Role of Complement Component C1q in the Onset of Preeclampsia in Mice

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Abstract—Preeclampsia (PE) is a life-threatening, pregnancy-induced disease and a leading cause of maternal and fetal morbidity and mortality. Despite considerable research, the causes of PE remain unclear, and there is no effective treatment. Studies in animal models that resemble this complex pregnancy-related disorder may help to identify possible therapies for PE. Complement component C1q has an important role in trophoblast migration, spiral arteries remodeling, and normal placentation. Here we show that pregnant C1q-deficient (C1q−/−) mice recapitulate the key features of human PE: hypertension, albuminuria, endotheliosis, decreased placental vascular endothelial growth factor (VEGF) and elevated levels of soluble VEGF receptor 1 (sFlt-1) that correlate with increased fetal death. In addition, decreased blood flow and increased oxidative stress are observed in placentas from C1q−/− mice. Treatment of C1q−/− mice with pravastatin restored trophoblast invasiveness, placental blood flow, and angiogenic balance and, thus, prevented the onset of PE. Serum-soluble receptors for VEGF-1 levels were reduced and placental VEGF levels were significantly increased in C1q−/− mice treated with pravastatin compared with untreated C1q−/− mice (VEGF: 1067±171 versus 419±194 pg/mL; P<0.01). Pravastatin treatment reduced hypertension (change in mean arterial pressure: 1±1 versus 18±3 mm Hg in C1q−/− untreated mice), and albuminuria (of creatinine) was reduced from 820±175 to 117±45 μg/mg (both P<0.01). Renal damage and endothelial dysfunction were significantly attenuated with pravastatin. This model that highlights the causative role of impaired trophoblast invasion in the pathogenesis of PE allowed us to identify pravastatin as a good therapeutic option to prevent PE. (Hypertension. 2011;58:716-724.) ● Online Data Supplement

Key Words: mouse model ■ preeclampsia ■ trophoblast invasion ■ complement ■ pravastatin

Preeclampsia (PE), a pregnancy-specific, multisystemic disorder, is a leading cause of maternal and perinatal mortality and morbidity.1 Because PE only occurs during pregnancy and its symptoms resolve after delivery, the placenta is thought to be crucial to the development of the disease. Indeed, several studies suggested that a defective trophoblast invasion and abnormal placentation are some of the underlying mechanisms of PE.2,3 Conversion of the maternal spiral arteries into larger competent vessels is one of the essential steps in the development of the normal placenta. This process is apparently dependent on the invasion by trophoblasts of the subendometrial area and the spiral arteries. PE is characterized by shallow trophoblast invasion and unconverted narrow spiral arteries that leads to placental dysfunction and endothelial injury that eventually manifest as maternal hypertension and proteinuria.4

The study of PE in women is of critical importance; however, studies in humans have obvious limitations that prevent investigation of many pathophysiological mechanisms and that often limit the ability to establish cause-and-effect relationships in pregnant women with PE. Thus, we studied a new animal model of PE that resembles this complex pregnancy-related disorder and helped us identify a possible treatment.

Several studies demonstrated a strong association between complement activation and PE.5–7 For the last 20 years, many studies reported the deleterious effects of complement activation on pregnancy outcomes. However, complement component C1q deserves special consideration for its role in promoting trophoblast invasion of deciduas, a crucial step in normal placentation development. C1q is widely distributed in human decidual stroma and is actively synthesized by migrating extravillous trophoblasts.8,9 In addition, we demonstrated previously that C1q deficiency was associated with impaired labyrinth development and decidual vessel remodeling and increased fetal death in mice.9 These results suggest that defective local production of C1q may be involved in PE.8–9 Indeed, here we demonstrate that pregnant C1q-deficient (C1q−/−) mice recapitulate the key features of human PE: hypertension, albuminuria, endotheliosis, and increased levels of soluble vascular endothelial growth factor (VEGF) receptor 1 (sFlt-1) that correlate with increased fetal death and diminished litter size.

Despite advances in the understanding of the disorder, therapeutic approaches to the treatment of PE are severely limited. In the last few years, several studies in animals support the use of statins to prevent PE.10–13 Using this new mouse model of defective trophoblast invasion-induced PE,
we also found that pravastatin prevents the onset of the disease. Collectively these data indicate that statins may be a good therapeutic approach for the management of PE and, thus, offer hope for an effective intervention to prevent maternal and fetal mortality and morbidity in humans.

Materials and Methods
An expanded Methods section is available in the online Data Supplement (please see http://hyper.ahajournals.org).

Mice Mating and Treatment Protocols
Mice deficient in C1q on C57BL/6 background (provided by Dr Marina Botto, Imperial College, London, United Kingdom) and wild-type (WT) C57BL/6 mice (purchased from the Jackson Laboratory, Bar Harbor, ME) were used in all of the experiments (please see the online Data Supplement).

Blood Pressure Measurements
Blood pressure was measured in the tail artery in pregnant C1q$^{-/-}$×WT mice, and WT×WT mice with and without pravastatin treatment at different times along pregnancy until postpartum. Measurements were performed using a computerized, noninvasive tail-cuff acquisition system (CODA System, Kent Scientific Corporation, Torrington, CT), as described previously10,14 (please see the online Data Supplement).

Assessment of Albumin: Creatinine Ratio
Albumin:creatinine ratio (ACR) in random urine specimens (accepted alternative to 24-hour urine collections) was used to monitor renal function (please see the online Data Supplement).

In Situ Zymography
Matrix metalloproteinase (MMP) activity was measured by in situ zymography, as described previously15 (please see the online Data Supplement).

Immunohistochemistry
Day 15 placental tissue was frozen in OCT (Sakura Finetek) and cut into 10-μm-thick sections. Endogenous peroxidase activity was quenched with Peroxo-block (Invitrogen Corporation, Camarillo, CA), and normal goat serum was used to block nonspecific binding (Cappel, Aurora, OH; please see the online Data Supplement).

Placental Perfusion Studies
Placental perfusion was examined by injecting day 15 pregnant mice with and without pravastatin were euthanized by cervical dislocation on day 15 of pregnancy. The abdominal-thoracic aorta was then excised, placed in cold PBS, and cleaned of adhering connective and adipose tissue. Aorta from each mouse was divided into 4 rings of 2-mm length. (please see the online Data Supplement).

Isoprostane Measurements
Isoprostane 8-iso-prostaglandin E2a (STAT-8) is a marker for oxidative stress that increases in PE.16 Decidual tissue at day 9 and day 15 were harvested and homogenized in 9 volumes of 0.1 mol/L of Tris (pH 7.4) containing 1 mmol/L EDTA and 10 μmol/L of indomethacin and stored at −80°C in the presence of 0.005% butylated hydroxytoluene before being assayed for free 8-isoprostane using a STAT-8-isoprostane enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI).

Isolation of Aortic Rings
WT×WT and C1q$^{-/-}$×C1q$^{-/-}$ mice untreated or treated with pravastatin were euthanized by cervical dislocation on day 15 of pregnancy. The abdominal-thoracic aorta was then excised, placed in cold PBS, and cleaned of adhering connective and adipose tissue. Aorta from each mouse was divided into 4 rings of 2-mm length. (please see the online Data Supplement).

Statistical Analysis
Data were processed using SigmaStat, version 3.5 (Systat, Point Richmond, CA), statistical program for Windows. Data are expressed as mean±SEM (please see the online Data Supplement).

Results
Placental C1q Is Crucial for Fetal Survival
To investigate the contribution of fetal-derived cell (trophoblasts) C1q to fetal death, we performed studies in C1q$^{-/-}$ females mated with WT males (C1q$^{-/-}$×WT). C1q$^{-/-}$ females mated with WT males showed normal pregnancies. Fetal resorption frequency and litter size in C1q$^{-/-}$×WT mice were not different from control matings (WT×WT; Figure 1A and 1B). The absence of pregnancy complications in C1q$^{-/-}$×WT indicates the important contribution of trophoblast C1q to fetal survival. On the other hand, WT females mated with C1q$^{-/-}$ males showed increased fetal resorption frequency comparable to C1q$^{-/-}$×C1q$^{-/-}$ mice (Figure 1A and 1B), suggesting that maternal-derived C1q is not required for normal placentation and fetal survival.

Diminished Collagenase Activity in Deciduas From C1q$^{-/-}$ Pregnant Mice
MMPs play a pivotal role in trophoblast invasion and placental angiogenesis processes by degrading collagen IV in basement membranes and extracellular matrix components.17 Robust MMP activity against collagen IV (green fluorescence) was observed in the giant trophoblast cells (TGCs; the outermost layer of the extraembryonic compartment that establishes direct contact and invades the maternal decidua) in the deciduas of WT×WT mice, suggesting active trophoblast migration (Figure 1C). In contrast, weak fluorescence (diminished MMP activity) was observed in the deciduas of C1q$^{-/-}$×C1q$^{-/-}$ mice. This observation is in agreement with the increased fetal resorption frequency observed in C1q$^{-/-}$×C1q$^{-/-}$ mice. In addition, the decidua was thicker in C1q$^{-/-}$×C1q$^{-/-}$ mice compared with WT×WT mice, confirming a defective trophoblast invasion of the maternal tissue by the TGC.

Increased Albuminuria and Glomerular Endotheliosis in C1q$^{-/-}$ Mice
The presence of proteinuria is used to confirm the diagnosis of PE.18 In C1q$^{-/-}$ pregnant mice, a time course increase in urine ACR that reaches statistical significance at day 9 of pregnancy was observed (Figure 2A). Albumin excretion in C1q$^{-/-}$ mice reached values 8 times higher than that measured in control mice (Figure 2A). Proteinuria was also observed in WT females mated with C1q$^{-/-}$ males but was not observed in C1q$^{-/-}$ mice mated with WT mice (data not shown).

Endotheliosis, an inflammation of the glomerular endothelium, is a frequent renal lesion observed in women with PE.18 Electron microscopic analysis was performed to identify endothelial injury in C1q$^{-/-}$ pregnant mice (C1q$^{-/-}$×C1q$^{-/-}$). Electron microscopic examination of renal glomerular capillaries in C1q$^{-/-}$×C1q$^{-/-}$ mice showed significant endothelial swelling with reduction of endothelial fenestrations (Figure 2B). Swollen glomerular endothelial cells occluded the
capillaries lumina in 50% of the glomeruli in C1q−/−×C1q−/− mice. In contrast, open capillary lumens and intact endothelial cells were observed in the control (WT×WT) mice (Figure 2C). Increased fibrin deposition, another characteristic of glomerular endotheliosis, was also observed in kidneys from C1q−/−×C1q−/− mice (Figure 2B) compared with WT×WT mice (Figure 2C).

C1q−/− mice in a hybrid (129×C57BL/6) genetic background have been shown to develop a lupus-like disease with glomerulonephritis and multiple apoptotic cell bodies.19 However, nonpregnant (NP) C1q−/− mice used in these experiments are fully backcrossed onto C57BL/6 and do not develop glomerulonephritis or any other histological evidence of renal disease.20 Electron microscopic analysis studies showed neither glomerular lesions nor fibrin deposition in NP C1q−/− females (data not shown). In addition, albuminuria was not observed in NP C1q−/− females (ACR: NP C1q−/−=85±18 μg/mg versus WT×WT day 15=97±18 μg/mg). These data suggest that the glomerular endotheliosis observed in C1q−/− mice develops during pregnancy.

Blood Pressure in C1q−/−×C1q−/− Mice
Hypertension is the most common diagnostic sign in PE.18 Increased mean arterial blood pressure (MAP) was observed in C1q−/−×C1q−/− mice when compared with control matings (WT×WT; Figure 2D). MAP was slightly higher in the C1q−/−×C1q females from day 8 to day 12 of pregnancy but did not reach statistical significance when compared with WT×WT mice (Figure 2D). At day 13, MAP significantly increased in pregnant C1q−/− mice when compared with controls. Hypertension persisted in pregnant C1q−/− mice until delivery (Figure 2D). WT×C1q−/− mice developed hypertension with the same pattern of blood pressure variation than C1q−/−×C1q−/− mice. In contrast, C1q−/−×WT mice did not show hypertension (data not shown).

Increased Sensitivity to Angiotensin II and Decreased Vasorelaxant Response to Acetylcholine in C1q−/−×C1q−/− Mice
An increased sensitivity of the arteries to vasoconstrictor agents like angiotensin II (Ang II) has been described in PE in women and mice.10,21 Thus, we investigated whether increased hypersensitivity to Ang II was present in the aorta
Capillary lumina, and no fibrin deposition were observed in kidneys from control WT/C1q 

Aortic rings from C1q−/− mice showed increased reduction of the inner diameter in response to Ang II and reduced increase of the inner diameter in response to AcH when compared with aortic rings from WT mice (Figure 2E). Aortic rings from C1q−/− mice showed increased reduction of the inner diameter in response to Ang II when compared with control WT×WT mice. N=6 to 8 mice per experimental group. *Different from untreated, WT mice. N increases reduction of the inner diameter in response to Ang II and reduced increase of the inner diameter in response to AcH when compared with control WT×WT mice. D, Mean arterial pressure (MAP) in C1q−/−×C1q−/− and WT×WT mice. Data were expressed as change in MAP from mating day. C1q−/−×C1q−/− mice showed increased MAP when compared with control WT×WT matings (P<0.01). #P<0.05 vs previous time within the same group. Ten to 12 mice were studied in each experimental group. E, Inner diameter of aortic rings from C1q−/−×C1q−/− and WT×WT mice incubated with angiotsin II (Ang II), norepinephrine (NE), and NE+acetylcholine (AcH). Aortic rings from pregnant C1q−/− mice showed increased reduction of the inner diameter in response to Ang II and reduced increase of the inner diameter in response to AcH when compared with control WT×WT mice. *Different from untreated, P<0.05; #Different from WT×WT, P<0.05.

Figure 2. Preeclamptic features in C1q-deficient (C1q−/−)×C1q−/− matings. A, Albumin:creatinine ratio (ACR) increases along pregnancy in C1q−/−×C1q−/− mice. Albuminuria was not observed in control matings of wild-type (WT)×WT mice. N=6 to 8 mice per experimental group (P<0.05 vs WT×WT at the same time; #P<0.05 vs previous time within the same group). B, Transmission electron micrographs of glomeruli from C1q−/−×C1q−/− mice (original magnification: ×10 000) show swollen glomerular endothelial cells (black arrows) occluding the glomerular capillaries and loss of fenestrations (top middle). Increased fibrin deposition was also observed in glomeruli from C1q−/−×C1q−/− mice (top right, C). No signs of endothelial injury, well-preserved fenestrations, widely patent glomerular capillary lumina, and no fibrin deposition were observed in kidneys from control WT×WT mice. C, Transmission electron micrographs of glomeruli from C1q−/−×C1q−/− mice (original magnification: ×10 000) show swollen glomerular endothelial cells (black arrows) occluding the glomerular capillaries and loss of fenestrations (top middle). Increased fibrin deposition was also observed in glomeruli from C1q−/−×C1q−/− mice (top right, C). No signs of endothelial injury, well-preserved fenestrations, widely patent glomerular capillary lumina, and no fibrin deposition were observed in kidneys from control WT×WT mice. D, Mean arterial pressure (MAP) in C1q−/−×C1q−/− and WT×WT mice. Data were expressed as change in MAP from mating day. C1q−/−×C1q−/− mice showed increased MAP when compared with control WT×WT matings (P<0.01). #P<0.05 vs previous time within the same group. Ten to 12 mice were studied in each experimental group. E, Inner diameter of aortic rings from C1q−/−×C1q−/− and WT×WT mice incubated with angiotsin II (Ang II), norepinephrine (NE), and NE+acetylcholine (AcH). Aortic rings from pregnant C1q−/− mice showed increased reduction of the inner diameter in response to Ang II and reduced increase of the inner diameter in response to AcH when compared with control WT×WT mice. *Different from untreated, P<0.05; #Different from WT×WT, P<0.05.

of C1q−/−×C1q−/− mice. According to our hypothesis, aortic rings from C1q−/−×C1q−/− mice showed increased contractile response to Ang II when compared with aortic rings from control WT×WT matings (Figure 2E). Aortic rings from C1q−/−×C1q−/− mice showed a 52±8% reduction in diameter when exposed to Ang II compared with 21±5% in aortic rings isolated from control matings (Figure 2E). In addition, norepinephrine-treated aortic rings from C1q−/−×C1q−/− mice showed attenuated vasorelaxant effect of acetylcholine (AcH) when compared with aortic rings from WT×WT mice (Figure 2E). This impaired AcH vasodilatation suggests an endothelial dysfunction in C1q−/−×C1q−/− mice.

Serum sFlt-1 Levels in C1q−/−×C1q−/− Mice
sFlt-1, a potent antiangiogenic molecule, has been associated with defective placentaion and PE.22,23 To substantiate that C1q−/−×C1q−/− mice develop the main characteristics of human PE, we measured serum sFlt-1. As expected, C1q−/−×C1q−/− mice showed increased serum levels of sFlt-1 when compared with control matings. At day 15 of pregnancy, a 2-fold increase in sFlt-1 levels was observed in C1q−/−×C1q−/− mice compared with WT×WT mice (Figure 3A).

Diminished Blood Flow and VEGF Expression in Placentas From C1q−/−×C1q−/− Mice
To detect blood perfusion defects secondary to defective placentation in C1q−/−×C1q−/− mice, fluorescein isothiocyanate-dextran was injected in the maternal circulation at day 15 of pregnancy. In WT×WT matings with normal pregnancies, the fluorescent tracer accumulated in the placental labyrinth indicating adequate placental blood flow (Figure 3B). In contrast, diminished blood perfusion (diminished fluorescent tracer accumulation) was observed in the labyrinth of C1q−/−×C1q−/− matings (Figure 3B). Quantification of fluorescein isothiocyanate-dextran in placental homogenates by luminescence spectrometry confirmed the histological findings (Figure 3B).
The high levels of sFlt-1 observed in C1q-deficient (C1q\(^{-/-}\))\times C1q\(^{-/-}\) mice led us to investigate the placental levels of VEGF, a crucial molecule in placental development.\(^{22}\) According to our hypothesis, low VEGF expression was detected by immunohistochemistry in the labyrinth in placentas from our experimental group. Four to 5 placentas were studied in each experimental group. The bar graph shows the quantification of VEGF (brown color) observed in the placental labyrinth in C1q\(^{-/-}\) mice. The bar graph shows the quantification of dextran-FITC (molecular weight: 2 000 000) injection in the maternal circulation. In control WT\times WT matings with normal pregnancies, the fluorescent tracer accumulated in the placental labyrinth (laboratory). Less blood perfusion was observed in the labyrinth of C1q\(^{-/-}\) mice, indicative of lower placental flow compared with control WT mice. The bar graph shows the quantification of dextran-FITC is a measure of increased placental flow. N = 6 to 8 mice in each experimental group. Four to 5 placentas were studied in each experimental group. *Different from WT\times WT, P < 0.01. C, Staining for VEGF was observed in placentas from control WT mice, WT\times WT mouse, and wild-type WT\times WT mice at day 15 of pregnancy. *Different from WT\times WT, P < 0.01. D, Increased isoprostane 8-iso-prostaglandin F\(_2\)a (STAT-8) levels were observed in day 9 deciduals and day 15 placentas from C1q\(^{-/-}\) mice vs control WT\times WT matings. Quantification of placental VEGF values by ELISA is shown in the bar graph (*different from WT\times WT mice, P < 0.01). The high levels of sFlt-1 observed in C1q\(^{-/-}\) mice led us to investigate the placental levels of VEGF, a crucial molecule in placental development.\(^{22}\) According to our hypothesis, low VEGF expression was detected by immunohistochemistry in the labyrinth in placentas from C1q\(^{-/-}\) mice (Figure 3C). In contrast, abundant staining for VEGF was observed in placentas from control WT\times WT matings with normal pregnancies (Figure 3C). Similar results were observed when placental VEGF levels were quantified by ELISA (Figure 3C). The high levels of sFlt-1 observed in C1q\(^{-/-}\) mice led us to investigate the placental levels of VEGF, a crucial molecule in placental development.\(^{22}\) According to our hypothesis, low VEGF expression was detected by immunohistochemistry in the labyrinth in placentas from C1q\(^{-/-}\) mice (Figure 3C). In contrast, abundant staining for VEGF was observed in placentas from control WT\times WT matings with normal pregnancies (Figure 3C). Similar results were observed when placental VEGF levels were quantified by ELISA (Figure 3C).

Increased Decidual and Placental Oxidative Stress in C1q\(^{-/-}\) Mice

Oxidative stress in placental tissue has been associated with PE.\(^{16,24}\) STAT-8 is not only a marker for oxidative stress but also a potent vasoconstrictor and an inhibitor of trophoblasts invasion.\(^{24,25}\) Increased STAT-8 levels were observed in day 9 decidual tissue and day 15 placentas from C1q\(^{-/-}\) mice compared with control WT\times WT matings with uneventful pregnancies (Figure 3D). Increased levels of STAT-8 preceded the onset of hypertension, suggesting that the vasoconstrictor effects of STAT-8 may contribute to the increased MAP observed in C1q\(^{-/-}\) mice.

Pravastatin Prevents Preeclamptic Features in C1q\(^{-/-}\) Mice

Knowing that pravastatin prevented the onset of PE in the CBA/J\times DBA/2 mouse model of PE and other animal models,\(^{10–13}\) we decided to study whether pravastatin can also prevent PE in this model. Pravastatin (5 mg/d) was given from day 6 to day 15. In agreement with previously reported studies, pravastatin prevented PE and rescued the pregnancies in C1q\(^{-/-}\) mice (Figure 4A). In addition, pravastatin attenuated glomerular injury and prevented albuminuria in C1q\(^{-/-}\) mice. Figure 4B shows that ACR levels at day 15 of pregnancy were reduced in C1q\(^{-/-}\) mice treated with pravastatin compared with untreated mice. Accordingly, electron microscopic analysis of kidneys from C1q\(^{-/-}\) mice treated with pravastatin revealed normal configuration of glomerulus (Figure 4C). The capillary lumina were not occluded, and there were no signs of endothelial injury in kidneys from C1q\(^{-/-}\) mice treated
Pravastatin increases placental VEGF levels in C1q<sup>−/−</sup> preeclamptic mice

Previous results from our laboratory showed that pravastatin increased VEGF release from trophoblasts, diminished the release of sFlt-1, and rescued pregnancies in the CBA/J×DBA/2 mouse model of PE. Thus, we investigated whether pravastatin can decrease serum sFlt-1 levels and increase placental VEGF levels in pregnant C1q<sup>−/−</sup> mice. Figure 5A and Supplemental Figure A illustrate the effects of pravastatin on placental VEGF levels. VEGF expression in placenta from C1q<sup>−/−</sup>×C1q<sup>−/−</sup> mice was increased by pravastatin treatment. In addition, the increased serum levels of sFlt-1 observed in C1q<sup>−/−</sup>×C1q<sup>−/−</sup> mice were not observed when the mice received pravastatin treatment (Figure 5B). In addition, pravastatin treatment increased MMP activity against collagen IV (Supplemental Figure B) in pregnant C1q<sup>−/−</sup> mice, suggesting that prava-
Pravastatin restored angiogenic balance and normal placentation in pregnant C1q-deficient (C1q<sup>−/−</sup>) mice. A, Placental VEGF quantification by ELISA demonstrates that pravastatin restored placental VEGF levels in pregnant C1q<sup>−/−</sup> mice; different from wild-type (WT)×WT mice, *P<0.01. B, Increased serum sFlt-1 levels observed in C1q<sup>−/−</sup>×C1q<sup>−/−</sup> mice at day 15 of pregnancy were not observed when pregnant C1q<sup>−/−</sup> mice were treated with pravastatin; *different from WT×WT, *P<0.05. C, Placental and decidual iso-prostan 8-iso-prostaglandin F2α (STAT-8) levels. Pravastatin prevented decidual and placental oxidative stress in C1q<sup>−/−</sup>×C1q<sup>−/−</sup> mice at days 9 and 15 of pregnancy (*<P<0.01). N=6 to 8 mice per experimental group.

Discussion

More than 200 000 American women per year develop PE (a number equal to the number of women affected by breast cancer). It is the most common reason for a woman to die during pregnancy and the leading cause of fetal mortality and morbidity. Although considerable research effort, very little is understood about its etiology and pathophysiology, which are complex and multifactorial. Furthermore, neither the incidence nor the treatment of the disease has changed substantially in the last century.

Human studies are complicated by the fact that it is difficult to determine in which patients PE will develop. Moreover, when the pathological features of PE are observed, it is usually too late, and at this time, premature delivery, associated with severe sequelae for the infant, is the only definitive treatment. Therefore, to establish cause-and-effect relationships to identify possible targets for therapies, we used an animal model that simulates the clinical scenario in women. In this study, we showed that C1q<sup>−/−</sup> mice develop the clinical symptoms of human PE.

We demonstrated previously that complement component C1q plays a crucial role in trophoblast migration and spiral arteries remodeling, contributing to placentation development. Thus, we hypothesized that mice deficient in C1q could develop PE. Indeed, we demonstrated an association among the absence of C1q, abnormal placentation, and onset of PE in mice. Mice deficient in C1q develop the characteristic features of human PE: hypertension, proteinuria, and glomerular damage.

That WT females mated with C1q<sup>−/−</sup> males develop PE clearly indicates that the lack of C1q on trophoblast cells, and not maternal C1q, is responsible for the development of PE. In our studies, the absence of C1q on trophoblast cells was associated with abnormal invasion of the maternal decidua. MMPs are important enzymes that contribute to extracellular matrix degradation, in which collagen IV is the major element. Elevated expression of MMPs is usually seen in invasive cells like highly tumorigenic cancers and trophoblasts. In WT×WT mice, we found that MMPs efficiently degraded collagen IV, assisting the invading TGCs to pass through the basement membranes. In contrast, poor collagenolytic activity was shown in C1q<sup>−/−</sup>×C1q<sup>−/−</sup> mice. The defective production of MMP was associated with an impaired invasion of maternal decidual tissue. It was reported that silencing the genes for MMP9, the protease responsible for collagen IV degradation, with small interfering RNA inhibited the growth and invasiveness of trophoblasts. Some MMPs also promote angiogenesis (a critical process required for tumor cell survival and normal placentation development) by degrading the vascular basement membrane interstitium and also by releasing sequestered angiogenic molecule VEGF.

Thus, the deficient MMP activity observed in the in pregnant C1q<sup>−/−</sup> mice might also affect placental angiogenesis leading to abnormal placentation. The increased levels of STAT-8, a marker of oxidative stress and inhibitor of trophoblast migration, observed in pregnant C1q<sup>−/−</sup> mice can also be responsible for the impaired trophoblast migration and deficient placentalation observed in these mice. Interestingly we were able to relate defective TGC migration, impaired invasion of decidua, and increased oxidative stress that occur during the early stages of placentation, with the onset of PE later on in pregnancy. These data are in agreement with other authors that suggested that PE in humans is caused by abnormal implantation and development of the placenta that occur early during the first trimester and that lead to the later manifestation of the maternal symptoms in the second and third trimesters. Therefore, our model showed to be useful in documenting the progression of events that cannot be followed with any confidence in human studies.

Because C1q<sup>−/−</sup> mice have a deficit in the clearance of apoptotic cells, we needed to consider that apoptotic tro-
phoblasts can accumulate in the placenta and affect placentation and pregnancy outcomes in C1q<sup>−/−</sup> × C1q<sup>−/−</sup> mice. However, that WT mothers mated with C1q<sup>−/−</sup> males still develop PE suggests that maternal C1q is not relevant to the onset of PE. In addition, C1q<sup>−/−</sup> mice on C57BL/6 mice used in our experiments do not develop autoimmune disease.

PE has been termed the “disease of theories,” reflecting the confusion that surrounds the causes and pathophysiology of PE. Because PE only occurs during pregnancy and its symptoms resolve after delivery, factors produced by the placenta are thought to initiate the maternal vascular response. Maternal hypertension is part of the maternal vascular response, and, thus, it is a key feature of PE in women. Pregnant C1q<sup>−/−</sup> mice developed hypertension that started at day 13 of pregnancy and persisted until delivery. These data suggest that a factor(s) released from the defective placenta into the maternal circulation may be responsible for the endothelial dysfunction underlying hypertension in PE. At day 18 of pregnancy we observed the maximal change in MAP from mating day in pregnant C1q<sup>−/−</sup> mice (18.2 ± 3 mm Hg). Vasoconstrictor STAT-8 produced in placental cells can also be responsible for the increased blood pressure observed in pregnant C1q<sup>−/−</sup> mice. The increase in blood pressure observed in C1q<sup>−/−</sup> × C1q<sup>−/−</sup> mice, although significant when compared with control matings, is moderate. Other important factors that can increase blood pressure in pregnant mice and, thus, have been related to PE, like endothelin 1 or angiotensin receptor agonistic autoantibodies, should be studied in this model.

In addition to hypertension, we demonstrated endothelial dysfunction characterized by increased vascular susceptibility to vasoconstriction and decreased vasorelaxation in aortas from pregnant C1q<sup>−/−</sup> mice. Increased sensitivity to Ang II and decreased Ach-induced relaxation was observed in aortic rings from pregnant C1q<sup>−/−</sup> mice when compared with aortic rings from control WT × WT matings.

Endotheliosis is a common renal lesion observed in patients with PE. Electron microscopy analysis of the kidneys from pregnant C1q<sup>−/−</sup> mice revealed significant glomerular damage when compared with control matings, including swelling of the glomerular endothelial cells, fibrin deposition, and occlusion of capillary lumens. These glomerular features are consistent with endotheliosis. The presence of glomerular damage is also reflected in the presence of proteinuria, frequently used in the clinic to confirm the diagnosis of PE. A time course increase of ACR, reaching maximum values at day 12 of gestation, was observed in pregnant C1q<sup>−/−</sup> mice. As we discussed before, NP C1q<sup>−/−</sup> mice on a C57BL/6 background do not develop renal disease. Therefore, the renal damage observed in pregnant C1q<sup>−/−</sup> mice is originated as a consequence of pregnancy.

In recent years, several studies demonstrated the beneficial effects of statins in preventing the onset of PE in different animal models. Here we found that pravastatin also prevented the onset of PE in C1q<sup>−/−</sup> mice. Treatment with pravastatin prevented the appearance of the key features of PE in pregnant C1q<sup>−/−</sup> mice. Hypertension, glomerular endothelial lesions and albuminuria were not observed in pregnant C1q<sup>−/−</sup> mice treated with pravastatin. In addition, the contractile response to Ang II of aortic rings from pregnant C1q<sup>−/−</sup> mice treated with pravastatin was not different from rings isolated from control WT × WT matings.

In addition, pravastatin restored the endothelial function in aortic rings from C1q<sup>−/−</sup> × C1q<sup>−/−</sup> mice. These results are in agreement with studies suggesting that statins lower blood pressure in hypertensive patients and studies in animals showing that statins improve vascular reactivity in PE. Pravastatin restored placental VEGF levels and collagenolytic activity in TGCs, improving placentation and pregnancy outcomes. Moreover, pravastatin restored placental blood flow and prevented oxidative damage. This is in accordance with previous results showing that pravastatin increases VEGF release and placental growth factor, a VEGF-like angiogenic factor, from mouse trophoblasts. In addition, pravastatin also diminished sFlt-1 levels in pregnant C1q<sup>−/−</sup> mice. Thus, by restoring placental angiogenic balance, pravastatin improved placentation, prevented PE, and improved pregnancy outcomes in C1q<sup>−/−</sup> × C1q<sup>−/−</sup> mice.

**Perspectives**

In conclusion, we described a new mouse model of PE, where the lack of complement component C1q causes defective trophoblast invasion and impaired placentation leading to the onset of PE. Pregnant C1q<sup>−/−</sup> mice recapitulate the complex disorder of human PE characterized by hypertension, albuminuria, and endotheliosis, suggesting that C1q<sup>−/−</sup> mice will be a good animal model to understand the pathophysiology of PE and identify new therapies. Indeed, using this new mouse model of PE, we identified pravastatin as a candidate therapy to prevent PE and its related complications. We suggested different pathways through which pravastatin acts in this model. Pravastatin restored the angiogenic balance in the placenta, increased trophoblast migration, improved placental blood flow, and reduced the levels of oxidative stress. To confirm that statins are beneficial for the treatment of PE in women, clinical trials should be performed.

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**Disclosures**

None.

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Role of Complement Component C1q in the Onset of Preeclampsia in Mice
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EXPANDED MATERIAL AND METHODS

Mice mating and treatment protocols

Mice deficient in C1q on C57BL/6 background (provided by Dr Marina Botto, Imperial College, London, UK) and wild type C57BL/6 mice (purchased from The Jackson Laboratory, Bar Harbor, ME) were used in all experiments. C1q-deficient mice in a hybrid (129 x C57BL/6) genetic background have been shown to develop a lupus-like disease with glomerulonephritis (GN). However, non pregnant C1q-/- mice used in these experiments are fully backcrossed onto C57BL/6 and do not develop GN or any other histological evidence of renal disease. Eight- to 10-week-old virgin female C1q-/- or C57BL/6 (WT) mice were mated with 8- to 14-week-old C1q-/- or C57BL/6 (WT) males. Females were inspected daily for vaginal plugs; sighting a vaginal plug was designated as day 0 of pregnancy.

A group of C1q-/- x C1q-/- mice and a group of WT x WT mice were treated with pravastatin (5mg/mouse, i.p.) daily from day 4 to 12 of pregnancy.

On day 9 of pregnancy a group of mice were sacrificed and the deciduas were harvested for immunohistochemical studies. Another group of mice was sacrificed on day 15. Blood samples, placental tissue and aortic rings were isolated. Kidneys were also harvested to investigate the presence of endotheliosis. Urine samples were collected daily from day 0 to day 15 of pregnancy. The frequency of fetal resorption was determined on day 15 as previously described. A group of mice were allowed to complete the pregnancy and the litter size was recorded. Serum sFlt-1 levels were determined by ELISA (R&D Systems Inc, Minneapolis, MN).

Procedures that involved mice were approved by the York College – CUNY- Committee on Animal Use in Research and Education and were conducted in strict accordance with guidelines for the care and use of laboratory research animals promulgated by the NIH.

Blood Pressure Measurements

Blood pressure was measured in the tail artery in pregnant C1q-/- x C1q-/- mice, and WT x WT mice with and without pravastatin treatment at different times along pregnancy until postpartum. Measurements were performed using a computerized, non-invasive tail-cuff acquisition system (CODA System, Kent Scientific Corporation, Torrington, CT, USA) as previously described. The CODA system utilizes volume-pressure recording technology to detect changes in tail volume that correspond to systolic and diastolic pressures and calculates mean arterial pressure (MAP) during each measurement cycle. Our protocol consisted of 8 acclimation cycles and 8 measurement cycles daily. Anaesthetized mice were placed in plastic restrainers. Mouse body temperatures were monitored closely and maintained between 34°C and 36°C using infrared heating. Data were expressed as change in MAP from mating day.

Assessment of albumin/creatinine ratio (ACR)

Albumin-to-creatinine ratio (ACR) in random urine specimens (accepted alternative to 24-hour urine collections) was used to monitor renal function. Urinary albumin was determined daily until the end of pregnancy by ELISA (Albuwell M (Exocell, Philadelphia, PA)). Creatinine in urine was quantified with the Creatinine Companion kit (Exocell, Philadelphia, PA), based upon the Jaffe' reaction of alkaline picrate with creatinine.

In situ Zymography

Metalloproteinases (MMPs) activity was measured by in situ zymography as previously described. Briefly, 10-μm- day 9 decidual sections were washed in PBS and then incubated for 2 hours in 20 μg/ml DQ collagen IV (Invitrogen, Carlsbad, CA) in 50 mM Tris-HCl, 50 mM NaCl, 10 mM CaCl2, pH 6.8 at 37°C in the dark. In parallel, control sections were preincubated with buffer containing 10 mM EDTA followed by 2 hour incubation in DQ-collagen IV solution supplemented with 10 mM EDTA to indicate the contribution of MMPs. The reaction was stopped by removing the substrate solution followed by 10 minutes incubation in 4% paraformaldehyde-PBS. Finally, mounting medium supplemented with DAPI (Vector Laboratories, Burlingame, CA) was applied. Sections were observed under a fluorescence microscope (Nikon Eclipse 50i,
Nikon Inc, Melville, NY) and photographs were taken using a Nikon DigiSight Color Digital Camera System and NIS-Elements Research Imaging Software. Increased fluorescence indicates increased collagen IV degradation by MMPs. Each experimental group consisted of 5-6 mice and 4 tissue sections per animal were studied.

**Immunohistochemistry**

Day 15 placental tissue was frozen in O.C.T. (Sakura Finetek, CA) and cut into 10 μm-thick sections. Endogenous peroxidase activity was quenched with Peroxo-block (Invitrogen Corporation, Camarillo, CA) and normal goat serum was used to block nonspecific binding (Cappel, Aurora, OH). VEGF164 expression was detected using a goat polyclonal antibody (R&D Systems Inc, Minneapolis, MN). The IHC reaction was developed using diaminobenzidine. Sections were counterstained with hematoxylin. To validate the immunohistochemical studies VEGF in placental homogenates was quantified by ELISA. 10% placental homogenates were stored overnight at -20°C and two freeze-thaw cycles were performed to break the cell membranes. The homogenates were then centrifuged for 5 min at 5000 x g and tested for VEGF using a commercial ELISA kit (R&D).

Kidneys from every experimental group were acquired at day 15 of gestation, frozen and cut into 10 μm-thick sections. Sections were subjected to endogenous peroxidase activity inhibition and incubation with normal rabbit serum followed by an incubation with rabbit anti-mouse fibrin (Dako North America Inc., Carpinteria, CA) and specific secondary IgG antibodies conjugated with HRP (Sigma Chemical, St Louis, MO). Bound IgG-HRP was detected with diaminobenzidine. Sections were counterstained with hematoxylin.

A group of kidneys were fixed in 2% paraformaldehyde/2% glutaraldehyde in 0.1M phosphate buffer for electron microscopy (EM). Kidney sections were evaluated for the presence of endotheliosis (loss of fenestrations, endothelial swelling and detachment of endothelial cells from the glomerular basement membrane).

**Placental perfusion studies**

Placental perfusion was examined by injecting day 15 pregnant females with 100 μL of 25 mg/mL FITC-labeled dextran (MW 2 000 000; Sigma-Aldrich, St Louis, MO) via the retro-orbital vein. After 15 minutes, the mice were killed and the placentas removed and flash frozen. Serial frozen sections were examined and photographed under a Nikon DigiSight Color Digital Camera System and NIS-Elements Research Imaging Software.

Some placentas were homogenized in 9 volumes of 0.1 M Tris (pH 7.4) and FITC-dextran content was measured with a Perkin-Elmer luminescence spectrometer (San Jose, CA, USA). A standard curve was constructed by plotting fluorescence (arbitrary units) against different concentrations of FITC-dextran.

**Isoprostane measurements**

Isoprostane 8-iso-prostaglandin F2a (STAT-8) is a marker for oxidative stress that increases in PE. Decidual tissue at day 9 and day 15 placental tissue were harvested and homogenized in 9 volumes of 0.1 M Tris (pH 7.4) containing 1 mM EDTA and 10 μM indomethacin and stored at -80°C in the presence of 0.005% BHT before being assayed for free 8-isoprostane using a STAT-8-Isoprostane EIA kit (Cayman Chemical, Ann Arbor, MI).

**Isolation of aortic rings**

WT x WT and C1q-/- x C1q-/- mice untreated or treated with pravastatin were euthanized by cervical dislocation on day 15 of pregnancy. The abdominal-thoracic aorta was then excised, placed in cold PBS, and cleaned of adhering connective and adipose tissue. Aorta from each mouse was divided into 4 rings of 2-mm length.

To study the contractile response to angiotensin II (Ang II), each of the aortic rings was incubated in 2 mL DMEM medium containing either Ang II (100 nmol/L) or vehicle (dH2O) for 60 min. To evaluate endothelium-dependent relaxation, aortic rings were contracted with norepinephrine (NE) (3 x 10^-6 mmol/L) and then exposed to acetylcholine (ACh) (3 x 10^-6 mmol/L). The inner diameters were calculated by measuring luminal diameter through the transverse section of the thin-vessel ring using the NIS-elements Research Imaging
software (Nikon Inc Tech Co, LTD, Tokyo, Japan). Luminal diameters were measured at three points separated by equal angles and averaged. 5 mice were studied in each experimental group.

**Statistical analysis**

Data were processed using SigmaStat, version 3.5 (Systat, Point Richmond, CA), statistical program for Windows. Data are expressed as mean plus or minus SEM. After confirming that the data were normally distributed (Kolomogorov-Smirnov test of normalcy), statistical analyses using Student t-test were conducted to compare differences in means. Associations were considered to be statistically significant if the value of P was less than .05. Blood pressure data were analyzed by paired 1-way repeated measures ANOVA with Dunnett’s *post hoc* test within group and 1-way ANOVA with Bonferroni’s *post hoc* test between groups.

**References**


Figure S1. (A) Histochemical detection of placental VEGF in C1q−/− x C1q−/− mice treated with pravastatin. (B) Pravastatin restores degradation of collagen IV on TGC. Green fluorescence indicates positive enzymatic activity on collagen IV. 4 slides per animal were stained and 6-8 animals were studied in each experimental group. (C) Adequate blood perfusion (fluorescent tracer accumulation) comparable to WT x WT control matings was observed in placentas from C1q−/− x C1q−/− mice treated with pravastatin. The bar graph shows the quantification of dextran-FITC in placental homogenates. Increased placental dextran-FITC is a measure of increased placental flow. 4 slides per animal were stained and 6-8 animals were studied in each experimental group.*different from WT x WT, p<0.01.