**Preeclampsia**

Effects of Pravastatin on Human Placenta, Endothelium, and Women With Severe Preeclampsia

Fiona C. Brownfoot, Stephen Tong, Natalie J. Hannan, Natalie K. Binder, Susan P. Walker, Ping Cannon, Roxanne Hastie, Kenji Onda, Tu’uhevaha J. Kaitu’u-Lino

**Abstract**—Preeclampsia is a major pregnancy complication where excess placental release of soluble fms-like tyrosine kinase-1 (sFlt-1) and soluble endoglin causes maternal endothelial and multisystem organ injury. Clinical trials have commenced examining whether pravastatin can be used to treat preeclampsia. However, the preclinical evidence supporting pravastatin as a treatment is limited to animal models, with almost no studies in human tissues. Therefore, we examined whether pravastatin reduced sFlt-1 and soluble endoglin secretion and decreased endothelial dysfunction in primary human tissues. Pravastatin reduced sFlt-1 secretion from primary endothelial cells, purified cytotrophoblast cells, and placental explants obtained from women with preterm preeclampsia. It increased soluble endoglin secretion from endothelial cells but did not change secretion from placental explants. The regulation of sFlt-1 by pravastatin seemed to be mediated via the 3-hydroxy-3-methylglutaryl-coenzyme A reductase cholesterol synthesis pathway. Pravastatin also reduced markers of endothelial dysfunction, including vascular cell adhesion molecule-1 expression and leukocyte adhesion on endothelial cells and increased endothelial cell migration and invasion. We also treated 4 patients with preterm preeclampsia presenting at <30 weeks of gestation with daily pravastatin. Pravastatin seemed to stabilize blood pressure, proteinuria, and serum uric acid levels. Furthermore, serum sFlt-1 levels decreased. We collected the placenta at delivery and found that pravastatin reduced sFlt-1 secretion. These results indicate that pravastatin reduced sFlt-1 and soluble endoglin production and decreased endothelial dysfunction in primary human tissues. We also present pilot data, suggesting that pravastatin can stabilize clinical and biochemical features of preterm preeclampsia. Our data obtained in human tissues support the concept that pravastatin is a candidate therapeutic for preeclampsia.

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Preeclampsia affects 5% to 8% pregnancies and is estimated to cause 60,000 maternal deaths annually and far greater numbers of perinatal deaths.1,2 A key step in the pathophysiology is placental release of antiangiogenic factors soluble fms-like tyrosine kinase-1 (sFlt-1)3 and soluble endoglin (sENG)4 into the maternal circulation. They cause widespread endothelial and multisystem organ injury.5–7 Currently, there is no treatment and delivery is the only way to stop disease progression. If preeclampsia occurs at an early gestation, then it is too late to stop disease progression. However, if preeclampsia occurs at an early gestation, then it is too late to stop disease progression. Therefore, the need to deliver the fetus preterm for maternal indications might lead to neonatal death and disability arising from prematurity.1 Thus, an efficacious therapeutic that can quench the disease process could improve maternal and perinatal outcomes.

There has been recent interest in the use of statins to treat preeclampsia. Notably, evidence has emerged that statins have vasoprotective properties independent of their effects in lowering serum cholesterol.8,9 Cudmore et al10 showed that simvastatin significantly reduced sFlt-1 secretion from placenta and endothelial cells. Unfortunately, simvastatin may not be acceptable for use during pregnancy. It has a category X rating in light of observational studies, demonstrating an association with fetal malformations if administered during the first trimester.11,12

Some have proposed that pravastatin may be a more promising therapeutic candidate given its safety profile could be better than simvastatin. Unlike simvastatin, which is hydrophobic, pravastatin is hydrophilic, meaning it may less readily cross through the placenta to the fetus.11,13 Cohort studies have shown that administration of lipophilic statins have increased fetal malformation risk; however, hydrophilic statins, including pravastatin, have not been associated with an increased risk.11 A multicenter randomized controlled trial has been undertaken to examine the effect of oral pravastatin on serum sFlt-1 levels in women with preterm preeclampsia,14 and a separate prevention trial has also started.15

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However, the evidence supporting the premise that pravastatin could treat preeclampsia is limited to data from 4 animal models of preeclampsia. 

Notably, atorvastatin increases sFlt-1 release in those diagnosed with an acute myocardial infarction. Therefore, it cannot simply be assumed that the decrease in sFlt-1 levels in human placenta treated with simvastatin will be the case for all statins.

We examined whether pravastatin can reduce sFlt-1 and sENG secretion in primary human endothelial and placental tissues, including placenta obtained from the cases of preterm preeclampsia. We examined whether the effects on sFlt-1 production were mediated through the 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase cholesterol synthesis pathway. In addition, we investigated the effect of pravastatin on primary endothelial cells and whether it decreases molecular markers of endothelial dysfunction.

We also report a pilot study of 4 women diagnosed with preterm preeclampsia at <30 weeks where we administered 40 mg of pravastatin daily orally. We examined trends in clinical markers of disease progression, including blood pressure and proteinuria. Furthermore, we measured serum sFlt-1 and sENG daily to see whether pravastatin increased, decreased, or stabilized levels. Finally, to obtain evidence that pravastatin may be directly acting on the placenta to affect sFlt-1 production in these patients, their placental explants were cultured ex vivo and treated with pravastatin.

**Methods**

A detailed description of methods is available in the online-only Data Supplement.

**Isolation and Culture of Placental Explants**

Human placental tissue was collected from 3 women with severe early onset preeclampsia (delivered at ≤32 weeks of gestation) defined using the 2013 American College of Obstetricians and Gynecologists guidelines. Villous explants (n=3) were prepared as previously described and treated with 0 or 2000 μmol/L of pravastatin.

Written informed consent was obtained from all women, and ethical approval was obtained from the Mercy Health Human Research Ethics Committee.

**Isolation and Treatment of Primary Human Umbilical Vein Endothelial Cells and Primary Human Trophoblast Cells**

Term placenta (n=4) were collected from women having elective cesarean sections and human umbilical vein endothelial cells (HUVECs), and trophoblasts were isolated as previously described and treated with 0, 20, 200, and 2000 μmol/L of pravastatin.

**Farnesyl Pyrophosphate Treatment**

Primary HUVECs (n=3) and primary trophoblasts (n=3) were treated with 2000 μmol/L of pravastatin for 30 minutes, and then, 50 μmol/L or 200 μmol/L of farnesyl pyrophosphate (Sigma) was added to HUVECs or trophoblasts, respectively.

**Endothelial Dysfunction**

Endothelial dysfunction was induced by treatment of primary HUVECs (n=4) for 6 hours with trophoblast-conditioned media (collected from primary trophoblasts cultured for 24 hours) or 10 ng/mL tumor necrosis factor-α (TNF-α; Sigma) and washed and treated with pravastatin at 2000 μmol/L for an additional 6 hours. For the leukocyte adhesion assay experiment, THP-1 cells were preincubated with calcein (Merk Millipore, Darmstadt, Germany) and applied to HUVECs as previously described.

**Endothelial Cell Migration and Invasion**

To assess changes in endothelial cell migration and invasion, the xCELLigence system (Roche diagnostic, Castle Hill, New South Wales, Australia) was used. Chemoattractant vascular endothelial growth factor (VEGF; Bio-Scientific, Kirrawee, New South Wales, Australia) was added to 12.5 nM/mL on the lower receiver plate. Platelets were added to the device either straight for migration or with growth factor–reduced matrigel (Biosciences) for invasion. HUVEC migration and invasion were assessed by determining electric impedance across the device every minute for 2 hours and then every hour for 48 hours.

**ELISA Analysis**

Concentrations of sFlt-1 and sENG were measured in conditioned cell/tissue culture media or patient serum using the DuoSet VEGF R1/Flt-1 kit (R&D systems; Biosciences, Waterlo, Australia) and a DuoSet Human Endoglin CD/105 ELISA kit (R&D systems). Concentrations of endothelin-1 (ET-1) were measured in human serum using the Quantikine ET-1 ELISA kit (R&D systems) as per manufacturer’s instructions.

**Reverse Transcription Polymerase Chain Reaction**

RNA was extracted from placental explants and HUVECs using a RNeasy mini kit (Qiagen, Valencia, CA) and quantified using the Nanodrop ND 1000 spectrophotometer (NanoDrop technologies Inc, Wilmington, DE). RNA (0.2 μg) was converted to cDNA using Superscript VILO cDNA synthesis kit (Life Technologies) as per manufacturer guidelines. Taqman gene expression assays for hemeoxygenase-1 and endoglin were used (Life Technologies). Primers for eL13 and i13 were designed as previously described, and sybr green–based gene expression assays were performed.

All data were normalized to glyceraldehyde 3-phosphate dehydrogenase as an internal control and calibrated against the average C of the control samples. Results expressed as fold change from the control.

**Immunofluorescence**

Localization of the transcription factor nuclear factor (erythroid-derived 2)–like 2 (Nrf2) was examined in trophoblasts treated with 2000 μmol/L of pravastatin. Anti-Nrf2 antibody (Nrf2[His-300]-sc-13032; Santa Cruz Biotechnology, Dallas, TX) was applied at 4 μg/mL (negative control isotype matched rabbit IgG [Santa Cruz]), and an alexafluor-488 goat antirabbit secondary antibody (Life Technologies) was applied at 1:200 dilution. Cells were counterstained with DAPI and mounted. Fluorescent staining was visualized using the EVOS FL (Life Technologies).

**Clinical Study**

Four women with preterm preeclampsia at 23+0 to 30+0 weeks of gestation were consented to receive 40 mg of pravastatin daily. Recruitment to this study was based on preeclampsia being defined as 2 blood pressures of >140/90 on 2 occasions 6 hours apart and proteinuria ≥20.5 g in a 24-hour urine collection and were unlikely to require delivery in the next 24 hours (determined by an independent clinical team). We examined the effects of pravastatin on the clinical features of preeclampsia and have documented mean systolic and diastolic blood pressures; mean atrial pressure recorded over each 24-hour period is shown in Figure 5 as the dot, with the error bars indicating the systolic and diastolic levels. Day 1 is the mean
blood pressure recorded during the first 24 hours of admission before commencing pravastatin, and day 2 onward is mean blood pressure collected after pravastatin administration. We have also assessed biochemical markers that may reflect the severity of preeclampsia, specifically sFlt-1, sENG, and ET-1. Safety data, including side effects and possible adverse events in the study participant and the fetus/neonate, were collected. We obtained placental explants (n=4) at delivery and treated them with pravastatin at 0, 200, or 2000 μmol/L. Conditioned media was collected to assess sFlt-1 and sENG and cell lysates collected for RNA extraction.

Results
Pravastatin Reduces sFlt-1 Production in Primary Human Tissues
We first examined the effects of pravastatin on sFlt-1 secretion from endothelial cells (HUVECs) and primary trophoblast cells. Pravastatin dose dependently reduced sFlt-1 secretion from both HUVECs (Figure 1A) and primary trophoblasts (Figure 1B). In addition, there was a dose-dependent reduction in sFlt-1 secretion when pravastatin was added to placental explants obtained from 3 patients diagnosed with preterm preeclampsia (Figure 1C).

The soluble form of the VEGF receptor 1 (sFlt-1) is a splice variant of the FLT-1 transcript VEGF receptor 1 (FLT-1). There are many sFlt-1 variants that differ in sequence at the C-terminal region. The e15a variant is a recently described primate-specific sFlt-1 and is by far, the dominant splice product of the FLT-1 transcript in placenta: ≈80% of all FLT-1 transcripts are spliced to become sFlt-1-e15a whereas <10% is spliced to FLT-1.26 As such, sFlt-1-e15a is likely the most important placentally derived sFlt-1 variant and may be the key sFlt-1 variant responsible for preeclampsia. We therefore examined the effects of pravastatin on sFlt-1-e15a transcript levels in primary HUVECs (Figure 1D) and preterm preeclamptic placental explants (Figure 1E) and found that mRNA expression was significantly decreased.

The other major sFlt-1 variant is sFlt-1-i13, which is generically expressed in many tissues, such as placenta, endothelium, brain, heart, and kidney.26 Interestingly, pravastatin did not alter sFlt-1-i13 mRNA expression in either HUVECs or preterm preeclamptic placental explants (Figure S1 in online-only Data Supplement). Therefore, we conclude that pravastatin decreases sFlt-1 secretion by reducing transcription of the placentally derived sFlt-1-e15a variant.

Pravastatin Has Opposing Effects on sENG Secretion From Endothelial Cells and Placental Explants
The effect of pravastatin on placental sENG secretion has not been thoroughly explored. We found that pravastatin induced a dose-dependent increase in sENG secretion from primary HUVECs (Figure 1F). In contrast, pravastatin did not upregulate sENG secretion from placental explants obtained from women diagnosed with preterm preeclampsia (Figure 1G).

Effects of Pravastatin on sFlt-1 and sENG Secretion Are Mediated via the HMG-CoA Reductase Pathway
Pravastatin was developed to block intracellular cholesterol synthesis by inhibiting HMG-CoA reductase (Figure S2). We examined whether the effects of pravastatin on sFlt-1 and sENG secretion were mediated via this pathway (Figure 2A). We did this by coadministering pravastatin (to block HMG-CoA reductase) and farnesyl pyrophosphate. Farnesyl pyrophosphate is an enzyme in the HMG-CoA reductase pathway downstream to the site of action of pravastatin. Therefore, if the decrease in sFlt-1 induced by pravastatin is indeed mediated through the HMG-CoA reductase pathway, the further addition of farnesyl pyrophosphate should rescue the decrease in sFlt-1 secretion. We found that pravastatin did reduce sFlt-1 secretion from HUVECs (Figure 2A) and trophoblasts (Figure 2B) and increase sENG secretion from HUVECs (Figure S2), and its effect on secretion was inhibited in the presence of farnesyl pyrophosphate. Therefore, we conclude that pravastatin decreased sFlt-1 in HUVECs and primary trophoblasts and increased sENG in HUVECs via the HMG-CoA reductase pathway.

Effects of Pravastatin on Upregulating Endogenous Antioxidant Pathways
Preeclampsia is associated with increased placental and generalized systemic oxidative stress.1 It would therefore be advantageous if a candidate therapeutic enhanced molecules involved in cellular antioxidant responses.

Nrf2 is a transcription factor that translocates into the nucleus and binds to genes with a transcriptional response element involved in cellular antioxidant response. Pravastatin administered into primary trophoblasts increased Nrf2 nuclear localization (Figure 2C). Hemeoxygenase-1 is a cytoprotective enzyme that is directly upregulated by Nrf2 and previously shown to be induced by simvastatin in endothelial cells.10 Pravastatin significantly induced hemeoxygenase-1 mRNA expression in HUVECs (Figure 2D), but surprisingly, it did not induce hemeoxygenase-1 expression in placental explants obtained from women with preterm preeclampsia (Figure 2E).

Pravastatin Reduces Endothelial Dysfunction In Vitro
Preeclampsia is associated with generalized maternal endothelial dysfunction, caused by factors released from the placenta, and further injury caused by increased systemic inflammation. One important cytokine likely to be involved in endothelial dysfunction in this disease is TNF-α, which is increased in preeclampsia.27 We next undertook functional assays to examine whether pravastatin can act directly on endothelial cells to decrease endothelial dysfunction.

Endothelial dysfunction is associated with increased expression of vascular cell adhesion molecule-1 (VCAM-1). VCAM-1 is a cell adhesion molecule that mediates leukocyte adhesion and is directly upregulated by TNF-α.28 We coadministered pravastatin and TNF-α into HUVECs and found that pravastatin blocked TNF-α-induced VCAM-1 expression (Figure S3). We devised a second in vitro model of endothelial dysfunction to model the biology of preeclampsia (ie, placental factors causing endothelial injury) ex vivo by adding conditioned media obtained from trophoblast culture to upregulate VCAM-1 on HUVECs. We coadministered pravastatin and found that pravastatin quenched VCAM-1 expression induced by trophoblast-conditioned media (Figure 3A). Thus, we conclude that pravastatin
suppresses VCAM-1 expression induced in HUVECs by either TNF-α or trophoblast-conditioned media.

It would be anticipated that decreased VCAM-1 expression would decrease monocyte adhesion. We therefore performed a leukocyte adhesion assay. Coadministration of monocytes (THP-1 cells) and either TNF-α (Figure S3) or trophoblast media (Figure 3B and 3C) into HUVECs increased monocyte adhesion. Pravastatin significantly decreased monocyte adhesion to HUVECs, caused by either TNF-α or trophoblast-conditioned media (Figure S3).

ET-1 is a potent circulating vasoconstrictor, increased in preeclampsia and secreted from endothelial cells. We found that pravastatin significantly decreased ET-1 mRNA expression in HUVECs treated with either TNF-α (Figure S3D) or trophoblast-conditioned media (Figure 3D).

We next examined whether pravastatin can alter HUVEC migration and invasion toward VEGF in the presence of sFlt-1. To do this, we used the xCELLigence system, an assay that monitors experiments continuously in real time. VEGF was used as the chemoattractant to induce cellular migration or invasion. We found that sFlt-1 impaired HUVEC migration (Figure 4A and 4B) and invasion (Figure 4C and 4D), and this was enhanced with pravastatin (Figure 4A–4D).

These data, obtained from 4 functional in vitro assays, provide evidence that pravastatin decreases endothelial dysfunction in primary human endothelial cells.

**Pravastatin Stabilizes Preterm Preeclampsia in Humans**

We performed a pilot clinical study to examine the effects of administering pravastatin into women diagnosed with preterm preeclampsia. There were 237 women presenting to Mercy Hospital for Women with preeclampsia for a 12-month period (October 1, 2013 to September 30, 2014). Two-hundred and four were excluded as they were at >32+6 weeks of gestation, 18 were delivered in 24 hours of presentation, 7 had fetoplacental Doppler abnormalities, 3 were twin pregnancies, and 1 women declined. We recruited 4 participants with preterm preeclampsia that presented between 24+5 and 29+4 weeks of gestation to Mercy Hospital for Women.

Table summarizes the clinical details of participants at admission and their subsequent clinical course. On the day of admission...
admission, participants were significantly hypertensive with labile blood pressures ranging between 155 and 200 mm Hg systolic and 90 to 105 mm Hg diastolic. The total amount of protein in a 24-hour urinary collection commenced at admission ranged between 840 and 2990 mg. All participants had growth-restricted fetuses in utero (estimated fetal weight, <10th centile) and 2 participants (participants 1 and 2) had severely growth-restricted fetuses (estimated fetal weight, <3rd centile). All patients were given 2 doses of 11.4 mg of betamethasone to reduce neonatal risks of extreme prematurity, and magnesium sulfate was commenced in each patient with possible disease stabilization. For 3 of 4 participants (participants 1, 3, and 4), hemoglobin, platelet, and liver function tests remained within normal limits. Participant 2 had mildly abnormal liver function tests (alanine aminotransferase, 69 U/L [reference range, <33 and γ-glutamyl transferase, 69 U/L [reference range, <40]) that improved 2 days after commencing pravastatin (27 U/L and 65 U/L, respectively). The urine protein/creatinine ratio stabilized or reduced in 3 of the 4 participants (participants 1–3; Figure 5F) after pravastatin administration, remained stable in participant 4 but progressively increased in participant 3.

Perhaps another indicator of maternal preeclamptic disease stabilization is the fact that the trigger for delivery for 3 of the 4 participants (participants 1, 2, and 4) was for fetal indications (because of abnormal fetal–placental blood flows...
indicating placental insufficiency) rather than evidence of worsening maternal organ system injury. Participant 3 was delivered after 15 hours of commencing pravastatin, as her blood pressure remained labile.

We next examined circulating levels of sFlt-1, sENG, and ET-1. It would be expected that sFlt-1 and sENG levels would increase acutely with advancing gestation, especially with the diagnosis of preeclampsia.30–32 Indeed, across the 24 hours between hospital admission and the commencement of pravastatin, sFlt-1 levels rose in 3 of the 4 participants (participants 1, 2, and 4; Figure 5G). After pravastatin was commenced, sFlt-1 levels seemed to stabilize in all participants. In 2 participants (participants 2 and 4) where admission to delivery was delayed for at least a week, sFlt-1 levels seemed to decline. Furthermore, circulating sENG levels (Figure 5H) and ET-1 levels (Figure 5I) also seemed to remain stable in all participants.
Pravastatin was well tolerated, and there were no obvious side effects attributable to pravastatin in our participants. At birth, there were no fetal or neonatal abnormalities. All babies were admitted to neonatal intensive care because of extreme prematurity, and there were no neonatal losses. There were complications consistent with extreme prematurity; 3 had evidence of chronic lung disease, 2 had grades 1 to 2 retinopathy of prematurity, and 1 neonate had a grade 1 intraventricular hemorrhage. All women recovered without complications and were discharged 4 days after their caesarean section.

**Table. Baseline Characteristics of Patients With Preterm Preeclampsia Recruited to Receive 40 mg of Pravastatin Daily**

<table>
<thead>
<tr>
<th>Baseline Characteristics</th>
<th>Participant 1</th>
<th>Participant 2</th>
<th>Participant 3</th>
<th>Participant 4</th>
</tr>
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<tbody>
<tr>
<td><strong>Characteristics at admission</strong></td>
<td></td>
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<tr>
<td>Age, y</td>
<td>30</td>
<td>37</td>
<td>33</td>
<td>24</td>
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<tr>
<td>BMI, kg/m²</td>
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<td>25</td>
<td>50</td>
<td>27</td>
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<tr>
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<td>29+4</td>
<td>28+0</td>
<td>26+4</td>
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<tr>
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<td>165</td>
<td>160</td>
<td>155</td>
</tr>
<tr>
<td>Diastolic BP, mm Hg</td>
<td>100</td>
<td>105</td>
<td>104</td>
<td>90</td>
</tr>
<tr>
<td>Regular antihypertensive therapy commenced on admission</td>
<td>Labetalol 200 mg QID</td>
<td>None</td>
<td>Labetalol 200 mg TDS</td>
<td>Labetalol 200 mg BD</td>
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<td><strong>Laboratory tests on day of admission</strong></td>
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<td></td>
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<tr>
<td>Hemoglobin (g/L) (g/dL)</td>
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<td>124 (12.4)</td>
<td>119 (11.9)</td>
<td>129 (12.9)</td>
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<tr>
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<td>201</td>
<td>380</td>
<td>243</td>
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<tr>
<td>Creatinine (μmol/L) (mg/dL)</td>
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<td>73 (0.83)</td>
<td>61 (0.69)</td>
<td>63 (0.71)</td>
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<tr>
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<td>17</td>
<td>10</td>
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<tr>
<td>γ-Glutamyl transpeptidase (&lt;40)</td>
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<td>75</td>
<td>6</td>
<td>14</td>
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<td>24-h urine protein at admission, mg/d</td>
<td>840</td>
<td>2990</td>
<td>2370</td>
<td>1920</td>
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<tr>
<td>Pretreatment sFlt-1 (highest value, ng/mL)</td>
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<td>176.5</td>
<td>69.6</td>
<td>228.5</td>
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<tr>
<td>Post-treatment sFlt-1 (lowest value, ng/mL)</td>
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<td>133.6</td>
<td>63.7</td>
<td>178.7</td>
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<td><strong>Ultrasound fetal growth at admission</strong></td>
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<tr>
<td>Estimated fetal weight, %</td>
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<td>&lt;3</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>Abdominal circumference, %; weight, g</td>
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<td>&lt;3; 912</td>
<td>15, 990</td>
<td>10, 788</td>
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<td>Umbilical artery systolic/diastolic ratio</td>
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<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
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<td>Amniotic fluid index</td>
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<tr>
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<tr>
<td>Days since admission</td>
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<td>7</td>
<td>2</td>
<td>7</td>
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<tr>
<td>Days of pravastatin treatment</td>
<td>3</td>
<td>6</td>
<td>1</td>
<td>6</td>
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<tr>
<td>Reason for delivery</td>
<td>Fetal well-being concern</td>
<td>Fetal well-being concern</td>
<td>Uncontrolled maternal hypertension</td>
<td>Fetal well-being concern</td>
</tr>
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BMI indicates body mass index; BP, blood pressure; and sFlt-1, soluble fms-like tyrosine kinase-1.

**Pravastatin Reduces sFlt-1 Secretion From Placental Explants Ex Vivo Obtained From Women Who Were Administered Pravastatin**

We hypothesized that the stabilization or decline in circulating sFlt-1 and sENG levels among participants with pravastatin treatment may have been mediated through direct effects on the placenta. To obtain support for this premise, we obtained placental explants from participants at delivery and treated them with increasing doses of pravastatin. We indeed found a significant dose-dependent reduction in sFlt-1 secretion (Figure 6A) with increasing doses of pravastatin. Similar to our experiments on placental tissues obtained from women who were not administered pravastatin (Figure 1E), we found a significant reduction in mRNA expression of the sFlt-1 isoform e15a (Figure 6B) but not sFlt-1 i13 (Figure S4). There was a nonsignificant reduction in sENG secretion (Figure 6C).

**Discussion**

Preeclampsia is one of the most serious complications of pregnancy where the health of both mother and fetus is at risk. There is no treatment other than delivery of the fetus and placenta. If severe disease occurs at preterm gestations, early delivery can inflict the risks of prematurity, including cerebral palsy, chronic lung disease, and neonatal demise. Severe disease can also occur at term gestation where the mother can become rapidly unwell.1,2,7 Hence, a medication that can quench preeclampsia disease progression has the potential to significantly improve morbidity and mortality of both mother and fetus.
Figure 5. Pravastatin (40 mg) daily stabilized clinical and biochemical features of preeclampsia in our participants. Blood pressure (the mean atrial pressure >24 hours is indicated by the point on each day, with mean systolic and diastolic blood pressure [mm Hg] indicated by the error bars; A–D), urinary protein/creatinine ratio (E), and serum urate concentrations (F) were stabilized in 3 of 4 participants. Pravastatin reduced soluble fms-like tyrosine kinase-1 (sFlt-1; G), stabilized soluble endoglin (sEng) levels (H) and endothelin-1 (ET-1) levels (I).
Pravastatin is currently being evaluated in 2 clinical trials. However, the move to clinical trials is perhaps surprising given all the preclinical mechanistic evidence pointing to pravastatin as a therapeutic has been performed in animal models. Work examining whether pravastatin has any biological effects in relevant human tissues is extremely limited. One recent study suggested that low-dose pravastatin has no effect on sFlt-1 or sENG secretion from placental explants obtained from 3 term preeclamptic women.

In this report, we have undertaken a comprehensive body of work to examine whether this statin may indeed be a potential therapeutic for preeclampsia. We performed a large body of preclinical studies exclusively in primary human tissues, including placentas from women with preterm preeclampsia. There were 3 themes to our preclinical studies. Firstly, we examined whether pravastatin reduced sFlt-1 and sENG production. Secondly, we investigated whether pravastatin could upregulate endogenous antioxidant defenses. Thirdly, we examined whether pravastatin could decrease endothelial dysfunction. Furthermore, we performed a pilot clinical study, administering pravastatin into 4 participants diagnosed with preterm preeclampsia and examined clinical, biochemical, and molecular markers of disease progression.

We demonstrated that pravastatin reduced sFlt-1 secretion from isolated cytotrophoblasts and HUVECs, as well as placental explants obtained from patients with preeclampsia. Pravastatin seemed to exert differential, tissue-specific effects on sENG secretion: it increased sENG secretion from HUVECs but stabilized sENG secretion from placental explants from women with severe preterm preeclampsia. We also showed that pravastatin seemed to be mediating its effects on sFlt-1 and sENG via the cholesterol synthesis pathway.

The increase in sENG secretion from endothelial cells has not been previously reported. Nachtigal et al found that atorvastatin-upregulated membrane bound endoglin in endothelium, and perhaps, this could cause an increase in sENG. It remains uncertain whether our finding of increased sENG release from endothelial cells may ultimately undermine the potential of pravastatin as a therapeutic for preeclampsia, especially given it seems to stabilize secretion from placenta. However, it is reassuring that circulating sENG levels seemed stable among our 4 women treated with pravastatin.

In our clinical study, we obtained evidence that administering 40 mg of pravastatin daily resulted in disease stabilization in 3 of 4 participants with preterm preeclampsia. During admission, the majority of these patients did receive regular antihypertensive therapy and all of our patients were given steroids for fetal maturation. Antihypertensive therapy and steroids have not been shown to affect preeclampsia disease progression or the secretion of antiangiogenic factors. The blood pressure seemed to stabilize without the need for increasing regular antihypertensive therapy in 3 of 4 patients although there were no new symptoms and no biochemical evidence of new onset involvement of other organ systems, such as the liver and the hematological system. This is a pilot study.
study of low numbers, and the results should be considered preliminary and interpreted with caution. Although our original intention was to recruit 12 participants in a small trial and publish the findings with the preclinical data, recruitment over the 1 year was slower than expected. Given we had completed our preclinical work and that it would likely take an additional 1 to 2 years to meet our recruitment target, we elected to publish the cohort recruited to date.

A novel aspect to our pilot study is that we interrogated serum molecular markers that may reflect disease severity. Interestingly, we found that serum sFlt-1 levels did not continue to rise and there was possibly a decline. sENG and ET-1 remained stable. These trends are reassuring, but we await validation from the much anticipated results from the Pravastatin to Ameliorate Early Onset Preeclampsia (STAMP) trial, a phase II randomized clinical trial of pravastatin to treat preterm preeclampsia. The stated primary outcome of this study is to examine whether pravastatin decreases serum sFlt-1 levels.

There has been a recent study where a woman with antiphospholipid syndrome was administered pravastatin from 23 weeks and delivered at term. Although this seems an impressive gain in gestation (which did not occur for any of our participants), there are reasons to view this case with caution. Although she had antiphospholipid syndrome and a past history of severe preeclampsia (both strong risk factors for subsequent preeclampsia), the diagnosis of preeclampsia was made on the basis of hypertension and borderline proteinuria (360 mg per 24 hours). This is perhaps a borderline diagnosis of preeclampsia. Furthermore, she was also receiving additional medication: enoxaparin and aspirin. In that study, serum sFlt-1 levels were not reported.

A novel aspect to our trial is that we performed functional assays on the placentas from trial participants at delivery. We indeed confirmed that the placental explants treated with pravastatin decreased sFlt-1 secretion, providing indirect evidence suggesting that the possible decline, or stabilization, in serum sFlt-1 observed in the trial may be caused directly by pravastatin on the preeclamptic placenta.

Delivery was required in 3 of 4 participants on fetal grounds because of severe growth restriction and abnormal fetal–placental blood flows. One patient required delivery after 15 hours of commencing pravastatin as her blood pressure remained labile. This suggests that pravastatin may have stabilized the maternal preeclamptic disease in 3 of 4 women, and potentially, their pregnancies could have progressed on maternal grounds. However, this pilot trial was too small to answer whether in fact pravastatin is able to safely prolong pregnancy when administered to women diagnosed with preterm preeclampsia.

**Perspectives**

We have undertaken preclinical studies using primary human tissues and a small pilot trial to examine whether pravastatin may be a potential therapeutic for preeclampsia. We found that pravastatin seems to decrease secretion of sFlt-1 and stabilizes sENG secretion from placenta and that it likely ameliorates the associated endothelial dysfunction. Furthermore, we obtained clinical and biochemical evidence that pravastatin may stabilize the preeclamptic disease in the mother. Collectively, our data support the contention that pravastatin may be a promising therapeutic for preeclampsia, but it should not be used until the findings of large robust clinical trials have been reported.

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**Disclosures**

None.

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Effects of Pravastatin on Human Placenta, Endothelium, and Women With Severe Preeclampsia

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Supplementary

Effects of Pravastatin on Human Placenta, Endothelium, and Women with Severe Preeclampsia

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Short title: Pravastatin treatment for preeclampsia

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Methods

Isolation and culture of placental explants

Human placental tissue was collected from three women with severe early onset preeclampsia (delivered at ≤32 weeks gestation). Preeclampsia was defined using the 2013 American College of Obstetricians and Gynecologists (ACOG) guidelines: the presence of hypertension >140/90 on two occasions 4 hours apart and any of the following: proteinuria >300mg/day, renal insufficiency, impaired liver function, thrombocytopenia or visual disturbance[1].

Written informed consent was obtained from all women and ethical approval was obtained from the Mercy Health Human Research Ethics Committee.

Villous explants (n=3) were prepared as previously described[2]. After 24 hours placental explants were treated with 0 or 2000 µmol/L (µM) pravastatin (Sigma, St Louis, United States) for 72h under 8% O₂ and 5% CO₂ at 37°C. To assess sFlt-1 and sENG secretion, protein levels were normalized against placental explant weights.

Isolation and treatment of primary human umbilical vein endothelial cells (HUVECs)

Umbilical cords (n=4) were collected from normal term placentas, the cord vein was infused with 10 ml (1mg/ml) of collagenase (Worthington, Lakewood, New Jersey) and cells isolated as previously described[2]. Cells were used between passages 2 to 4 and plated at 24,000 /cm² and treated at 80% confluency with 0, 20, 200, 2000 µM pravastatin for 24 hours. Conditioned media was collected for assessment of sFlt-1 and sENG secretion and cell lysates collected for RNA extraction.

Isolation and treatment of primary human trophoblast cells

Term placentas were collected from women having elective caesarean sections. Human trophoblasts (n=4) were isolated and plated as previously described[2, 3]. Cells were treated with 0, 20, 200 or 2000 µM pravastatin for 72 hours under 8% O₂ and 5% CO₂ at 37°C. Conditioned media was collected to assess sFlt-1 secretion.

Farnesyl pyrophosphate treatment

Primary HUVECs (n=3) and primary trophoblasts (n=3) were treated with 2000 µM pravastatin for 30 minutes and then 50 µM or 200 µM of farnesyl pyrophosphate (Sigma) was added to HUVECs and trophoblasts respectively and treatments continued for 24 hours.

Endothelial dysfunction

Endothelial dysfunction was induced by treatment of primary HUVECs (n=4) for 6 hours with 10ng/ml TNFα (Sigma) or trophoblast conditioned media (collected from primary trophoblasts cultured for 24 hours), washed and treated with pravastatin at 2000 µM for a further 6 hours. For the leucocyte adhesion assay experiment, THP-1 cells were pre-incubated with calcein (Merk Millipore, Darmstadt, Germany) for 30 minutes at 37°C and applied to HUVECs as previously described[4]. Fluostar omega fluorescent plate reader (BMG labtech, Victoria, Australia) was used to detect fluorescence (quantify adhesion) and an EVOS FL microscope (Life Technologies) was used to capture images.
**Endothelial cell migration and invasion**

To assess changes in endothelial cell migration and invasion, the xCELLigence system (Roche diagnostic, New South Wales, Australia) was used. Vascular endothelial growth factor (VEGF) (Bio-scientific, New South Wales, Australia) 12.5 ng/ml was the chemoattractant and 40,000 cells per well were plated and treated with ± 100 ng/ml sFlt-1 and 0 µM or 200 µM pravastatin. Electrical impedance was measured in each well every minute for 2 hours then every hour for 48 hours.

The invasion assay consisted of the same treatments as outlined for migration, however culture wells were coated with growth-factor reduced matrigel (Biosciences).

**ELISA analysis**

Concentrations of sFlt-1 and sENG were measured in conditioned cell/tissue culture media or patient serum using the DuoSet VEGF R1/Flt-1 kit (R&D systems by Bioscience, Waterloo, Australia) and a DuoSet Human Endoglin CD/105 ELISA kit (R&D systems). Concentrations of endothelin-1 were measured in human serum using the Quantikine endothelin-1 ELISA kit (R&D systems) as per manufacturer’s instructions.

**RT-PCR**

RNA was extracted from placenta explants and HUVECs using an RNeasy mini kit (Qiagen, Valencia, CA) and quantified using the Nanodrop ND 1000 spectrophotometer (NanoDrop technologies Inc, Wilmington, DE). 0.2ug of RNA was converted to cDNA using Superscript VILO cDNA synthesis kit (Life Technologies) as per manufacturer’s instructions.

Taqman gene expression assays for heme-oxygenase 1 (HO1) and endoglin were used (Life Technologies). RT-PCR was performed on the CFX 384 (Bio-Rad, Hercules, CA) using FAM-labeled Taqman universal PCR mastermix (Life Technologies) with the following run conditions: 50 °C for 2 minutes; 95 °C for 10 minutes, 95 °C for 15 seconds, 60 °C for 1 minute (40 cycles). Sybr green-based 22 gene expression assays for e15a and i13 primers were used. Primers were designed as previously described (Geneworks, South Australia, Australia)⁵. RT-PCR was performed using the following run conditions: 95 °C for 20 minutes; 95 °C for 0.01 minutes, 60 °C for 20 minutes, 95 °C for 1 minute (39 cycles), melt curve 65 °C to 95 °C at 0.05 °C increments at 0.05 seconds.

All data were normalized to GAPDH as an internal control and calibrated against the average C_t of the control samples. Results expressed as fold change from control.

**Immunofluorescence**

Localisation of the transcription factor Nuclear factor (erythroid-derived 2) – like 2 (Nrf2) was examined in trophoblasts treated with 2000 µM pravastatin. Primary trophoblasts were plated on fibronectin-coated chamber slides. After 24h, cells were washed and treated with 0 µM or 2000 µM pravastatin for 24 hours. Cells were washed with ice-cold PBS and fixed with 70% ethanol for 10 minutes. Cells were rehydrated in PBS for 5 minutes, 0.5% triton-X was added for 10 minutes to
permeabilize cell and nuclear membrane. Protein block (Life Technologies) was applied for 30 minutes at room temperature. Anti-Nrf2 antibody (Nrf2(H-300):sc-13032) (Santa Cruz Biotechnology, Dallas, Texas) at 4µg/ml was applied, (negative control iso-type matched rabbit IgG (Santa Cruz)) cells were incubated at 4°C for 12 hours. Cells were washed and alexafluor-488 goat anti-rabbit secondary antibody (Life Technologies) was applied at 1:200 in 1% BSA/PBS for 45 minutes at room temperature. Cells were washed and counter stained with DAPI and mounted. Fluorescent staining was visualized using the EVOS FL (Life Technologies).

Clinical study
We obtained ethics approval from The Mercy Health Human Research Ethics Committee to commence a trial examining the effect of pravastatin on treating women with preterm preeclampsia (approved 9th April 2013). This trial was registered prospectively with the Australian and New Zealand Clinical Trial Registry at http://www.anzctr.org.au ACTRN12613000268741.

Four women with preterm preeclampsia at 23+0 to 30+0 weeks gestation were consented to receive 40 mg pravastatin daily. Recruitment to this study was based on preeclampsia being defined as two blood pressures of >140/90 on two occasions 6 hours apart and proteinuria ≥0.5 g in a 24 h urine collection and were unlikely to require delivery in the next 24 hours (determined by an independent clinical team). We examined the effects of pravastatin on the clinical features of preeclampsia, including daily blood pressure (mean systolic and mean diastolic reading) and proteinuria (determined by daily urinary protein:creatinine ratio). We also investigated the effects of pravastatin on biochemical markers that may reflect the severity of preeclampsia, specifically sFlt-1, sENG and endothelin-1. Safety data including side effects and possible adverse events in the study participant and the fetus/neonate were collected. We obtained placental explants at delivery and treated them with pravastatin at 0, 200 or 2000 µM for 72 hours. Conditioned media was collected to assess sFlt-1 and sENG and cell lysates collected for RNA extraction.

Statistical analysis
Triplicate technical replicates were performed for each experiment, with a minimum of three separate experiments repeated for each in vitro study. Data was tested for normal distribution and statistically tested as appropriate. When three or more groups were compared a 1-way ANOVA (for parametric data) or Kruskal-Wallis test (for non-parametric data) was used. Post-hoc analysis was carried out using either the Tukey (parametric) or Dunn’s test (non-parametric). When two groups were analysed, either an unpaired t-test (parametric) or a Mann-Whitney test (non-parametric) was used. All data is expressed as mean ±SEM. P-values <0.05 were considered significant. Statistical analysis was performed using GraphPad Prism 6 software (GraphPad Software, La Jolla, CA).

We used descriptive statistics to report baseline clinical characteristics and analyte values.
Supplementary references


Supplementary Figure S1: Pravastatin does not change expression of the $i13$ isoform in HUVECs (A) or placental explants (B).

*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.
Supplementary Figure S2: Statins upregulate sENG via HMG CoA Reductase. Statins block HMG CoA reductase, an enzyme involved in cholesterol synthesis (A). Pravastatin up-regulates sENG secretion from HUVECs and this effect is reduced in the presence of farnesyl pyrophosphate (C). *p<0.05
Supplementary Figure S3: Pravastatin decreases markers of endothelial dysfunction induced by TNF-α. To further assess whether pravastatin can reduce endothelial dysfunction, primary HUVECs were treated with TNF-α (10 ng/ml) +/- pravastatin and the effect on markers of endothelial dysfunction assessed. TNF-α up-regulated HUVEC vascular cell adhesion molecule (VCAM-1) and this effect was significantly reduced with the addition of pravastatin (200 and 2000 µM) (A). TNF-α also up-regulated the adhesion of fluorescently labeled monocytes and this effect was also significantly reduced with the addition of pravastatin 2000 µM (B-C). ET-1 expression was up regulated by TNF-α and this effect was significantly reduced with the addition of pravastatin (200µM, 2000µM) (D). *p<0.05, ****p<0.0001. Representative images are shown at (5X magnification for all images).
Supplementary Figure S4: Pravastatin (200 and 2000 µM) did not affect sFlt1-i13 expression from placental explants obtained from study participants.