.4 (high salt washing buffer) and three times with 20 mM HEPES, 140 mM NaCl, 5 mM CaCl2, 0.1% Tween-20, pH 7.4 (low salt washing buffer). C4 purified from fresh human serum (0.02 mg/ml, defined by the functionally active amount of C4 after purification) in 20 mM HEPES, 140 mM NaCl, 5 mM CaCl2, pH 7.4 (C4 buffer) was added to each well and incubated for 1 hour at 37°C. Following three washes with blocking buffer, 50 µl of goat anti-human C4 (Diasorin 81905, Stillwater, Minnesota, USA) diluted 1:1000 in blocking buffer was added and incubated for 1 hour at room temperature. The wells were washed three times with blocking buffer, and 50 µl of anti-goat peroxidase (Sigma–Aldrich M7504, St. Louis, USA) diluted 8000-fold in blocking buffer was added and incubated for 1 hour at room temperature.

After three washing steps with blocking buffer, the wells were developed using 50 µl of OPD (orthophenylenediamine, Dako S2045, Glostrup, Denmark) for 5 minutes. The reaction was stopped using 50 µl of 0.5 M H2SO4. Absorbance was read at 492/620 nm using a microplate reader. The extent of MBL–MASP2 mediated C4 fixation was determined by photometry and expressed as a percentage of standard normal human serum values.

3.8. Determination of other laboratory parameters

Standard laboratory parameters were measured by Cell-Dyn 3500 hematology analyzer (platelet count) or by a particle-enhanced immunoturbidimetric assay on an autoanalyzer with the manufacturer's kits (Cobas Integra 800, Roche, Mannheim, Germany, Cat. No.: 20764930).

3.9. Statistical analysis

The normality of continuous variables was assessed using the Shapiro–Wilk test. Since the continuous variables were not normally distributed, nonparametric statistical methods were employed. The Mann–Whitney U test was used to compare continuous

variables between two groups, and the Kruskal–Wallis analysis of variance (ANOVA) by ranks test was used for comparisons among multiple groups. Post-hoc tests for multiple comparisons of mean ranks were also conducted. Categorical variables were compared using Fisher's exact test and Pearson's χ^2 test. The Spearman rank-order correlation was used to calculate correlation coefficients.

All statistical analyses were performed using STATISTICA (version 8.0; StatSoft, Inc., Tulsa, Oklahoma, USA) and the Statistical Package for the Social Sciences (version 15.0 for Windows; SPSS, Inc., Chicago, Illinois, USA). A p-value of <0.05 was considered statistically significant.

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4. Results

4.1. The assessment of plasma ADAMTS13 activity, von Willebrand Factor Antigen levels, and von Willebrand Factor multimer structure in preeclampsia

4.1.1. Patient characteristics and standard laboratory parameters

The clinical characteristics and standard laboratory parameters of the study participants are comprehensively detailed in Table 1. An analysis of the data revealed that there was no statistically significant difference in the age distribution between the preeclamptic patients and the healthy pregnant and non-pregnant women, indicating that age was not a confounding factor in this study.

However, Table 1. highlights that a majority of the clinical features and laboratory parameters measured exhibited significant differences across the three study groups. These differences underscore the distinct physiological and biochemical profiles associated with preeclampsia compared to healthy pregnancy and non-pregnant states. Despite these variations, one notable exception was observed: there was no significant difference in the percentage of individuals with blood group 0 between the preeclamptic group and the healthy pregnant and non-pregnant groups. This detail, although not shown in the table, suggests that blood group 0 distribution was consistent across the groups, further emphasizing that the observed clinical and laboratory differences were not influenced by blood group distribution.

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Table 1. Overview of clinical features, standard laboratory metrics, plasma ADAMTS13 activity, and von Willebrand factor antigen (VWF) levels among healthy non-pregnant women, healthy pregnant women, and patients with preeclampsia

	Healthy Non-Pregnant Women (n=59)	Healthy Pregnant Women (n=70)	Preeclamptic Patients (n=67)
Age (years)	28 (23–35)	30 (28–32)	29 (26–33)
BMI at blood draw (kg/m²)	20.8 (19.6–22.9)	25.9 (24.2–27.7)\(b\)	30.0 (27.7–33.3)(b,d)
Smokers	14 (23.7%)	0 (0%)\(b\)	3 (4.5%)(a)
Primiparas	n.a.	45 (64.3%)	43 (64.2%)
Systolic blood pressure (mmHg)	115 (110–120)	110 (105–120)\(a\)	160 (154–180)(b,d)
Diastolic blood pressure (mmHg)	80 (70–80)	70 (60–80)\(b\)	100 (97–110)(b,d)
Gestational age at blood draw (weeks)	n.a.	35 (30–36)	38 (36–39)(d)
Gestational age at delivery (weeks)	n.a.	39 (38–40)	38 (37–40)(c)
Fetal birth weight (grams)	n.a.	3500 (3200–3800)	3200 (2450–3600)(d)
Fetal growth restriction	n.a.	0 (0%)	11 (16.4%)(d)
Platelet count (cells/µl)	194 (170–225)	192 (168–221)	214 (186–254)(a,c)
Serum BUN level (mM)	4.1 (3.5–4.8)	2.7 (2.1–3.2)\(b\)	3.4 (2.7–4.1)(a,d)
Serum creatinine level (µM)	66 (61–72)	47 (41–51)\(b\)	63 (55–70)(d)
Serum bilirubin level (µM)	9.3 (6.6–12.4)	5.1 (3.8–6.6)\(b\)	7.4 (5.8–9.3)(a,d)
Serum AST activity (U/l)	17 (15–20)	16 (12–21)	19 (15–24)
Serum ALT activity (U/l)	14 (12–17)	11 (7–16)	15 (11–21)(c)
Serum LDH activity (U/l)	154 (126–174)	159 (131–174)	192 (155–317)(b,d)
Serum CRP level (mg/l)	0.7 (0.5–1.8)	3.6 (1.7–7.3)\(b\)	6.7 (3.0–12.5)(b,c)
Plasma ADAMTS13 activity (%)	91.6 (78.5–104.4)	96.3 (85.6–116.2)	98.8 (76.5–112.8)
Plasma VWF:Ag level (%)	70.0 (60.2–87.3)	129.3 (105.1–182.8)\(b\)	187.1 (145.6–243.1)(b,d)

Data are presented as median (25–75 percentile) for continuous variables and as number (percent) for categorical variables. n.a.: not applicable; ADAMTS13: A Disintegrin-like And Metalloprotease with ThromboSpondin type 1 motif, member 13; BMI: body mass index; BUN: blood urea nitrogen; AST: aspartate aminotransferase; ALT: alanine aminotransferase; LDH: lactate dehydrogenase; CRP: C-reactive protein; VWF:Ag: von Willebrand factor antigen.
Statistical significance:
a $p<0.05$ versus healthy non-pregnant women
b $p<0.001$ versus healthy non-pregnant women
c $p<0.05$ preeclamptic patients versus healthy pregnant women
d $p<0.001$ preeclamptic patients versus healthy pregnant women

4.1.2. Plasma ADAMTS13 activity, VWF:Ag levels and VWF multimeric pattern

The ADAMTS13 and VWF:Ag assay results are summarized in Table 1 and depicted in Figure 1A and B. Notably, there was no significant difference in plasma ADAMTS13 activity among the three study groups. However, plasma VWF antigen levels were markedly higher in preeclamptic patients compared to both healthy pregnant and nonpregnant women. Adjustment for age, body mass index (BMI), and gestational age at blood draw using analyses of covariance (ANCOVA) did not alter these findings. To account for differences in gestational age at blood collection, we further divided the preeclamptic and healthy pregnant groups into gestational age subgroups. In these gestational age categories, no significant differences in plasma ADAMTS13 activity were observed between preeclamptic patients and healthy pregnant women. However, preeclamptic patients consistently exhibited significantly elevated plasma VWF:Ag levels compared to normotensive, healthy pregnant women across all gestational age categories. Within the preeclamptic patient group, no statistically significant differences were found in plasma ADAMTS13 activity or VWF:Ag levels based on disease severity (mild vs. severe preeclampsia), onset (late vs. early), or the presence of fetal growth restriction (data not shown).Additionally, we assessed the multimeric pattern of VWF in matched groups of preeclamptic patients, healthy pregnant women, and healthy non-pregnant women. Interestingly, the VWF multimeric pattern was normal in all studied individuals. The percentage of large multimers did not significantly differ between preeclamptic patients and healthy pregnant/non-pregnant women (median [range]: 24.7% [18.5–33.7], 27.3% [22.2–30.2], and 25.5% [20.2–28.8], respectively; $p = 0.45$). Representative examples of the intact multimeric structure of VWF are shown in Figure 2.

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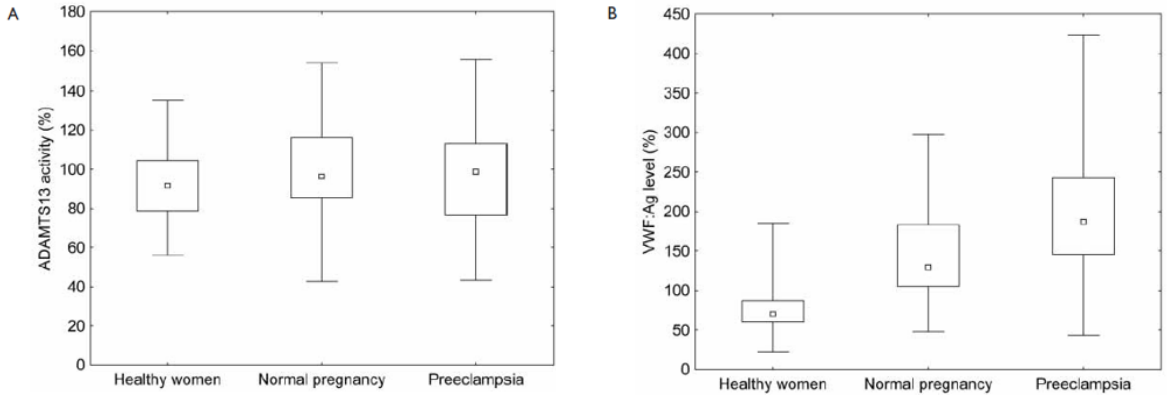

Figure 1. Plasma ADAMTS13 activity (A) and von Willebrand factor antigen (VWF:Ag) levels (B) of healthy non-pregnant and pregnant women and preeclamptic patients. Middle point: median; Box: interquartile range (25–75 percentile); Whisker: range. (52)

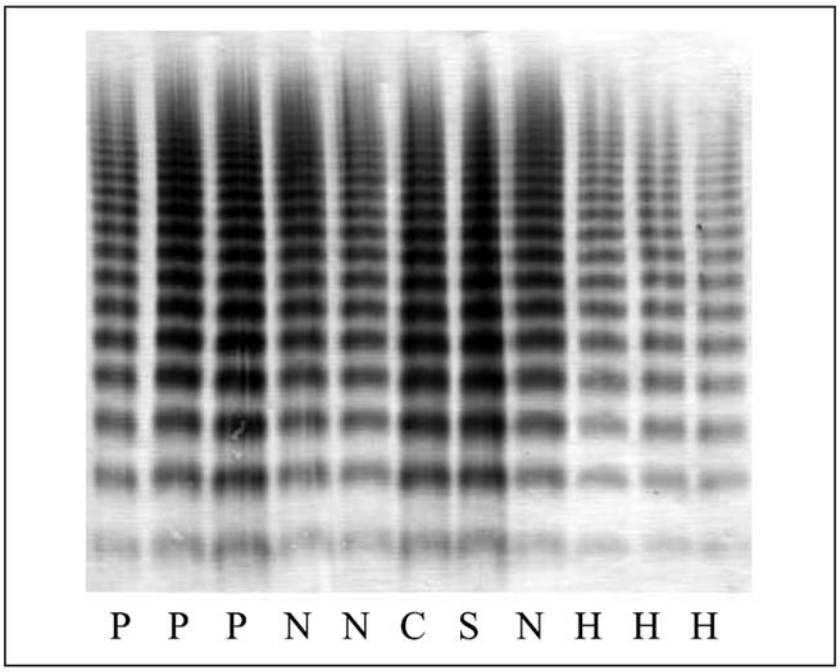

Figure 2. Representative examples of the intact multimeric structure of von Willebrand factor (VWF) in healthy non-pregnant women (H), healthy pregnant women (N) and preeclamptic patients (P) (C: fresh pooled plasma of (5) healthy volunteers; S: Dade Behring Standard Human Plasma), (52).

4.1.3. Relationship of clinical characteristics, standard laboratory parameters and plasma VWF:Ag levels to plasma ADAMTS13 activity

We also examined the relationships between clinical characteristics, standard laboratory parameters, and plasma VWF:Ag levels with plasma ADAMTS13 activity in our study groups. We observed a trend where primiparous women had lower plasma ADAMTS13 activity compared to multiparous women in both the healthy pregnant and preeclamptic groups. This difference was statistically significant when considering all pregnant women in the study (median 25–75 percentile): 92.6 (75.8–110.6) % for primiparas versus 104.2 (92.1–120.8) % for multiparas; p=0.011. In preeclamptic patients, there was a significant negative correlation between plasma VWF:Ag levels and ADAMTS13 activity (Spearman R=-0.26; p=0.040), but this significance was lost after adjusting for maternal age, primiparity, BMI, and gestational age at blood draw in a multiple linear regression analysis (standardized regression coefficient (β)=-0.11; p=0.48). No significant correlations between VWF:Ag levels and ADAMTS13 activity were found in the healthy pregnant and non-pregnant groups, or in the overall study population (Fig. 3). Additionally, no other clinical or laboratory parameters were significantly associated with plasma ADAMTS13 activity in any of the study groups.

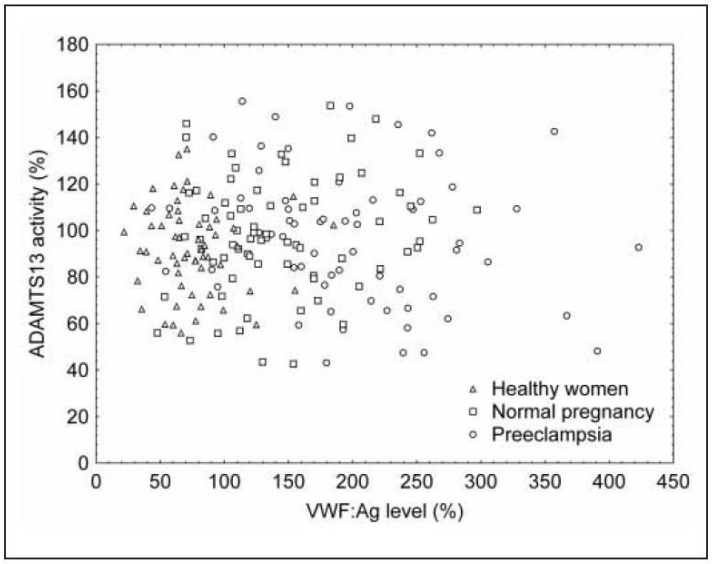

Figure 3. Scatterplot of plasma ADAMTS13 activity versus von Willebrand factor antigen (VWF:Ag) levels in healthy nonpregnant and pregnant women and preeclamptic patients (52).

4.2. Assessment of the complement system in preeclampsia

4.2.1. Patient characteristics

The clinical characteristics of the study participants are outlined in Table 2. There were no statistically significant differences in age among the three study groups. Additionally, the gestational age at blood collection and the percentage of primiparas did not differ significantly between preeclamptic patients and healthy pregnant women. However, all other clinical characteristics listed in Table 2 showed significant differences among the study groups.

Table 2. Clinical characteristics of healthy non-pregnant and pregnant women and preeclamptic patients.

	Healthy non-pregnant women (n = 59)	**Healthy pregnant women (n = 60)**	**Preeclamptic patients (n = 60)**
Age (years)	28 (23–35)	30 (28–32)	29 (26–32)
BMI at blood draw (kg/m²)	20.8 (19.6–22.9)	25.8 (24.3–27.9)[a]	29.9 (26.9–33.3) [a, b]
Smokers	14 (23.7%)	0 (0%) [a]	3 (5.0%) [c]
Primiparas	n.a.	37 (61.7%)	38 (63.3%)
Systolic blood pressure (mmHg)	115 (110–120)	110 (107–120)	162 (155–180) [a, b]
Diastolic blood pressure (mmHg)	80 (70–80)	70 (60–80) [a]	100 (97–110) [a, b]
Gestational age at blood draw (weeks)	n.a.	36 (36–37)	37 (36–39)
Gestational age at delivery (weeks)	n.a.	39 (38–40)	38 (37–39) [b]
Fetal birth weight (g)	n.a.	3450 (3150–3700)	3125 (2450–3475) [b]
Fetal growth restriction	n.a.	0 (0%)	11 (18.3%) [b]

Data are presented as median (25–75 percentile) for continuous variables and as number (percent) for categorical variables. n.a.: not applicable; BMI: body mass index

[a] $p < 0.001$ versus healthy non-pregnant women

[b] $p < 0.001$ preeclamptic patients versus healthy pregnant women

[c] $p < 0.05$ versus healthy non-pregnant women

4.2.2. Circulating levels of C-reactive protein, complement proteins, their activation fragments and regulatory factors in healthy non-pregnant and

pregnant women and preeclamptic patients

The findings from the C-reactive protein and complement assays are presented in Table 3. Healthy pregnant women exhibited significantly higher levels of CRP, C4d, C3a, SC5b9, C3, C9, and factor H antigen, along with significantly lower levels of C1-inhibitor compared to non-pregnant women. Preeclamptic patients showed significantly elevated levels of CRP, C4d, C3a, and SC5b9, and significantly reduced C3 concentrations compared to healthy pregnant women. When compared to healthy non-pregnant women, preeclamptic patients had higher levels of CRP, C4d, C3a, SC5b9, C4, C3, C9, and factor H antigen, and lower levels of C1-inhibitor. There were no significant differences in Bb and C4b-binding protein levels among the three study groups. Subgroup analysis revealed that preeclamptic patients with fetal growth restriction had significantly higher plasma SC5b9 levels than those without IUGR (median (interquartile range): 112.3 (59.1–147.5) ng/ml versus 69.9 (46.7–107.8) ng/ml, respectively; $p < 0.05$).

Table 3. Circulating levels of C-reactive protein and complement components in healthy non-pregnant and pregnant women and preeclamptic patients.

	Healthy non-pregnant women (n = 59)	Healthy pregnant women (n = 60)	Preeclamptic patients (n = 60)
Serum CRP (mg/l)	0.7 (0.5–1.8)	3.6 (1.7–6.6) [a]	6.8 (2.7–12.1) [a, b]
Plasma C4d (µg/ml)	0.04 (0.02–0.06)	0.11 (0.08–0.15) [a]	0.16 (0.10–0.21) [a, b]
Plasma Bb (µg/ml)	0.12 (0.10–0.14)	0.11 (0.09–0.15)	0.12 (0.10–0.14)
Plasma C3a (ng/ml)	85.5 (29.7–173.8)	751.6 (194.6–1660) [a]	1358 (854.8–2142) [a, b]
Plasma SC5b9 (ng/ml)	32.5 (20.5–52.8)	59.9 (42.1–86.6) [a]	75.9 (50.8–116.3) [a, b]
Serum C4 (g/l)	0.26 (0.24–0.31)	0.28 (0.24–0.35)	0.31 (0.25–0.40) [c]
Serum C3 (g/l)	1.48 (1.28–1.64)	1.79 (1.60–1.91) [a]	1.56 (1.45–1.78) [b, c]
Serum C9 (NHS%)	102 (91–119)	131 (115–150) [a]	127 (113–143) [a]
Serum C1-inhibitor (g/l)	0.21 (0.19–0.23)	0.18 (0.16–0.20) [a]	0.19 (0.17–0.22) [c]
Serum C4b-binding protein (µg/ml)	836.7 (625.5–1105)	823.9 (610.2–1004)	790.1 (614.5–980.8)
Serum factor H antigen (NHS%)	125.1 (99.3–163.0)	181.5 (143.3–230.1) [a]	193.3 (171.0–234.7) [a]

Data are presented as median (25–75 percentile) for continuous variables. CRP: C-reactive protein; NHS: normal, pooled human serum. [a] $p < 0.001$ versus healthy non-pregnant women, [b] $p < 0.05$ preeclamptic patients versus healthy pregnant women, [c] $p < 0.05$ versus healthy non-pregnant women

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The relative levels of complement regulatory factors in proportion to the corresponding complement proteins are depicted in Fig. 4 a–c. Serum C1-inhibitor levels were significantly lower in relation to serum C4 concentrations in both healthy pregnant women and preeclamptic patients compared to healthy non-pregnant women. There was a trend for healthy pregnant women to have lower serum C4b-binding protein concentrations relative to C4 levels compared to healthy non-pregnant women. Preeclamptic patients had significantly lower C4b-binding protein/C4 ratios than healthy non-pregnant women. However, there were no significant differences in the C1-inhibitor/C4 and C4b-binding protein/C4 ratios between preeclamptic and healthy pregnant women.

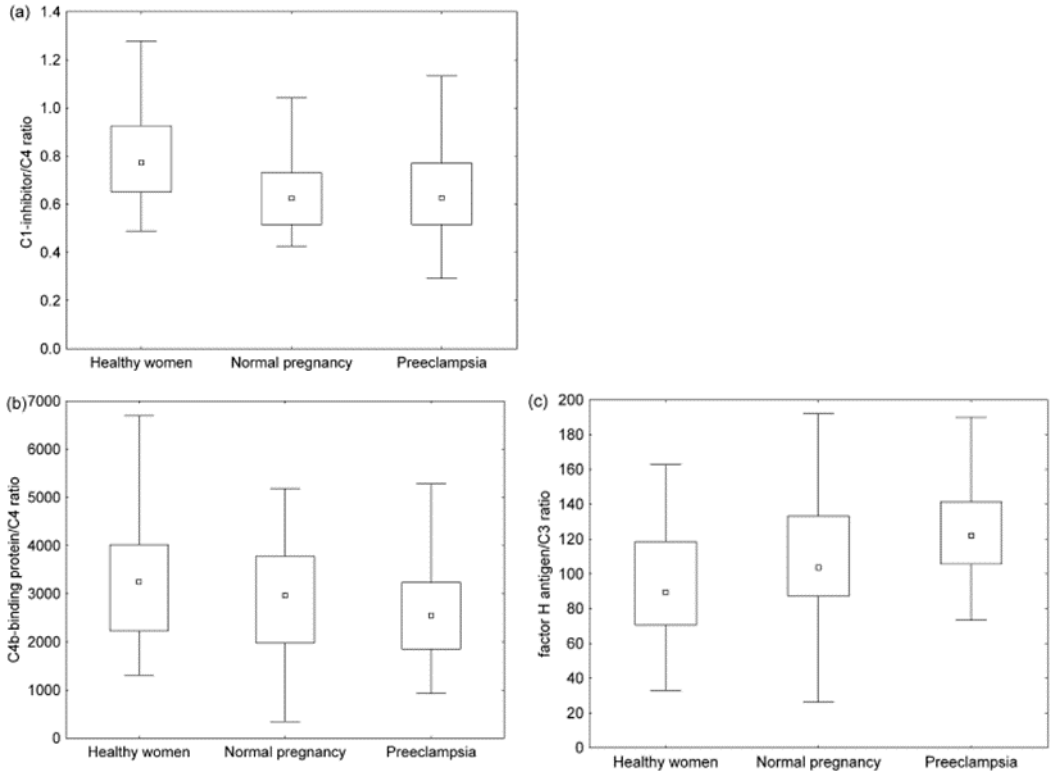

Fig. 4. C1-inhibitor/C4 (a), C4b-binding protein/C4 (b) and factor H antigen/C3 ratios (c) of healthy non-pregnant and pregnant women and preeclamptic patients. Middle point: median; Box: interquartile range (25–75 percentile); Whisker: range. (53)

The factor H antigen/C3 ratios were significantly higher in both healthy pregnant women and preeclamptic patients compared to healthy non-pregnant women. This ratio was also significantly different between preeclamptic and healthy pregnant women. The levels of classical, lectin, or alternative pathway split products in relation to their parent proteins are shown in Fig. 5 a and b, respectively. Fig. 5c illustrates the relative levels of C3a to C3 in our study groups. Both preeclamptic and healthy pregnant women had significantly higher C4d/C4 and C3a/C3 ratios compared to healthy non-pregnant women. Additionally, preeclamptic patients had significantly higher C4d/C4 and C3a/C3 ratios than healthy pregnant women.

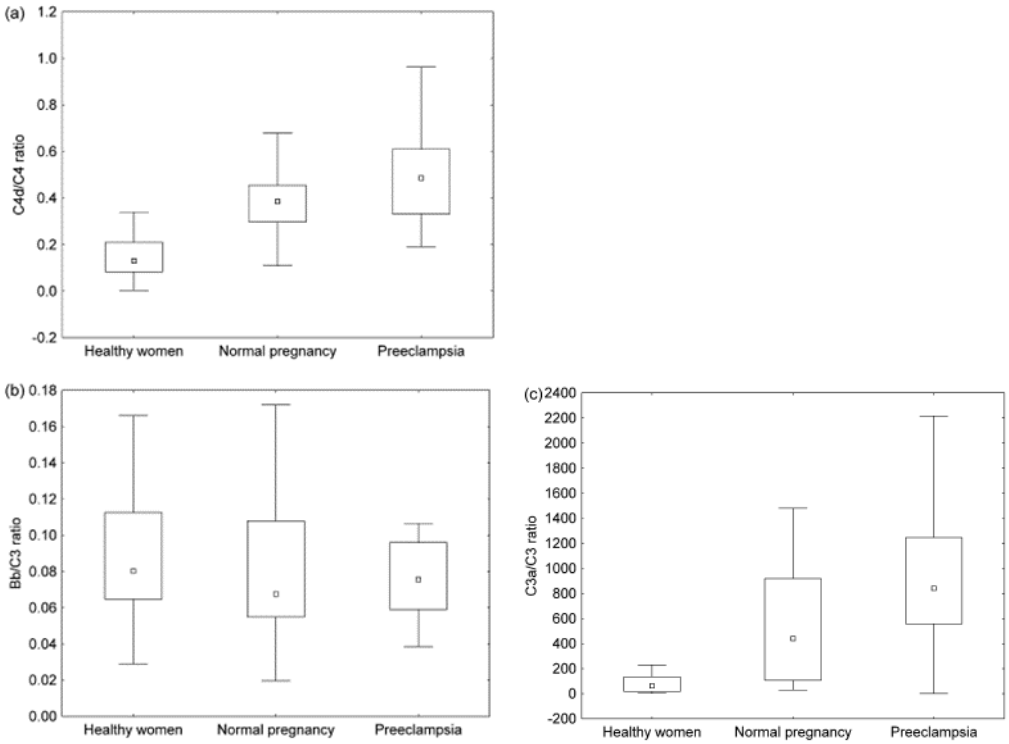

Fig. 5. C4d/C4 (a), Bb/C3 (b) and C3a/C3 ratios (c) of healthy non-pregnant and pregnant women and preeclamptic patients. Middle point: median; Box: interquartile range (25–75 percentile); Whisker: range (53).

Nevertheless, Bb/C3 ratios did not differ among the three study groups. As shown in Fig. 6, activation of the classical or lectin pathway (C4d) positively correlated with C3 activation (C3a) in both healthy pregnant women and preeclamptic patients (for healthy pregnant women: Spearman $R = 0.51$, $p < 0.001$, Fig. 6a; for preeclamptic patients:

Spearman R = 0.47, p < 0.001, Fig. 6b). The correlation between C3 and terminal pathway activation (SC5b9 versus C3a concentrations) was significant only in preeclamptic patients (R = 0.44, p < 0.001, Fig. 6d), but not in healthy pregnant women (R = 0.19, p = 0.14, Fig. 6c). We also explored the relationship between serum CRP levels and concentrations of complement components in our study groups. In healthy non-pregnant women, there was a significant positive correlation between serum CRP and C9 concentrations (Spearman R = 0.42, p < 0.05). In healthy pregnant women, there was a trend towards increasing serum C3 concentrations with rising serum CRP levels (Spearman R = 0.29, p = 0.06). In preeclamptic patients, serum CRP levels positively correlated with concentrations of C3a, C4, C9, C1-inhibitor, and C4b-binding protein (Spearman R = 0.38, 0.32, 0.52, 0.45, and 0.36, respectively, p < 0.05 for each). No other significant relationships were found between CRP levels and complement variables in any study group.

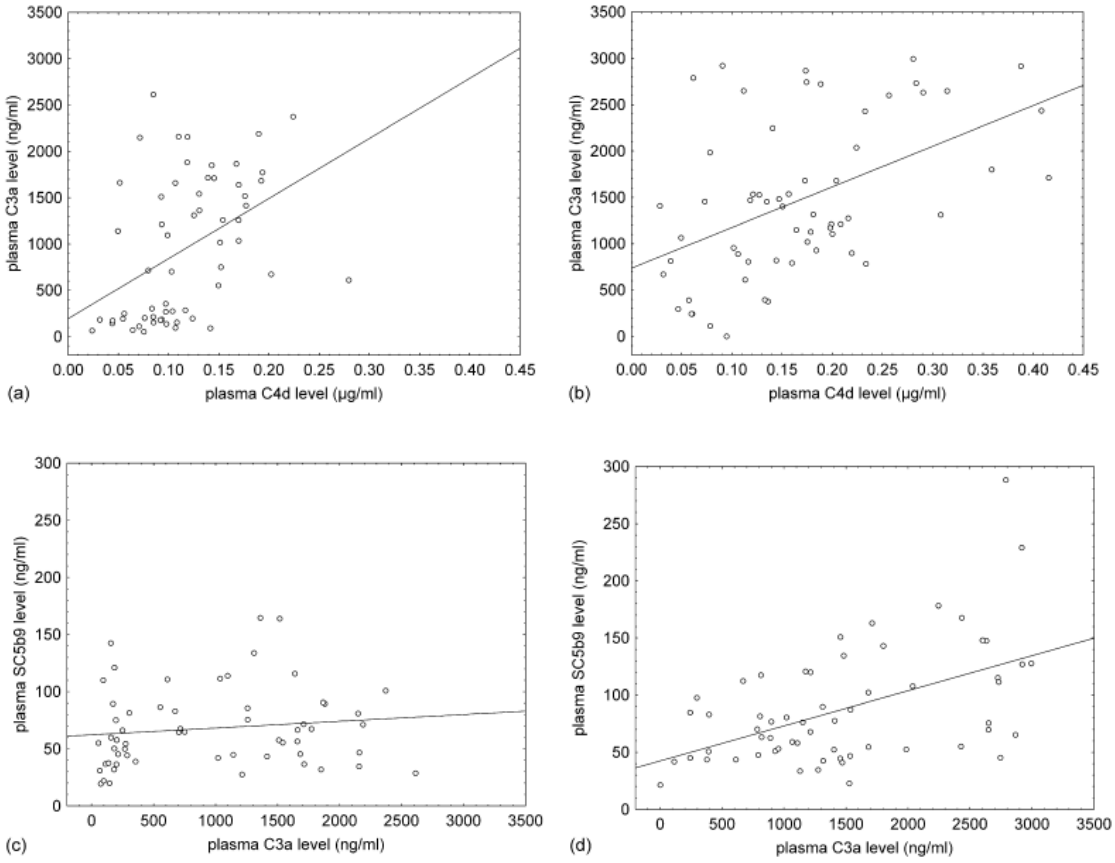

Fig. 6. Scatterplots of C3a versus C4d and SC5b9 versus C3a concentrations in healthy pregnant women (a and c) and preeclamptic patients (b and d), (53).

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4.2.3. Mannose-binding lectin complement pathway activity in normal pregnancy and preeclampsia

The MBL–MASP2 complex is the key in the early activation of the mannose-binding lectin pathway and is characterized by its C4 splitting activity. We found higher activity level of the MBL–MASP2 complex in healthy pregnant women compared to non-pregnant women [126.6 (81.2–142.7)% vs. 92.1 (6.8–113.1)%, $p < 0.001$]. In preeclamptic patients, we measured similar MBL–MASP2 activity levels to those of healthy pregnant women [113.9 (0.0–140.3)% vs. 126.6 (81.2–142.7)%, $p > 0.05$]. As illustrated in Fig. 7, a larger proportion of both preeclamptic and healthy pregnant women exhibited elevated MBL–MASP2 activity compared to healthy non-pregnant women, although there was no significant difference between the two pregnant groups. Additionally, MBL–MASP2 activity did not correlate with clinical characteristics of preeclamptic patients, such as disease onset, severity, growth restriction, or birth weight.

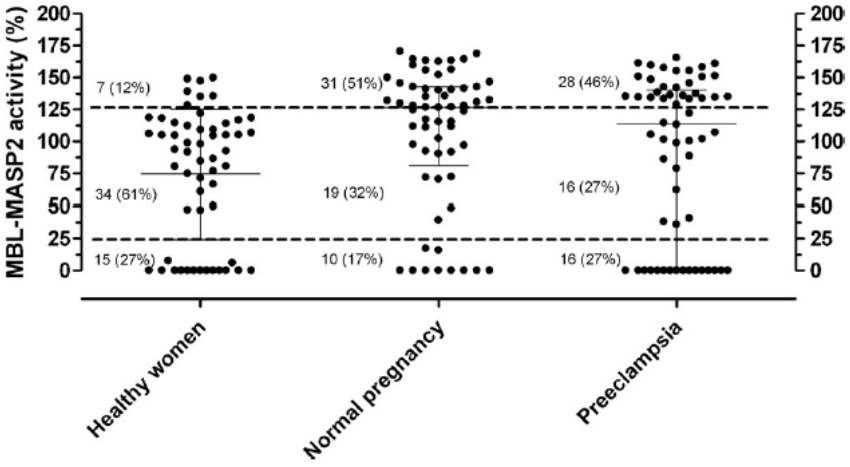

Fig. 7. illustrates the functional activity of the mannose-binding lectin pathway in healthy non-pregnant, healthy pregnant, and preeclamptic patients, measured by the activity of the MBL–MASP2 complex. The dashed horizontal lines indicate 1 SD below and above the mean of the healthy non-pregnant group, used to define deficient, normal, and high MBL–MASP2 activity ranges. The total number (with percentages) of subjects in each category is indicated. $p < 0.0001$ (chi-square test), (54).

4.2.4. Lack of relationship between mannose-binding lectin pathway activity and pathological complement activation in preeclampsia

To further examine the potential role of the mannose-binding lectin pathway in the pathological complement activation observed in preeclampsia, categories for deficient, normal, and high MBL–MASP2 activity were established. Using 1 SD below and above the mean of the healthy non-pregnant group as cut-off points, the corresponding values (25% and 125% activity values, respectively) were used to define deficient (0–25%), normal (25–125%), and high activity (>125%) groups. These cut-off points were intended to separate the groups rather than define strict "cut-off" levels. Typically, the mean plus 2 SDs is used to define "positivity," but in this case, due to sample variance and the presence of deficient, sufficient, and elevated subgroups, the mean plus 1 SD rule was applied. Tables 4 A–4C display results for various complement proteins, regulators, and activation products based on the functional activity of the MBL–MASP2 complex. No association was found between deficient, normal, or high activity and complement activation products (C4d, Bb, C3a, SC5b9), complement proteins (C4, C3, C9), complement regulatory factors (C1-inhibitor, C4b-binding protein, factor H antigen), and the acute phase reactant CRP in healthy non-pregnant (Table 4A), healthy pregnant (Table 4B), and preeclamptic (Table 4C) groups.

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Table 4 A. The levels of complement components and CRP in healthy non-pregnant women (n=56), in subgroups defined on the basis of MBL–MASP2activity.

	MBL–MASP2 activity		
	Deficient (<25%)	**Normal (25–125%)**	**High (>125%)**
CRP (mg/l)	0.56 (0.43–1.49)	1.05 (0.60–2.32)	0.62 (0.35–0.96)
C4d (μg/ml)	0.05 (0.02–0.06)	0.04 (0.02–0.07)	0.03 (0.03–0.06)
Bb (μg/ml)	0.11 (0.10–0.15)	0.12 (0.10–0.14)	0.13 (0.06–0.15)
C3a (ng/ml)	38.0 (16.4–173.8)	114.0 (29.7–204.9)	92.0 (21.4–136.1)
SC5b9 (ng/ml)	37.2 (24.0–71.6)	32.9 (21.2–55.6)	19.0 (8.4–43.5)
C4 (g/l)	0.27 (0.25–0.31)	0.26 (0.23–0.31)	0.31 (0.26–0.33)
C3 (g/l)	1.53 (1.32–1.69)	1.40 (1.26–1.56)	1.53 (1.38–1.75)
C9 (NHS%)	109 (84–122)	102 (96–113)	91 (81–146)
C1-inhibitor (g/l)	0.20 (0.18–0.22)	0.22 (0.20–0.23)	0.20 (0.18–0.28)
C4b-binding protein (μg/ml)	769.4 (623.2–1294)	810.7 (559.7–1044)	1081 (856.7–1164)
Factor H antigen (NHS%)	130.1 (93.3–165.6)	125.1 (106.6–167.4)	113.2 (91.1–159.4)

No significant differences were found using the Kruskal–Wallis analysis of variance by ranks test.

Table 4 B. The levels of complement components and CRP in healthy pregnant women (n=60), in subgroups defined on the basis of MBL–MASP2 activity.

	MBL–MASP2 activity		
	Deficient (<25%)	**Normal (25–125%)**	**High (>125%)**
CRP (mg/l)	2.12 (1.41–3.72)	3.15 (1.68–6.47)	4.11 (2.99–6.89)
C4d (μg/ml)	0.10 (0.08–0.15)	0.10 (0.07–0.12)	0.12 (0.09–0.17)
Bb (μg/ml)	0.11 (0.09–0.12)	0.11 (0.09–0.19)	0.11 (0.09–0.22)
C3a (ng/ml)	884.9 (354.3–1640)	1236 (182.9–1683)	611.4 (194.6–1542)
SC5b9 (ng/ml)	65.7 (42.1–89.4)	46.1 (34.6–81.0)	66.3 (49.9–86.6)
C4 (g/l)	0.26 (0.24–0.31)	0.28 (0.22–0.35)	0.31 (0.26–0.36)
C3 (g/l)	1.79 (1.50–1.85)	1.79 (1.60–1.85)	1.79 (1.60–1.97)
C9 (NHS%)	129 (120–141)	129 (115–160)	131 (111–146)
C1-inhibitor (g/l)	0.16 (0.15–0.18)	0.18 (0.16–0.19)	0.19 (0.16–0.21)
C4b-binding protein (μg/ml)	941.7 (777.1–1029)	660.7 (417.6–861.0)	853.4 (618.4–1064)
Factor H antigen (NHS%)	210.7 (176.6–230.9)	178.0 (77.7–227.3)	182.9 (146.5–284.3)

No significant differences were found using the Kruskal–Wallis analysis of variance by ranks test.

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Table 4 C. The levels of complement components and CRP in preeclamptic women (n=60), in subgroups defined on the basis of MBL–MASP2 activity.

	MBL–MASP2 activity		
	Deficient (<25%)	**Normal (25–125%)**	**High (>125%)**
CRP (mg/l)	7.83 (4.56–13.08)	5.26 (2.90–11.73)	7.78 (1.67–10.75)
C4d (µg/ml)	0.16 (0.10–0.24)	0.13 (0.09–0.16)	0.18 (0.11–0.22)
Bb (µg/ml)	0.12 (0.10–0.15)	0.11 (0.09–0.17)	0.12 (0.10–0.14)
C3a (ng/ml)	1607 (1244–2515)	1405 (642.2–1760)	1117 (813.0–1875)
SC5b9 (ng/ml)	83.3 (43.4–135.0)	72.8 (52.4–109.1)	75.9 (52.1–105.0)
C4 (g/l)	0.35 (0.28–0.45)	0.29 (0.24–0.37)	0.31 (0.24–0.45)
C3 (g/l)	1.62 (1.48–1.75)	1.56 (1.45–1.69)	1.54 (1.39–1.84)
C9 (NHS%)	128 (113–154)	124 (114–137)	131 (111–143)
C1-inhibitor (g/l)	0.19 (0.18–0.20)	0.18 (0.16–0.20)	0.21 (0.17–0.22)
C4b-binding protein (µg/ml)	824.7 (704.9–1007)	763.4 (554.3–971.2)	782.9 (663.2–1013)
Factor H antigen (NHS%)	188.1 (173.1–213.3)	178.1 (133.4–209.2)	202.3 (189.8–251.5)

No significant differences were found using the Kruskal–Wallis analysis of variance by ranks test.

5. Discussion

5.1. The assessment of plasma ADAMTS13 activity, von Willebrand Factor Antigen levels, and von Willebrand Factor multimer structure in preeclampsia

We observed no significant difference in plasma ADAMTS13 activity between preeclamptic patients and healthy pregnant or non-pregnant women. However, plasma VWF antigen levels were significantly higher in the preeclamptic group compared to the healthy groups. The VWF multimeric pattern appeared normal in all groups. Significantly lower plasma ADAMTS13 activity were reported in patients with HELLP syndrome compared to healthy pregnant and non-pregnant women (55). This reduction was not due to inactivating autoantibodies and normalized upon remission. The higher plasma VWF antigen levels, ristocetin cofactor, and collagen binding activities in HELLP syndrome patients are also known, without the presence of ultralarge VWF multimers. It was proposed, that elevated VWF levels may lead to reduced ADAMTS13 activity due to the increased need to process the excess VWF. Preeclamptic patients were not included in that study.

These findings were later confirmed (56), showing increased plasma VWF antigen and active VWF levels, along with moderately decreased ADAMTS13 activity in preeclamptic patients with HELLP syndrome. In preeclamptic patients without HELLP syndrome, only plasma VWF antigen levels were elevated compared to healthy pregnant women, while active VWF levels, ristocetin cofactor, and ADAMTS13 activities were similar to controls, aligning with our findings. However, their study focused mainly on HELLP syndrome, with only six non-HELLP preeclamptic patients included. They concluded that acute endothelial cell activation and decreased ADAMTS13 activity in HELLP syndrome lead to increased active VWF, which may explain thrombocytopenia and platelet-rich thrombi formation.

The main observation from our study is that, despite higher plasma VWF antigen levels, plasma VWF cleaving protease activity is normal in preeclampsia. While low ADAMTS13 activity has been noted in other conditions with elevated VWF antigen levels, the biological significance is unclear (57-64). It might be due to the protease being consumed or sequestered by the endothelial cells (65-67). However, our results indicate that ADAMTS13 activity is not always low when VWF:Ag levels are high. In

preeclampsia, ADAMTS13 activity appears to be regulated differently from VWF:Ag levels, without severe deficiency indicated by normal ADAMTS13 activity and absence of ultralarge VWF multimers. This suggests that VWF:Ag levels' consumptive regulation is secondary in preeclampsia, as liver and renal failure, DIC, and significant inflammation were not observed in our preeclamptic group.

HELLP syndrome, a severe condition distinct from preeclampsia, involves more intense systemic inflammation and endothelial injury, influencing ADAMTS13 activity. Previous studies link ADAMTS13 deficiency with severe inflammatory responses. In HELLP syndrome, acute endothelial activation might result in increased active VWF levels, contributing to thrombocytopenia and microthrombi formation. This hypothesis is supported by our findings and the previously mentioned studies investigating the ADAMTS13 activity in HELLP syndrome (55, 56), suggesting that a combination of high VWF levels and low ADAMTS13 activity could lead to HELLP syndrome. In addition to plasma exchange applied in refractory postpartum cases, recombinant ADAMTS13 could be a potential treatment, pending further research.

5.2. Assessment of the complement system in preeclampsia

We conducted a large cross-sectional study to measure the circulating levels of various complement components to explore the role of the complement system in preeclampsia. This study included healthy non-pregnant and pregnant women as controls. Our results indicate that the complement system is activated through the classical and/or lectin pathways, leading to increased terminal complex formation in the third trimester of normal pregnancy and even more so in preeclampsia, as evidenced by elevated concentrations of activation markers in the bloodstream.

There are several potential reasons for the activation of the classical pathway in both healthy pregnancies and preeclampsia. During normal pregnancy, circulating immune complexes are present in significant amounts, with a further increase observed in preeclampsia, suggesting an imbalance between the production and removal of these immune complexes (68, 69). We also observed a stepwise increase in serum CRP levels in healthy pregnant women and preeclamptic patients, with CRP levels correlating positively with C3a concentrations in preeclamptic patients, supporting the activation of the classical pathway in preeclampsia. Additionally, double-stranded DNA can activate

the classical pathway (70), and cell-free DNA from both maternal and fetal origins have been detected in the maternal plasma during normal pregnancy, with higher levels in preeclampsia (71, 72). Our previous studies have shown that heat shock protein 70 (Hsp70) is a potent activator of the classical pathway (73), and elevated serum Hsp70 levels have been observed in preeclamptic patients compared to healthy pregnant women (74-76). Apoptotic cells can also activate the classical pathway; in normal pregnancies and more so in preeclamptic pregnancies, increased trophoblast apoptosis and the shedding of trophoblast debris into the maternal circulation may contribute to systemic complement activation (77-79).

The activity of the lectin pathway has also been shown to increase during normal pregnancy (80). Elevated levels of circulating mannose-binding lectin (MBL) and certain MBL genotypes have been associated with preeclampsia, although some contradictory data exist (81-83). Ficolins, another set of pattern recognition proteins involved in the lectin pathway, have also been implicated in preeclampsia (84). Various factors can regulate the levels of native complement components such as C3, C4, and C9, as well as complement regulatory proteins in normal pregnancy and preeclampsia, which might explain conflicting reports in the literature (85-88). In our study, positive correlations between serum CRP and levels of C3, C4, C9, C1-inhibitor, and C4b-binding protein suggest increased synthesis of these components as part of the acute phase reaction characteristic of both healthy pregnancy in the third trimester and preeclampsia. However, the concurrent consumption of complement factors might result in unchanged or decreased levels of these proteins, as observed with C4b-binding protein, C3, and C1-inhibitor. The relative deficiency of C1-inhibitor and C4b-binding protein in healthy pregnant women and preeclamptic patients, indicated by decreased C1-inhibitor/C4 and C4b-binding protein/C4 ratios, suggests decompensation of classical and/or lectin pathway regulatory mechanisms, allowing further activation of the complement cascade leading to terminal complex formation. As long as the maternal system can control complement activation, no pathological symptoms manifest. However, an imbalance favoring complement activation might contribute to the development of preeclampsia.

Elevated levels of the complement activation fragment Bb in the first 20 weeks of pregnancy were independently associated with preeclampsia later in gestation (89),

suggesting early pregnancy complement activation is involved in preeclampsia's pathogenesis. Although apoptotic processes can also activate the alternative complement pathway, we did not detect elevated Bb levels in overt preeclampsia (90). The relative abundance of factor H in the third trimester, indicated by increased factor H antigen/C3 ratios, might prevent this complement activation route despite the presence of placental apoptotic debris in maternal circulation. Further studies should investigate complement regulators and activation to show the dynamics of complement activation and dysregulation during normal and pathological pregnancies.

An interesting finding of our study is that preeclamptic patients with fetal growth restriction had significantly higher plasma SC5b9 levels than those without. Preeclampsia with fetal growth restriction involves reduced placental perfusion (91). Excessive terminal complement activation in these cases might reflect increased release of apoptotic trophoblast debris from the ischemic placenta into maternal circulation. Complement activation with enhanced terminal complex formation might also contribute to fetal growth restriction. Complement deposits are common in acute atherosis, strongly associated with IUGR (92). The membrane attack complex colocalized with fibrin deposits at injury sites in villous trophoblast, especially in placentas from IUGR or preeclampsia pregnancies, and MAC binding enhanced apoptosis in cytotrophoblasts under low oxygen tension (93). In a mouse model of spontaneous miscarriage and IUGR, complement activation disrupted angiogenic factors needed for normal placental development (94).

Systemic complement activation may connect placentally derived inflammatory stimuli and the maternal syndrome of preeclampsia. Several clinical symptoms and pathogenetic features of preeclampsia could be explained by complement activation (95). Anaphylatoxins (C3a, C4a, and C5a) can enhance vascular permeability and induce smooth muscle contraction (96), and they are chemotactic factors for leukocytes (97). C5a and the terminal complex can activate monocytes and neutrophils, releasing biologically active inflammatory mediators like proteases, free oxygen radicals, and pro-inflammatory cytokines (98-102). Recent data on thrombotic microangiopathy associated with ADAMTS13 deficiency show complement activation can directly cause endothelial injury (103).

Activation markers in the systemic circulation may indicate systemic complement activation. However, the lack of correlation between C3a and SC5b9 levels in normal pregnancy, and isolated excess SC5b9 levels in preeclampsia with fetal growth restriction, suggests increased terminal complex concentrations may also result from local placental activation and release of SC5b9 into maternal circulation with syncytiotrophoblast-derived microparticles (STBM).

Since our study could not differentiate between the roles of the classical and mannose-binding lectin pathways in pathological complement activation in preeclampsia, we aimed to clarify this issue. To investigate the mannose-binding lectin (MBL) complement pathway in a disease, several approaches can be used. These include measuring recognition components like MBL or serine protease (MASP-2) as antigens in immunoassays or ligand assays. The C4 splitting activity of the functional MBL-MASP2 complex can also be measured (50), which is indicative of both allelic variation-based deficiency and alterations in the concentrations of affected proteins.

We observed increased mannose-binding lectin pathway activity in women with normal pregnancies compared to healthy non-pregnant controls, a finding consistent with previous reports. The increase in MBL concentration and lectin pathway activity during healthy pregnancy has been linked to various factors, including human growth hormone, placental growth hormone, cortisol, and fetal production.

However, we did not find an association between increased mannose-binding lectin pathway activity and preeclampsia. Previous studies on MBL in preeclampsia were largely based on genetic analyses, with only a few examining the amount or functional activity of MBL(81, 104, 105). Certain genetic variations associated with higher serum MBL levels were more frequent in women with preeclampsia, while variations leading to lower serum MBL levels seemed protective against the disorder (83). Our results, showing no association between MBL-MASP2 activity and preeclampsia, are consistent with the notion that only reduced MBL levels might offer protection against preeclampsia (83).

Our findings align with other studies (81, 84), suggesting that the mannose-binding lectin complement pathway has a minor role, if any, in the development of preeclampsia. Specifically, we found no link between MBL pathway activity and the clinical features of preeclampsia (onset, severity, fetal growth restriction, birth weight),

systemic inflammation (as indicated by CRP levels), or complement activation products and regulators.

Based on previous studies and our current observations, it appears that complement activation in preeclampsia likely occurs via the classical pathway. During normal pregnancy, immunocomplexes form with fetal trophoblast products as antigens. In preeclampsia, an excess of these immunocomplexes versus their removal triggers a proinflammatory cascade involving trophoblast apoptosis/necrosis and oxidative stress, leading to the disease's clinical symptoms. This classical pathway activation is evidenced by increased anaphylatoxin and membrane attack complex formation in preeclampsia.

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6. Conclusions

1. Despite the high level of von Willebrand factor antigen, the ADAMTS13 activity in blood plasma does not decrease in preeclampsia. The multimer structure of the von Willebrand factor remains intact in preeclampsia.

2. The complement system activation in preeclampsia occurs via the classical pathway and the mannose-binding lectin pathway may play only a minor role. The activation of the complement system leads to an increased formation of the terminal complex (SC5b9). The excessive amount of the terminal complex measured in the maternal circulation is associated with intrauterine growth restriction in pregnant women with preeclampsia.

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7. Summary

We investigated the activity of the plasma von Willebrand factor cleaving protease (ADAMTS13), von Willebrand factor (VWF) antigen levels, and the structure of von Willebrand factor multimers in preeclampsia. Additionally, we aimed to analyze the systemic activation of the complement system via the classical, lectin, and alternative pathways in preeclampsia. We found no significant differences in plasma ADAMTS13 activity among the three groups studied: preeclamptic pregnant women, healthy pregnant women, and healthy non-pregnant women. The plasma levels of von Willebrand factor antigen were significantly higher in preeclamptic pregnant women compared to healthy pregnant and non-pregnant women. We also examined the multimer structure of von Willebrand factor in 5 preeclamptic women, 5 healthy pregnant women, and 5 healthy non-pregnant women, matched for age, gestational age, and smoking status. The multimer structure of von Willebrand factor was intact in all groups, with no significant differences in the percentage of large multimers among the preeclamptic, healthy pregnant, and healthy non-pregnant women.

Our study on the activation of the complement system showed that the levels of CRP, C4d, C3a, SC5b9, C3, C9 and factor H antigen were significantly higher, while those of C1-inhibitor were significantly lower in healthy pregnant than non-pregnant women. In addition, preeclamptic patients had significantly higher CRP, C4d, C3a, SC5b9 levels and significantly lower C3 concentrations as compared to healthy pregnant women. Their CRP, C4d, C3a, SC5b9, C4, C3, C9 and factor H antigen levels were significantly higher, while C1-inhibitor concentrations were significantly lower compared with healthy non-pregnant women. Preeclamptic patients with fetal growth restriction had significantly higher plasma SC5b9 levels than those without IUGR.

Activation of the classical or lectin pathway(C4d) showed significant positive correlation to C3 activation (C3a) both in healthy pregnant women and preeclamptic patients. In our further study we could prove the subordinate role of the lectin pathway, indicating systemic activation of the complement system via the classical pathway, leading to increased formation of the terminal complex (SC5b9). The excessive amount of the terminal complex in the maternal circulation was associated with intrauterine growth restriction in preeclamptic pregnancies.

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