Vascular damage and dysfunction in hypertensive emergencies
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Citation for published version (APA):
Amraoui, F. (2017). Vascular damage and dysfunction in hypertensive emergencies
Sphingolipid metabolism in pre-eclamptic and normotensive pregnancies


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MANUSCRIPT IN PREPARATION
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ABSTRACT

Background: Ceramide is a sphingolipid with anti-angiogenic and pro-apoptotic properties that has recently been shown to be elevated in pre-eclampsia. In addition, pre-clinical and clinical studies suggest that ceramide may contribute to the development of hypertension and proteinuria. We aimed to compare sphingolipid metabolism in pre-eclamptic and normotensive pregnant women and to assess whether ceramide is related to hypertension and proteinuria in pre-eclampsia.

Methods: We measured sphingolipid content in plasma and placental tissue of pre-eclamptic and normotensive pregnant women using electrospray tandem mass spectrometry. Participants were recruited from the department of obstetrics at a university hospital in Amsterdam, The Netherlands.

Results: In total 92 pregnant women were included, 58 were normotensive and 34 had pre-eclampsia. Total plasma ceramide was elevated in pre-eclamptic women (9111±3090 nmol/ml) compared to normotensive women (7359±1674 nmol/ml, p=0.02). Plasma ceramide was higher in pre-eclamptic women with HELLP syndrome (11162 ± 2478 nmol/ml) compared to pre-eclamptic women without HELLP (7375 ± 2466 nmol/ml, p<0.01) and normotensive women (p<0.01). Placental ceramide was similar in pre-eclamptic (55±26 nmol/mg) and normotensive pregnant women (56±25 nmol/mg, N=32-58, p=0.81). Plasma ceramide was correlated with proteinuria in women with pre-eclampsia (r=0.62, p<0.01), but not with blood pressure and inversely correlated with platelet count (r=0.51, p=0.01) and gestational age (r=0.66, p<0.01).

Conclusions: Plasma ceramide is elevated in women with pre-eclampsia and HELLP syndrome. The correlation of plasma ceramide with proteinuria suggests a possible role in the pathogenesis of kidney injury associated with pre-eclampsia.
INTRODUCTION

Pre-eclampsia is characterized by hypertension and proteinuria in the second half of pregnancy and is one of the major causes of maternal death worldwide. Long after pregnancy, women with a history of pre-eclampsia remain at increased risk for developing cardiovascular and renal disease. Chronic hypertension and proteinuria, which may persist in up to 30% of women after pregnancy, likely contribute to the increased risk of future cardiovascular and renal disease.

Several placenta-derived factors have been linked to pre-eclampsia, including the anti-angiogenic factor sFLT-1, which scavenges vascular endothelial growth factor (VEGF) from the circulation thereby contributing to both blood pressure (BP) elevation and proteinuria. Blockade of VEGF signaling induces a shift in intracellular sphingolipid metabolism towards increased ceramide production, a pro-apoptotic lipid which induces BP elevation and has been linked to the loss of glomerular barrier integrity. Ceramide has recently been shown to be elevated in plasma and placental tissue of women with pre-eclampsia. We have previously shown that placental expression and enzyme activity of glucosidase beta acid (GBA), which hydrolyzes glycosylceramide into ceramide, is increased in placental tissue of women with pre-eclampsia.

Ceramide belongs to the sphingolipid family, which are bioactive components of cell membranes and are involved in key cellular processes such as cell growth, differentiation, barrier function, migration and apoptosis. Ceramide can be synthesized de novo, but can also result from either hydrolysis of sphingomyelin or breakdown of more complex sphingolipids (salvage pathway). Ceramide can be used to synthesize sphingomyelin, which is abundantly present in virtually all cells. Ceramide can also be metabolized to yield sphingosine and sphingosine-1 phosphate (S1P). Within this backbone of sphingolipid metabolism, S1P and ceramide have multiple opposing actions, including pro- and anti-angiogenic effects respectively. Alternative mechanisms to regulate ceramide levels, in addition to the ceramide-S1P rheostat, are provided by glycosylation and phosphorylation of ceramide to yield more complex sphingolipids.

In the current study, we aimed to assess whether sphingolipid metabolism is altered in women with pre-eclampsia compared to normotensive pregnancies and whether changes are related to hypertension and proteinuria in pre-eclampsia.
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METHODS

Study population
Clinical data and biosamples were obtained from participants in the Pre-eclampsia And Non pre-eclampsia DATabase (PANDA) program of the department of Obstetrics & Gynecology of the Academic Medical Center in Amsterdam, The Netherlands. Definitions and clinical criteria used in the biobank program have been published previously. Briefly, pre-eclampsia was defined by systolic blood pressure (BP) ≥ 140 mmHg or diastolic BP ≥ 90 mmHg recorded on two occasions at least 4 hours apart, after 20 weeks' gestation in a previously normotensive woman combined with new-onset proteinuria with urinary protein excretion ≥ 300 mg/24-hours. BP was measured manually in the sitting position at the right upper arm using an aneroid sphygmomanometer. Diastolic BP was determined at Korotkoff sound V. HELLP syndrome was defined by lactate dehydrogenase ≥ 600 U/L or haptoglobin < 0.2 g/L, aspartate or alanine aminotransferase ≥ 70 U/L, and platelet count < 100 * 10^9/L. Experiments were carried out in accordance with the declaration of Helsinki after informed consent from the participants was obtained. Experiments were approved by an independent ethics committee.

Liquid chromatography - mass spectrometry of plasma and placental tissue
For plasma sphingolipid analysis, lipids were extracted from 50 μL plasma as described. Briefly; 50 μL of plasma was diluted with 150 μL of phosphate buffered saline, followed by the addition of 1 mL methanol and 0.5 mL chloroform containing an internal standard mixture added as a cocktail of 500 pmol each. Standards for sphingoid bases and sphingoid base 1-phosphates were 17-carbon chain length analogs: C17-sphingosine, (2S,3R,4E)-2-aminoheptadec-4-ene-1,3-diol (d17:1-So); C17-sphinganine, (2S,3R)-2-aminoheptadecane-1,3-diol (d17:0-Sa); C17-sphingosine 1-phosphate, heptadecasphing-4-enine-1-phosphate (d17:1-So1P); and C17-sphinganine 1-phosphate, heptadecasphinganine-1-phosphate (d17:0-Sa1P). Standards for N-acyl sphingolipids were C12-fatty acid analogs: C12-Cer, N-(dodecanoyl)-sphing-4-enine (d18:1/C12:0); C12-Cer 1-phosphate, N-(dodecanoyl)sphing-4-enine-1-phosphate (d18:1/C12:0-Cer1P); C12-sphingomyelin, N-(dodecanoyl)sphing-4-enine-1-phosphocholine (d18:1/C12:0-SM); and C12-glucosylceramide, N-(dodecanoyl)-1-β-glucosyl-sphing-4-eine. The mixture was sonicated followed by incubation for 4 h at 48°C. Following this incubation, the extracts were transferred to a new glass tube, dried down, and reconstituted in methanol (600 μl) by vortexing and incubating at 48°C for 15 min. The reconstitution in methanol and incubating at 48°C is a new addition to our previously published method, and was incorporated into the existing method to ensure proper solubilisation of the long-chain sphingolipids. The lipid extract thus obtained, contained insignificant levels of proteins as measured by the Bradford assay (data not
shown) and was used in the analysis of the sphingolipids C1P, ceramide, sphingomyelin, and monohexyleramid. The lipids were separated using a Kinetix C18 column (50 × 2.1 mm, 2.6 µm; Phenomenex) on a Nexera UPLC system (Shimadzu) and eluted using a linear gradient (solvent A, 58:41:1 CH₃OH/water/HCOOH 5 mm ammonium formate; solvent B, 99:1 CH₃OH/HCOOH 5 mm ammonium formate, 20–100% B in 3.5 min and at 100% B for 4.5 min at a flow rate of 0.4 ml/min at 60°C). ESI-MS/MS using a 6500 QTRAP® (Sciex) mass spectrometer was used to detect sphingolipids via multiple reaction monitoring under positive ionization.

The placenta samples were weighed and an amount of PBS was added to obtain a 10% w/v solution. The resultant mixture was homogenized using an Omni tissue grinder and the material was ground until a fine suspension was obtained. Sphingolipid internal standards as done for the plasma samples were added to 200 microliters of the resultant suspension, and lipids were extracted via the method of Bligh and Dyer. Following phase separation, the bottom organic layer was transferred to a new glass tube, dried via a vacuum dryer followed by resuspension in 600 microliters of methanol. Sphingolipid measurements were obtained from these samples via liquid chromatography mass spectrometry method described above. Analysts were blinded for patient data during these experiments.

**Statistical analysis**

Continuous variables were expressed as mean and standard deviation (SD) or median and interquartile range (IQR) for variables with a skewed distribution. Categorical data are expressed as number and percentages. Between group differences were assessed by t-test for parametric and Mann–Whitney U test for non-parametric distributions. Chi-square statistics were used for categorical variables. Comparison of plasma and placenta sphingolipids between pre-eclamptic women with and without HELLP and normotensive women was carried out by a one-way ANOVA with post-hoc Bonferroni correction. Linear regression analysis was used to assess correlations among sphingolipid content in plasma, placental tissue and clinical parameters. For statistical analyses, SPSS software was used (Statistical Package for the Social Sciences, version 19.0, Inc. Chicago, Illinois, USA). P-values were considered to indicate a significant difference if p<0.05.

**RESULTS**

**Clinical characteristics of pre-eclamptic and normotensive women**

In total 92 pregnant women were included in the study, 58 were normotensive and 34 had pre-eclampsia. Clinical characteristics with comparison of women with and without pre-eclampsia are summarized in Table 1. HELLP syndrome was present in 15 (44%) women with
pre-eclampsia. Magnesiumsulphate (MgSO4) was administered to 14 (41%) pre-eclamptic women. Antihypertensive treatment was administered to 24 (71%) women with pre-eclampsia, 14 (41%) received 1 antihypertensive agent, 7 (21%) received 2 antihypertensive agents and treatment with 3 different antihypertensive drugs was required in 3 (9%) pre-eclamptic women. For BP lowering therapy, a calcium-antagonist (nifedipine retard) was used in 20 (59%) women with pre-eclampsia, 12 (35%) received a central α1 agonist (methyldopa) and 5 (14%) received a combined α1 and β-blocking agent (labetalol). Nine (26%) women with pre-eclampsia were not treated with any type of antihypertensive medication, of these women, one received MgSO4 prior to delivery. A calcium antagonist (nifedipine retard) was administered to 7 (12%) normotensive as a tocolytic agent to delay preterm labour.

Table 1. Clinical characteristics with comparison of normotensive pregnant women and women with pre-eclampsia

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Pre-eclampsia</th>
<th>Normotensive</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients, N</td>
<td>34</td>
<td>58</td>
<td></td>
</tr>
<tr>
<td>Age, years</td>
<td>31±4</td>
<td>29±6</td>
<td>0.06</td>
</tr>
<tr>
<td>Caucasian*</td>
<td>14 (47%)</td>
<td>15 (41%)</td>
<td>0.62</td>
</tr>
<tr>
<td>Body Mass Index, kg/m2</td>
<td>28±6</td>
<td>24±6</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Systolic BP, mmHg</td>
<td>153±20</td>
<td>116±12</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Diastolic BP, mmHg</td>
<td>96±9</td>
<td>70±11</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Proteinuria, g/24hrs</td>
<td>1.95 [0.60-5.11]</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td>Platelet count, x10^9/L</td>
<td>138±89</td>
<td>238±52</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Lactate dehydrogenase*</td>
<td>321 [249-603]</td>
<td>188 [151-254]</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Nulliparous§</td>
<td>23 (70%)</td>
<td>36 (64%)</td>
<td>0.60</td>
</tr>
<tr>
<td>Gestational age at delivery, days</td>
<td>247±24</td>
<td>254±31</td>
<td>0.26</td>
</tr>
<tr>
<td>Birth weight, grams</td>
<td>2210±894</td>
<td>2530±892</td>
<td>0.10</td>
</tr>
<tr>
<td>Antenatal steroids†</td>
<td>12 (40%)</td>
<td>15 (27%)</td>
<td>0.23</td>
</tr>
<tr>
<td>Delivery by caesarean section</td>
<td>16 (47%)</td>
<td>12 (21%)</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Numbers represent mean ± standard deviation, median with [interquartile range], or number of subjects with percentages. *Data on ethnicity missing for 4 women with pre-eclampsia and for 21 normotensive women. §Data missing for 1 woman with pre-eclampsia and for 2 normotensive women. † Data missing for 4 women with pre-eclampsia and 3 normotensive women.

Sphingolipids in plasma

Total ceramide was higher in plasma of pre-eclamptic women compared to normotensive women, while sphinganine was significantly lower (Table 2 & Figure 1A). There were no significant differences with regard to other sphingolipid components in plasma. Ceramide subtypes C16, C18:0, C18:1, C20, C24:1 were significantly higher in pre-eclamptic plasma (Figure 1A). Comparison of total plasma ceramide levels among normotensive women (N=24) and pre-eclamptic women with (N=11, 46%) and without HELLP (N=13, 54%)
showed higher ceramide content in pre-eclamptic women with HELLP (11162 ± 2478 nmol/ml) compared to pre-eclamptic women without HELLP (7375 ± 2462 nmol/ml, p<0.01) and normotensive women (7359 ± 1674 nmol/ml, p<0.01, Figure 3). Plasma ceramide was similar in normotensive women and pre-eclamptic women without HELLP. Plasma sphinganine was not significantly different when compared among pre-eclamptic women with and with HELLP and normotensive pregnant women. Total plasma sphingomyelin was higher in pre-eclamptic women with HELLP (658398 ± 98149 nmol/ml) compared to those without HELLP (547442 ± 89043 nmol/ml, p=0.03) and normotensive women (555592 ± 109321 nmol/ml, p=0.2). Total plasma ceramide and plasma sphingomyelin were strongly correlated in both normotensives (r=0.62, p<0.01) and women with pre-eclampsia (r=0.76, p<0.01).

Figure 1. Sphingolipid metabolism alterations in plasma and placental tissue of women with pre-eclampsia

Human sphingolipid metabolic network was populated with the relevant lipid data for plasma (A) and placental tissue (B) for women with pre-eclampsia compared to their matched normotensive pregnant controls. The nodes are generated such that the magnitude of the fold change for the measured species is proportional to the diameter of the corresponding node of the metabolic network. Where these differences are statistically significant (p<0.05), those nodes are highlighted via a dashed border. The magnitude of a node for a fold change of one are exampled in the legend above panel A.
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Table 2. Sphingolipids in plasma of normotensive pregnant women and women with pre-eclampsia

<table>
<thead>
<tr>
<th>Sphingolipids (nmol/ml)</th>
<th>Pre-eclampsia (N=24)</th>
<th>Normotensive (N=24)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sphingomyelin</td>
<td>598297±107305</td>
<td>555592±109321</td>
<td>0.18</td>
</tr>
<tr>
<td>Ceramide</td>
<td>9111±3090</td>
<td>7359±1674</td>
<td>0.02</td>
</tr>
<tr>
<td>Monohexylceramide</td>
<td>26713±11646</td>
<td>28957±5640</td>
<td>0.40</td>
</tr>
<tr>
<td>Sphingosine</td>
<td>63±35</td>
<td>83±50</td>
<td>0.13</td>
</tr>
<tr>
<td>Sphingosine-1 Phosphate</td>
<td>1150±433</td>
<td>1178±533</td>
<td>0.84</td>
</tr>
<tr>
<td>Sphinganine</td>
<td>27±25</td>
<td>48±37</td>
<td>0.02</td>
</tr>
</tbody>
</table>

Numbers represent mean ± standard deviation.

Sphingolipids in placental tissue

Total monohexylceramide and sphinganine were significantly increased in placental tissue of pre-eclamptic women compared to normotensive pregnant women (Table 3 & Figure 1A). Total sphingomyelin and S1P tended to be higher in pre-eclampsia, but this was not statistically significant.

Placental monohexylceramide was higher in pre-eclamptic women with HELLP (7.4 ± 5.7 nmol/mg) compared to normotensive women (4.9 ± 2.2 nmol/ml, \( p=0.02 \)), but not compared to pre-eclamptic women without HELLP (6.6 ± 2.5 nmol/ml, \( p=1.00 \)). Placental monohexylceramide was similar in normotensive women and pre-eclamptic women without HELLP. Placental sphinganine was significantly higher in pre-eclamptic women with HELLP (13.5 ± 8.1 nmol/mg) compared to normotensives (8.1 ± 6.1 nmol/mg, \( p=0.02 \)), but not compared to pre-eclamptic women without HELLP (10.3 ± 6.9 nmol/mg, \( p=0.51 \)). Sphinganine was not significantly different among normotensives and pre-eclamptic women without HELLP (\( p=0.70 \)).

Pre-eclamptic women with HELLP had significantly higher placental S1P levels (0.11 ± 0.09 nmol/mg) compared to normotensives (0.06 ± 0.05, \( p=0.03 \)), but not compared to pre-eclamptic women without HELLP (0.07 ± 0.06, \( p=0.39 \)). Placental S1P was not significantly different between normotensives and pre-eclamptic women without HELLP. Comparison of total sphingomyelin and ceramide in placental tissue of pre-eclamptic women with and without HELLP and normotensives showed no significant differences (Table 3).

Table 3. Sphingolipids in placental tissue of normotensive pregnant women and women with pre-eclampsia

<table>
<thead>
<tr>
<th>Sphingolipids (nmol/mg)</th>
<th>Pre-eclampsia (N=32)</th>
<th>Normotensive (N=58)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sphingomyelin</td>
<td>353±123</td>
<td>311±92</td>
<td>0.07</td>
</tr>
<tr>
<td>Ceramide</td>
<td>55±26</td>
<td>56±25</td>
<td>0.81</td>
</tr>
<tr>
<td>Monohexylceramide</td>
<td>6.96±4.15</td>
<td>4.90±2.15</td>
<td>0.01</td>
</tr>
<tr>
<td>Sphingosine</td>
<td>3.62±2.49</td>
<td>3.55±3.51</td>
<td>0.92</td>
</tr>
<tr>
<td>Sphingosine-1 Phosphate</td>
<td>0.09±0.08</td>
<td>0.06±0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>Sphinganine</td>
<td>0.58±0.38</td>
<td>0.41±0.31</td>
<td>0.02</td>
</tr>
</tbody>
</table>

Numbers represent mean±standard deviation.
Ceramide is Elevated in Pre-eclampsia and Correlated with Proteinuria

Figure 2. Plasma ceramide in normotensive women and in pre-eclamptic women with and without HELLP syndrome

Boxes and whiskers represent mean range from minimum to maximum value. Plasma ceramide concentration is higher in pre-eclamptic women with HELLP (PE + HELLP, N=11) compared to pre-eclamptic women without HELLP (PE, N=13) and normotensive women (NT, N=24). * Indicates $p<0.01$ with one-way ANOVA and post-hoc Bonferroni correction.

Figure 3. Plasma ceramide is correlated with proteinuria in women with pre-eclampsia

Plasma ceramide concentration is correlated with urinary protein excretion in women with pre-eclampsia.
**Correlation of plasma ceramide with proteinuria and blood pressure in pre-eclampsia**

Total plasma ceramide was strongly correlated with proteinuria in women with pre-eclampsia ($r=0.62, p<0.01$), with higher urinary protein excretion in women with higher plasma ceramide values (Figure 4). Plasma ceramide was not correlated with systolic BP ($r=0.09, p=0.67$) and diastolic BP ($r=0.21, p=0.34$). Total placental ceramide was not correlated with proteinuria ($r=0.06, p=0.75$) or systolic and diastolic BP ($r=0.04, p=0.82$ for both).

**Figure 4. Plasma ceramide is correlated with gestational age at delivery in women with pre-eclampsia**

![Graph showing inverse correlation between total plasma ceramide and gestational age at delivery in women with pre-eclampsia.](image)

Plasma ceramide concentration is inversely correlated with gestational age in women with pre-eclampsia.

**Plasma ceramide is inversely correlated with gestational age**

Total plasma ceramide was inversely correlated with gestational age at delivery in women with pre-eclampsia ($r=0.66, p<0.001$), showing lower ceramide values with increasing gestational age at delivery (Figure 5). This correlation was absent in normotensive pregnant women ($r=0.24, p=0.26$). Total plasma ceramide was also inversely correlated with neonatal birth weight ($r=0.57, p<0.01$) and placental weight ($r=0.79, p=0.02$) in pre-eclamptic women, but not after adjustment for gestational age at delivery ($p=0.19$ and $p=0.08$ respectively). Plasma ceramide was not correlated with neonatal birth weight in normotensives ($r=0.24, p=0.25$). Placental ceramide was not correlated with gestational age at delivery or neonatal birth weight in pre-eclamptic women and normotensives.
Figure 5. Plasma ceramide is correlated with platelet count in women with pre-eclampsia.

**Plasma ceramide is correlated with platelet count**
Plasma ceramide was inversely correlated with platelet count in women with pre-eclampsia ($r=0.51$, $p=0.01$, Figure 6), but not in normotensive women ($r=0.17$, $p=0.68$). Placental tissue ceramide was not correlated with platelet count in women with pre-eclampsia ($r=0.04$, $p=0.85$).

**Plasma ceramide is correlated with placental FLT1 mRNA in HELLP syndrome**
To assess whether plasma ceramide levels are correlated with placental expression of sFLT-1, previously published real-time qPCR data on placental *FLT1* transcripts were reused. Total plasma ceramide was not correlated with total placental *FLT1* mRNA expression in women with pre-eclampsia ($r=0.47$, $p=0.24$) and normotensive pregnant women ($r=0.56$, $p=0.62$). Separate analysis of pre-eclamptic women with HELLP showed a strong correlation of plasma ceramide with placenta *FLT1* mRNA expression ($r=0.99$, $p<0.01$), but not in pre-eclamptic women without HELLP ($r=0.09$, $p=0.91$). Total placental ceramide was not correlated with placental *FLT1* mRNA expression in pre-eclamptic women with HELLP ($r=0.40$, $p=0.51$), pre-eclamptic women without HELLP ($r=0.19$, $p=0.68$) and normotensive pregnant women ($r=0.03$, $p=0.88$).
Ceramide in plasma and placental tissue are not correlated with GBA enzyme activity

To assess whether placental GBA enzyme activity is related to excess ceramide levels in pre-eclampsia, we used recently published data on GBA enzyme activity.\textsuperscript{10} Plasma ceramide was not correlated with placental GBA enzyme activity in pre-eclamptic women with HELLP ($r=0.02$, $p=0.96$), pre-eclamptic women without HELLP ($r=0.65$, $p=0.08$) and normotensive women ($r=0.25$, $p=0.75$). Total ceramide in placental tissue was also not correlated with placental GBA enzyme activity in pre-eclamptic women with HELLP ($r=0.43$, $p=0.15$) and normotensive women ($r=0.15$, $p=0.36$).

DISCUSSION

In the present study we show that plasma ceramide is elevated in women with pre-eclampsia and HELLP syndrome compared to normotensive pregnant women. In addition, higher plasma ceramide levels are associated with increased urinary protein excretion and lower platelet count.

To our knowledge, only one previous study addressed sphingolipid metabolism in women with pre-eclampsia and normotensive pregnant women.\textsuperscript{9} In this comprehensive study by Melland-Smith et al, a comparable elevation in plasma ceramide was observed in women with pre-eclampsia. The excess plasma ceramide was believed to originate from the placenta, which also showed increased ceramide content. We could not confirm the elevation in placental tissue ceramide in the present study. This discrepancy might be due to differences in gestational age, which appears to be inversely correlated with plasma ceramide. Mean gestational age was considerably higher in the present study compared to the study by Melland-Smith et al, possibly masking differences in placental ceramide content.

Elevation of placental ceramide in the study by Melland-Smith et al. was due to increased de novo synthesis and reduced lysosomal degradation of ceramide, but not by hydrolysis of sphingomyelin. Interestingly, blockade of VEGF signaling, which is part of the pathogenesis of pre-eclampsia, has also been shown to induce increased ceramide production via de novo synthesis.\textsuperscript{6} We observed a strong correlation between placental \textit{FLT1} mRNA and plasma ceramide in women with HELLP syndrome, suggesting that VEGF inhibition associated with pre-eclampsia could indeed contribute to increased ceramide levels. We recently showed that placental GBA enzyme activity was increased in placental tissue of pre-eclamptic compared to normotensive women, potentially augmenting ceramide levels via hydrolysis of glucocyslceramide.\textsuperscript{10} We did not observe elevated ceramide content in placental tissue nor any correlation between sphingolipids in plasma and in placental tissue of the same women, suggesting that excess plasma ceramide may not originate from the placenta.
Ceramide is Elevated in Pre-eclampsia and Correlated with Proteinuria

However, absence of these correlations does not rule out the possibility that excess plasma ceramide is released from placenta derived extracellular vesicles. Syncytiotrophoblast derived extracellular vesicles are released into the maternal circulation have been linked to the pathogenesis of pre-eclampsia via their pro-inflammatory and anti-angiogenic action. In 2008 Trajkovic et al. showed that ceramide is essential for exosome biogenesis and that purified exosomes are enriched in ceramide, potentially explaining the excess plasma ceramide in pre-eclampsia. Alternatively, platelets are a well known source of ceramide. Although S1P is immediately released in the circulation upon platelet activation, there are some conflicting data with regard to platelet ceramide content showing either a decrease or no change upon platelet activation. Our observation that ceramide increases with decreasing platelet count could indicate that elevated plasma ceramide level is platelet derived. However, we observed no difference in plasma S1P levels to support this theory.

Consumption of platelets may also be a marker of endothelial damage in women with pre-eclampsia, who display an increased amount of circulating endothelial cells and other markers of endothelial damage. Therefore, the observed inverse correlation between platelet count and plasma ceramide content may also point towards the endothelium as source of excess plasma ceramide. Activation of the endothelium by pro-inflammatory cytokines and oxidative stress leads to increased endothelial ceramide production via activation of acid or neutral sphingomyelinase. The strong correlation between plasma ceramide and sphingomyelinase observed in the present study, supports a role for sphingomyelinase in the excess ceramide production.

Elevated plasma ceramide was correlated with higher urinary protein excretion in women with pre-eclampsia. Whether ceramide is causally related to proteinuria in pre-eclampsia remains to be determined. In mice, a high-fat diet has been shown to induce obesity, glomerular changes and proteinuria, which coincide with an elevation in plasma and kidney ceramide content via the sphingomyelinase pathway. High fat diet-induced elevation of ceramide content and proteinuria was attenuated in acid sphingomyelinase knock-out mice and also after silencing of acid sphingomyelinase gene expression in wild type mice, suggesting that augmented ceramide generation is causally related to proteinuria. In line, plasma ceramide is elevated in patients with type 2 diabetes. A genetic variant in CERS2, encoding for ceramide synthase, has been associated with a higher rate of proteinuria in over 3000 diabetic patients participating in ONTARGET and TRANSCEND trials, with the highest rate in homozygotes and intermediate risk in heterozygotes. Metabolites of ceramide have been implicated in the pathogenesis of glomerular injury and proteinuria in genetic sphingolipid storage diseases such as Fabry. Finally, ceramide has been shown to increase endothelial permeability, potentially contributing to loss of glomerular barrier integrity and proteinuria in pre-eclampsia.
Although no correlation with BP was observed in the present study, elevation of plasma ceramide has previously been linked to hypertension. We have previously shown that plasma ceramide is elevated in patients with essential hypertension, and that plasma ceramide content was related to severity of hypertension. Importantly, pharmacological elevation of ceramide with dimethylsphingosine raised BP in spontaneously hypertensive rats, but not in normotensive control rats. Accordingly, elevation of ceramide via either dimethylsphingosine or with sphingomyelinase induced vasoconstriction *ex vivo* in vessels of spontaneously hypertensive rats due to increased thromboxane A2 production. This vasocontractile effect of ceramide was absent after mechanical removal of the endothelium and was not observed in normotensive control rats, indicating that ceramide is only vasocontractile in a state of endothelial dysfunction. Hypertension precedes endothelial dysfunction in spontaneously hypertensive rats, suggesting that elevated ceramide was not initially responsible for BP elevation in these animals, but may eventually have contributed to sustained BP elevation. In line with this hypothesis, dietary fish oil has been shown to lower BP and mitigate ceramide-induced vasoconstriction in spontaneously hypertensive rats via reduction of plasma ceramide content and thromboxane A2 generation. Endothelial dysfunction is also present in women with pre-eclampsia and may even precede pre-eclampsia, suggesting that elevated vascular ceramide content may contribute to BP elevation in pre-eclampsia. Interestingly, pre-eclampsia is associated with increased thromboxane A2 production, which could partly be explained by excess ceramide.

In conclusion, the current study corroborates the recent observation of elevated plasma ceramide content in women with pre-eclampsia compared to normotensive pregnant women. In contrast, we observed no differences in ceramide content of placental tissue nor any correlation between plasma and placenta sphingolipid content, suggesting that excess plasma ceramide does not originate from the placenta. Release of ceramide from placenta derived extracellular vesicle can however not be ruled out. We show for the first time that plasma ceramide is correlated with proteinuria in women with pre-eclampsia. Clinical and pre-clinical studies suggest that ceramide could be causally related to proteinuria, indicating that excess ceramide may be a potential novel target for treatment in pre-eclampsia.
REFERENCES


