**Preeclampsia**

**Preeclampsia Is Characterized by Placental Complement Dysregulation**

Aletta Buurma, Danielle Cohen, Kimberley Veraar, Dorrith Schonkeren, Frans H. Claas, Jan A. Bruijn, Kitty W. Bloemenkamp, Hans J. Baelde

See Editorial Commentary, pp 1114–1116

**Abstract**—Increasing evidence suggests that preeclampsia is associated with complement dysregulation. The origin of complement dysregulation in preeclampsia is unknown, and further unraveling this mechanism could provide both diagnostic tools and therapeutic targets. Because the placenta is believed to play a crucial role in the pathogenesis of preeclampsia, we investigated placentas from preeclamptic women (n=28) and controls (n=44) for the presence of complement activation products. Immunohistochemistry was performed for C1q, mannose-binding lectin, properdin, and C4d. Staining patterns were related to pregnancy outcome. Possible causes of complement activation were investigated, including the presence of immune deposits at the syncytiotrophoblast and changes in the placental mRNA expression of complement regulatory proteins. C4d was rarely present in placentas from healthy controls (3%), whereas it was observed in 50% of placentas obtained from preeclamptic women (P=0.001). In these placentas, C4d was observed in a focal (9/14) or diffuse (5/14) staining pattern at the syncytiotrophoblast. With respect to C1q, mannose-binding lectin, and properdin, no differences were observed between cases and controls. In preeclamptic women, diffuse placental C4d was associated with a significantly lower gestational age at delivery. Furthermore, the mRNA expression of the complement regulatory proteins CD55 and CD59 was significantly upregulated in preeclampsia. In conclusion, there is evidence for increased classical pathway activation and altered complement regulation in preeclampsia. The relation between C4d and lower gestational age at birth suggests that the extent of complement dysregulation is associated with the severity of preeclampsia. Inhibiting excessive complement activation may be a promising therapeutic approach in the management of preeclampsia. (Hypertension. 2012;60:1332-1337.) • Online Data Supplement

**Key Words:** preeclampsia • classical complement pathway • mannose-binding lectin pathway

• alternative complement pathway • placenta • fetal-maternal interface • complement regulatory proteins

Preeclampsia (PE) is a devastating pregnancy-specific syndrome, complicating 2% to 8% of pregnancies and contributing significantly to maternal and fetal morbidity and mortality. The syndrome is characterized by endothelial dysfunction, presenting clinically as maternal hypertension and proteinuria after 20 weeks of gestation.

Since the late 1980s, it became apparent that PE is relatively common among pregnant women with autoimmune diseases such as systemic lupus erythematosus and the antiphospholipid syndrome. Antiphospholipid antibodies can directly bind to trophoblast and activate the classical complement cascade, causing placental dysfunction, trophoblast injury, and impaired pregnancy outcome. In human placentas of systemic lupus erythematosus and antiphospholipid syndrome patients, activation of the classical pathway of complement at the fetal-maternal interface can be detected in ≤70% of patients and is related to intrauterine fetal loss and PE.

The association between PE and systemic lupus erythematosus/antiphospholipid syndrome, combined with the observation that complement activation mediates pregnancy complications in these autoimmune diseases, suggests that the complement system could also be involved in the pathogenesis of PE in women without underlying autoimmune diseases. Indeed, preeclamptic women have elevated levels of circulating complement activation products and increased quantities of complement components in their placentas. Additionally, mutations in complement regulatory proteins predispose to PE. Altogether, there is increasing evidence indicating that dysregulation of the complement system might play a role in the development of PE. However, it is unknown what triggers complement activation at the fetal-maternal interface and...
which of the complement pathways is activated during PE. More insight in this mechanism is essential to develop better prevention and treatment strategies aimed at the immunological aspects of this serious pregnancy complication.

The placenta is believed to play a crucial role in the pathogenesis of PE. Therefore, we studied complement activation in placentas obtained from preeclamptic women and control subjects, using markers covering all complement activation pathways. Furthermore, possible associations between placental complement activation and the clinical manifestations of PE were investigated, as well as possible causes of placental complement dysregulation, including changes in the mRNA expression of complement regulatory proteins and the presence of immune deposits at the fetal-maternal interface.

Methods
Patient Selection and Placenta Collection
We studied 72 placentas obtained from women who delivered at the Leiden University Medical Center Department of Obstetrics between 2002 and 2011. For the case group, 28 placentas were obtained from women with PE. This group contained several women with superimposed PE (n=8). For the first control group, we included 30 placentas obtained from healthy women with uncomplicated pregnancies that resulted in live births. As a second control group, placentas were obtained from women whose pregnancy was affected by intrauterine growth restriction (IUGR, defined as birth weight below fifth percentile for gestational age) but not by PE. All preeclamptic women in this study delivered by cesarean section. Because it cannot be excluded that lengthy delivery may affect the extent and distribution of complement deposits, we aimed to mainly include controls who also delivered by cesarean section (63%). Detailed information on patient characteristics in each group can be found in Table 1. Tissue samples from a central part of the placenta were collected immediately after delivery. The study was approved by the ethics committee of the Leiden University Medical Center, and informed consent was obtained from all patients.

Immunohistochemistry
To investigate complement activation, immunohistochemistry was performed for the following complement components: C4d (a component of both the classical and mannose-binding lectin [MBL] pathway), C1q (specific for classical pathway activation), MBL (representing MBL pathway activation), properdin (specific for alternative pathway activation), C3d, and the neocantigen of the membrane attack complex or MAC (can both be formed by activation of any of the 3 pathways). Sections were deparaffinized and antigen retrieval was performed. After blocking for endogenous peroxidase, sections were incubated with antibodies to C1q (DakoCytomation; 1:800), C4d (Biomedica Gruppe; 1:50), MBL (Sigma-Aldrich Biotechnology; 1:500), properdin (1:800), C3d (Abcam; 1:800), and SC5b-9 (Quidel, San Diego, CA; 1:150). Binding of the primary antibody was visualized with appropriate secondary antibodies and hematoxylin. The IUGR group was included to determine how specific immunohistochemical and immunofluorescent staining were.

Immunofluorescence
To investigate the presence of immune deposits at the syncytiotrophoblast, double immunofluorescent staining was performed to identify cytokeratin-7 (KRT7, specifically staining the syncytiotrophoblast) concomitant with human immunoglobulins. Frozen sections of placentas were washed and fixed in a mixture of acetone and alcohol. Sections were incubated for 1 hour with rabbit antihuman IgA/IgG/IgM (DakoCytomation; 1:100) and mouse antihuman KRT7 (Dako Cytomation; 1:100). Subsequently, sections were incubated for 30 minutes with fluorescein isothiocyanate-labeled goat antirabbit (Sigma-Aldrich Biotechnology; 1:200) and tetramethyl rhodamine isothiocyanate-labeled goat antimouse (Sigma-Aldrich Biotechnology; 1:100) antibodies. For negative controls, the primary antibodies were substituted with normal mouse serum (DakoCytomation) and normal rabbit immunoglobulin (DakoCytomation) in the same concentrations as the primary antibodies. When immune deposits were observed in the combined IgA/IgM/IgG staining, immunofluorescence was performed separately for IgA, IgM, and IgG to determine which isotype was predominantly present.

Quantification of Immunohistochemical and Immunofluorescent Staining
Positivity for immunohistochemical staining was scored semiquantitatively by 2 independent observers. The staining intensity at the surface of the syncytiotrophoblast was scored as absent (<10%), focal (10%–50%), or diffuse (>50%). For immunofluorescence, slides were blindly scored by 2 independent observers for the absence or presence of immune deposits on the syncytiotrophoblast using a fluorescent microscope (DM5500B, Leica Instruments). When immune deposits were observed, we scored which immunoglobulin isotype was most prominently present.
RNA Extraction and Quantitative PCR Analysis
Quantitative PCR was performed to quantify the placental mRNA expression of the membrane-associated complement regulatory proteins CD55 (decay accelerating factor), CD46 (membrane cofactor protein), and CD59. RNA was isolated and reversed to cDNA using an AMV cDNA synthesis kit (Roche, Indianapolis, IN). SYBR Green quantitative PCR was performed according to the manufacturer’s protocol. Expression of complement regulatory proteins was measured by the comparative threshold cycle method and normalized to hypoxanthine phosphoribosyltransferase and GAPDH expression. The primer pairs (Table S1 in the online-only Data Supplement) were designed to span 21 intron to avoid amplification of genomic DNA along with cDNA. To verify the accuracy of amplification, a melting curve analysis was performed. All cDNA samples were treated in duplicate.

Statistical Analysis
Categorical variables were compared using either the χ² test or the Fisher exact test. Differences in quantitative parameters between groups were assessed using 1-way ANOVA (for data normally distributed) or Kruskal–Wallis H 1-way analysis (for data not normally distributed). Differences in means were compared using either the unpaired t test or the Mann–Whitney U test. All analyses were performed using SPSS statistical software package (version 17.0; Chicago, IL). A P value <0.05 was considered statistically significant.

Clinical Data
Maternal ages were comparable between PE and control subjects. As expected, blood pressure and proteinuria were significantly higher in the PE group. Additionally, the average gestational age at delivery, birth weight, and placenta weight were significantly lower as compared with the controls. These and additional clinical characteristics are provided in Table 1.

Immunohistochemistry
C4d was observed in 14 (50%) of 28 placentas obtained from women with PE, whereas it was rarely present in placentas from healthy subjects (1 of 30, 3%). In the IUGR group, 3 (21%) of 14 subjects showed placental C4d. Of these 3 women, 2 experienced gestational hypertension. When present, C4d was observed at the syncytiotrophoblast in either a focal or a diffuse staining pattern (Figure 1). Table 2 shows the incidence of the different C4d staining patterns; this is visualized in Figure 2. χ² analysis indicated a strong association between C4d and PE (P=0.001). No relation was observed between the moment of onset of PE and placental C4d staining patterns. Among women with superimposed PE (n=8), 3 did not show placental C4d, 4 had focal, and 1 had diffuse placental C4d.

C1q was observed at the syncytiotrophoblast and in intravillous endothelial cells (Figure 1). C1q was never completely negative, neither in cases nor in control subjects (data not shown). In cases of diffuse C4d, C1q and C4d colocalized. Properdin was not observed at the syncytiotrophoblast, but occasionally in intravillous endothelial cells (Figure 1), both in cases and healthy subjects (no statistically significant difference, data not shown). When subdividing women with PE according to their C4d staining pattern, no differences were observed in the amount and distribution of properdin deposits. MBL was absent in all placentas, whereas liver tissue that served as a positive control was clearly positive. C3d was frequently observed at the syncytiotrophoblast in a focal staining pattern. However, no relation was observed between the presence of C4d and the presence of C3d, and no significant differences were observed between cases and controls (data not shown). Staining for MAC was observed in areas of villous injury. It was rarely observed at the fetal-maternal interface, and, as a consequence, colocalization

Table 2. C4d in PE Placentas vs Controls

<table>
<thead>
<tr>
<th>C4d Staining Pattern</th>
<th>PE (n=28)</th>
<th>Healthy Controls (n=30)</th>
<th>IUGR Controls (n=14)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No C4d, %</td>
<td>14 (50)</td>
<td>29 (97)</td>
<td>11 (79)</td>
</tr>
<tr>
<td>Focal C4d, %</td>
<td>9 (32)</td>
<td>0 (0)</td>
<td>3 (21)</td>
</tr>
<tr>
<td>Diffuse C4d, %</td>
<td>5 (18)</td>
<td>1 (3)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

PE indicates preeclampsia; IUGR, intrauterine growth restriction. χ² test (overall comparison): P=0.001

Results

Figure 1. Sequential sections of placentas stained immunohistochemically for C4d, C1q, mannose-binding lectin (MBL), and properdin. Each column represents an individual placenta; the immunohistochemical stainings are shown horizontally. The first column shows a C4d-negative placenta obtained from a woman whose pregnancy was uncomplicated. The middle column shows a placenta obtained from a woman with preeclampsia (PE), showing a focal C4d staining pattern. In the third column, a placenta of a patient with PE is shown, which is diffusely positive for C4d. A to C show the different C4d staining patterns. B and C illustrate that C4d is present at the surface of the syncytiotrophoblast (arrowheads), and not within villi (*). D to F show examples of placental C1q staining. C1q was not exclusively present in C4d-positive placentas but also in placentas in which C4d was absent. This phenomenon is illustrated in D, which shows a C4d-negative placenta that is evidently C1q positive (arrowhead). However, E and F demonstrate that C1q (arrowheads) does colocalize with C4d in C4d-positive placentas. G to I show that MBL was never observed. J to L show typical examples of properdin staining, demonstrating that properdin is exclusively localized on endothelial cells of the fetal vessels (arrowhead).
Immunofluorescence

In placentas obtained from preeclamptic women, immune deposits were observed at the syncytiotrophoblast in 10 of 27 placentas. Staining for KRT7 confirmed that immune deposits were located at the syncytiotrophoblast (Figure 3). In 23% of the placentas with no or minimal C4d (n=13), immune deposits were present. Of the placentas with focal C4d (n=9), 44% showed immune deposits. Placentas with diffuse C4d (n=5) showed immune deposits in 60%. Although a trend was observed between the presence of immune deposits and C4d, this was not statistically significant (Figure 4). In most placentas that showed immune deposits, the predominant isotype was IgM (Figure 2 and Table S2).

Placental mRNA Expression of Complement Regulatory Proteins

The mRNA levels of CD59 were on average 4-fold increased in preeclamptic women as compared with controls (P<0.01). The placental mRNA expression of CD55 was also significantly higher in preeclamptic women, with on average an ≈2-fold increase as compared with healthy subjects (P<0.05). The mRNA expression levels of CD46 were comparable between groups. The relative mRNA expression levels are illustrated in Figure 5.

Relation Between C4d and Clinical Manifestations

The different C4d staining patterns were not associated with the height of blood pressure or the amount of urinary protein (Table S3). Within the PE group, diffuse placental C4d was associated with a significantly (P=0.03) lower gestational age at delivery, as compared with cases with focal or no C4d (Figure 6). All preeclamptic women delivered by cesarean section. Therefore, gestational age depended on a clinical decision rather than on a spontaneous onset of labor. Overall, no relationships were observed between the indications to end pregnancy and C4d staining patterns. Among preeclamptic women, diffuse C4d was also associated with lower birth weight (P=0.04). However, with respect to birth weight percentiles, differences between groups were not statistically significant.
Evidence of classical complement activation in a subset of women with PE raises several questions. First, one may wonder why not all preeclamptic women develop placental complement deposits. PE is considered a complex disease, in which many different variables affect the risk to develop the disorder. Consequently, attempts have been made to subdivide cases of PE into different phenotypical and etiological subgroups. Although speculative, women with placental C4d may represent such a specific etiological subgroup, which raises the question: what causes classical pathway activation in these placentas?

Generally, complement deposits can be the consequence of either excessive activation or inadequate regulation of the complement system. In the case of PE, classical pathway activation could result from the binding of circulating antibodies, comparable with pregnancy complications in patients with antiphospholipid syndrome, in which antiphospholipid antibodies bind to the syncytiotrophoblast and thereby activate the classical pathway. However, classical pathway activation is not exclusively triggered by immune complexes; it can also be induced by binding of C1q to apoptotic cells. In the current study, a trend was observed between placental C4d and the presence of immune deposits. This difference was not statistically significant. However, our groups were probably too small to reach statistical significance, and, therefore, the difference could be statistically significant when investigating larger samples. Importantly, in these immune deposits, IgM was the predominant isotype. IgM is known to bind to damaged tissue in the setting of ischemia-reperfusion injury, thereby activating the complement system. Therefore, the predominant presence of IgM suggests that immune deposits on the syncytiotrophoblast may represent a nonspecific reaction to injury rather than a specific antibody-mediated response against fetal antigens.

Because mutations in complement regulatory proteins predispose to PE, apparently not only excessive activation but also inadequate inhibition of the complement system may be involved in the development of PE. Deficient complement inhibition could possibly explain why in controls the classical pathway stops at the level of C1q, whereas in a subset of women with PE the classical pathway progresses beyond this level, resulting in the deposition of C4d. Because both C1 inhibitor and factor H can inhibit classical pathway activation, a (relative) shortage of these complement inhibitors could be responsible for classical pathway activation beyond the level of C1q. To investigate whether placental complement deposits in case of PE could be because of insufficient local regulation, we measured the placental mRNA expression of the complement regulatory proteins CD55 and CD59. In conclusion, placental classical pathway activation may be a novel diagnostic tool and therapeutic target in PE.

In the present study, C4d was observed at the fetal-maternal interface in approximately half of the placentas obtained from women with PE. In contrast, C4d was rarely present in control placentas. C4d appears to be relatively specific for hypertensive disorders of pregnancy, because it was infrequently observed in placentas from normotensive women with IUGR (1 of 11). When it was observed in IUGR placentas, these pregnancies were often (2 of 3) also complicated by gestational hypertension. Because C4d is a component of both the classical and MBL pathways, we investigated the presence of C1q and MBL in order to determine which pathway is responsible for C4d deposits. MBL was never observed, making it very unlikely that C4d deposits are a result of MBL pathway activation. In accordance with previous reports, C1q was observed at the syncytiotrophoblast both in physiological and pathologica pregnancies. When C4d was present in a diffuse staining pattern, it colocalized with C1q, indicating that C4d deposits are most likely the result of classical pathway activation.

Evidence of classical complement activation in a subset of women with PE raises several questions. First, one may wonder why not all preeclamptic women develop placental complement deposits. PE is considered a complex disease, in which many different variables affect the risk to develop the disorder. Consequently, attempts have been made to subdivide cases of PE into different phenotypical and etiological subgroups. Although speculative, women with placental

**Figure 6.** Gestational age (in days) in preeclamptic women with no, focal, or diffuse placental C4d. *P<0.05.
gestational age (27+1 weeks) at delivery. Because all PE pregnancies were ended by cesarean section, the initiation of birth was based on a clinical decision rather than on a spontaneous onset of labor. Apparently, in the cases with diffuse C4d, the fetal or maternal condition was severely threatened and did not allow for longer expectant management. This excessive placental complement activation consequently provides a lead for therapeutic intervention. Indeed, murine models have shown that complement inhibition is effective in abrogating PE manifestations.18,19 Additionally, heparin, which is known to inhibit complement activation,20 is effective in preventing recurrent early onset PE in women with inherited thrombophilia.21

Perspectives

In conclusion, we have demonstrated that PE is associated with the presence of placental C4d and with an upregulation of the mRNA expression of complement regulatory proteins. Placental C4d deposits are likely a result of excessive classical complement activation. Importantly, the presence of placental C4d is associated with the severity of PE. Altogether, the current data suggest that the complement system plays an essential role in the pathogenesis of PE and may be a novel therapeutic target in the management of PE.

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None.

References
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PREECLAMPSIA IS CHARACTERIZED BY PLACENTAL COMPLEMENT DYSREGULATION

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Table S1. Primers used for quantitative PCR

Figure S1. Images of placental C3d and MAC

Table S2. Predominant isotype in placental immunoglobulin deposits

Figure S2. Images of placental IgM, IgG and IgA deposits

Table S3. Clinical characteristics of women with preeclampsia and no, focal or diffuse placental C4d deposits
<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequences (F: forward, R: reverse)</th>
<th>Product size</th>
</tr>
</thead>
</table>
| CD46 | F: TTCCTGGCGGCTTTTCTGGGT  
R: GTTGGTGCCCTCCTCACAGGC | 84           |
| CD55 | F: TCCTGGCGAGAAGGACTCAGTGA  
R: AGCCTTGTGGGCACCTCGCA | 96           |
| CD59 | F: TGTCGCTCCITTCCAGGTTCTGT  
R: TGACGGCTTGTTTGCGAGTCAGC | 161          |
| HPRT | F: AGATGGTCAAGGTCGCAAGC  
R: TCAAGGGCATATCCTACAAACAAAC | 115          |
| GAPDH | F: TTCCAGGAGCGAGATCCCT  
R: CACCCATGACGAACATGGG | 175          |
<table>
<thead>
<tr>
<th>Predominant isotype</th>
<th>Number of placentas (n=10)</th>
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<tr>
<td>IgM (%)</td>
<td>9 (90)</td>
</tr>
<tr>
<td>IgA (%)</td>
<td>1 (10)</td>
</tr>
<tr>
<td>IgG (%)</td>
<td>0 (0)</td>
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Table S3. Clinical characteristics of the women with preeclampsia and absent, focal or diffuse placental C4d deposits

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>C4d absent (n = 14)</th>
<th>Focal C4d (n = 9)</th>
<th>Diffuse C4d (n = 5)</th>
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<tbody>
<tr>
<td>Mean maternal age in years (SD)</td>
<td>32.2 (5)</td>
<td>32.2 (4)</td>
<td>28.8 (9.7)</td>
</tr>
<tr>
<td>Mean maternal BMI (kg/m^2) (SD)</td>
<td>27.7 (5.9)</td>
<td>24.0 (11)</td>
<td>28.1 (6.2)</td>
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<tr>
<td>Mean gravidity (SD)</td>
<td>1.9 (1.2)</td>
<td>2.1 (1.6)</td>
<td>2.8 (3.5)</td>
</tr>
<tr>
<td>Mean parity (SD)</td>
<td>0.6 (1.1)</td>
<td>0.8 (0.8)</td>
<td>1.0 (2.2)</td>
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<td>Highest diastole (mmHg) (SD)</td>
<td>110 (17)</td>
<td>104 (10)</td>
<td>106 (8)</td>
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<tr>
<td>Proteinuria (g/24h) (SD)</td>
<td>6.5 (5.8)</td>
<td>3.5 (3.8)</td>
<td>4.6 (5.3)</td>
</tr>
<tr>
<td>Mean gestational age at delivery</td>
<td>31+0 (11)</td>
<td>31+0 (13)</td>
<td>27+1 (8) *†</td>
</tr>
<tr>
<td>(weeks + days; SD in days)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Birth weight (g) (SD)</td>
<td>1278 (296)</td>
<td>1150 (326)</td>
<td>887 (226) *</td>
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<tr>
<td>Birth weight percentile</td>
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<td>&lt;5 (%)</td>
<td>0 (0)</td>
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<td>50-80 (%)</td>
<td>3 (21.4)</td>
<td>2 (22.2)</td>
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<tr>
<td>Gender</td>
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<td></td>
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<tr>
<td>Female (%)</td>
<td>9 (64)</td>
<td>4 (44)</td>
<td>1 (20)</td>
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<tr>
<td>Male (%)</td>
<td>5 (36)</td>
<td>5 (56)</td>
<td>4 (80)</td>
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<tr>
<td>Indication to end pregnancy</td>
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<tr>
<td>Fetal</td>
<td>5 (36)</td>
<td>3 (33)</td>
<td>3 (60)</td>
</tr>
<tr>
<td>Maternal</td>
<td>6 (43)</td>
<td>4 (44)</td>
<td>1 (20)</td>
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<tr>
<td>Combination</td>
<td>3 (21)</td>
<td>2 (22)</td>
<td>1 (20)</td>
</tr>
</tbody>
</table>

* denotes a statistically significant difference compared with C4d absent
† denotes a statistically significant difference compared with focal C4d
Figure S1: Images of placental C3d and MAC

Legend Figure S1: Example images of C3d and MAC staining patterns. A to C show C3d staining patterns. When C3d was present, it was observed at the syncytiotrophoblast (arrowheads). This observation suggests that placental complement activation may progress beyond the level of C4d. D to F show typical MAC staining patterns. MAC was rarely observed at the syncytiotrophoblast. Therefore, co-localization with C4d was rare. This observation indicates that classical pathway activation does not necessarily result in the activation of the entire complement cascade, confirming that complement regulatory mechanisms may prevent placental complement-mediated damage.
Figure S2. Images of placental IgM, IgG and IgA deposits

Legend Figure S2: Images of separate IgM (left), IgA (middle) and IgG (right) staining in placentas obtained from women with preeclampsia. The upper row shows the various immunoglobulin-stained sections. Arrows indicate immunoglobulin deposits at the syncytiotrophoblast. The lower row shows cytokeratin-7 (KRT7) staining in the same sections.