Plasma S1P and sphingosine are not different prior to preeclampsia in women at high risk of developing the disease

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Abstract (250 words)

Introduction
Sphingolipids like sphingosine-1-phosphate (S1P) have been implicated in the pathophysiology of preeclampsia. We hypothesised that plasma S1P would be increased in women at high risk of developing preeclampsia who subsequently develop the disease. Low circulating placental growth factor (PIGF) is known to be associated with development of preeclampsia so further we hypothesised that increased S1P would be associated with concurrently low PIGF.

Materials and Methods
Case control study using stored maternal blood samples from 14-24 weeks pregnancy, collected from 95 women at increased risk of preeclampsia. Pregnancy outcome was classified as uncomplicated, preterm preeclampsia (<37 weeks) or term preeclampsia. Plasma lipids were extracted and analysed by ultraperformance liquid chromatography coupled to electrospray ionisation tandem mass spectrometry to determine concentrations of S1P and sphingosine.

Results
Median plasma S1P was 0.339nmol/ml and sphingosine was 6.77nmol/l. There were no differences in the plasma concentrations of S1P or sphingosine in women who subsequently developed preeclampsia, no effect of gestational age, fetal sex, ethnicity, or presence of pre-existing hypertension. There was a correlation between S1P and sphingosine plasma concentration (p<0.0001). There was no relationship between S1P or sphingosine with PIGF.

Discussion
Previous studies have suggested that plasma S1P maybe a biomarker of preeclampsia. In our larger study we failed to demonstrate this is women at high risk of developing the disease. We did not show a relationship with known biomarkers of the disease, suggesting that S1P is unlikely to be a useful predictor of the development of preeclampsia later in pregnancy.

Key words
S1P; Sphingosine; Pregnancy; Preeclampsia; PlGF

Introduction

Sphingolipids affect a diverse array of cellular processes and functions in multiple biological systems. Deranged signalling has been linked to multiple diseases (1). Sphingosine-1-Phosphate (S1P) is a pleiotropic sphingolipid found in the circulation that has been extensively investigated in the context of multiple pathologies in part due to its potent effects on cell motility (2). Circulating S1P levels are tightly controlled by sphingosine kinase 1 and 2, S1P phosphatases (S1PP)(1), lipid phosphate phosphatases (2) and S1P lyase which degrades it into sphingosine and ethanolamine as part of the so-called sphingolipid rheostat (3). S1P affects cell movement via interactions with five G-protein linked cell surface receptors (S1PR1-5) and in vitro has been shown to affect the migratory cells of the placenta, extra-villous trophoblast (EVT), by interaction with S1PR2 (4). Sphingosine kinase deficient mice demonstrate impaired placental development (5). Impaired placental development and impaired EVT migration have been implicated in the pathophysiology of preeclampsia which affects ~3% of pregnancies and causes significant maternal and infant morbidity and mortality worldwide. S1P is potential mediator of the disease. S1P concentrations may also be altered in inflammatory states (6) further supporting its involvement in the disease, as preeclampsia is also, in part, an inflammatory disorder (7). Sphingosine is present at a much lower fraction in plasma than S1P (8) and little is known about control of plasma concentrations.

Studies using HPLC methods in non-pregnant healthy adult populations of men and women have demonstrated plasma S1P levels of 0.75 ± 0.16 nmol/mL (9) to 1nmol/ml (10). Concentrations increase in inflammatory and infectious disease as part of the body’s immune response (11, 12). In pregnant women studies have reported conflicting data on S1P concentrations in normal pregnancy and diseases associated with placental dysfunction. Melland-Smith et al. (13) demonstrated reduced serum S1P in women with preeclampsia versus normal pregnancy. This contrasted with findings by Dobierzewska et al. (9) who showed no significant difference in plasma S1P, at any gestation, in women who went on to develop preeclampsia versus controls. These studies are both small (n=10 and 7 respectively) and contrasting results may be explained by their potentially unrepresentative
sample size; the Melland-Smith study differed in that the investigators analysed samples taken nearer to diagnosis of preeclampsia. A larger study of 57 women with a less well-defined preeclampsia phenotype suggested an increase in plasma S1P in women with diagnosed preeclampsia (14).

Circulating levels of sphingosine are 8.0nmol/l in non-pregnant subjects (8). To our knowledge there is only one previous study examining plasma sphingosine concentration in preeclampsia and this suggested that the plasma concentrations were significantly higher than in the non-pregnant population (14).

In this study we examined second and early third trimester S1P and sphingosine plasma concentrations in a case-control study which included women at high risk of developing preeclampsia. All women included were at increased risk of preeclampsia either because of a history of a hypertensive pregnancy disorder and/or because of chronic pre-pregnancy hypertension. Based on the observed inhibitory effects of S1P on EVT migration in vitro (4) we hypothesised that S1P concentrations would be increased in women that went on to develop preeclampsia. We investigated the relationship between measurements of S1P, sphingosine and the preeclampsia biomarker, placental growth factor (PIGF), and compared different phenotypes of preeclampsia (preterm and term). In a subset of women with longitudinal samples, we compared measurements between the second and early third trimester.

Materials and Methods
This case control study used plasma samples collected from the Manchester Antenatal Vascular Service (The MAVIS clinic (15)). Collection of samples was approved by the NRES Committee North West 11/NW/0426 in accordance with the Declaration of Helsinki principles; all women gave written informed consent to donate samples for research studies. Women recruited had either current hypertension or a hypertensive disorder in a prior pregnancy. Clinical data and blood samples in EDTA were collected during routine visits between 14-17+6 and 18+0-24+6 weeks; for a small number of women longitudinal samples were available. For women included in this case-control study, pregnancy
outcomes were categorised as uncomplicated (birth ≥37 weeks, birthweight centile ≥10th with no hypertensive complications) or preeclampsia (defined using ISSHP (16) guidelines). Women with preeclampsia were divided into preterm preeclampsia (women who developed the disease before 37 weeks and who were delivered by this gestation) and term preeclampsia (women who developed preeclampsia after 37 weeks and were delivered after this gestation). In women with pre-pregnancy hypertension, pre-eclampsia was defined as worsening hypertension associated with evidence of placental dysfunction, proteinuria and/or multiorgan disease. All samples were taken prior to a diagnosis of preeclampsia. There were 127 measurements in total in 95 women; 65 women has a single measurement and 30 women had two measurements.

**Sphingolipid extraction and UPLC-MS/MS analysis**

Lipid extractions were carried out as previously described (17) using a single-phase system to maximise recovery (18). Briefly, plasma (50 µl) was added to ice-cold ethyl acetate: isopropanol: water (6:3:1, v/v/v) and spiked with 4 ng each of deuterated internal standards for sphingosine (sphingosine-d7; Avanti Polar Lipids, Alabaster, AL, USA) and S1P (S1P-d7; Avanti Polar Lipids). Samples were then incubated on ice for 30 min, centrifuged to pellet out the denatured proteins, and the supernatant was collected and dried down under a gentle stream of nitrogen. The resulting lipid residues were resuspended in methanol containing 0.1 % (v/v) formic acid (mobile phase B) and stored at -20°C until analysis. Assay recoveries for sphingosine-d7 and S1P-d7 were 87.7 % and 90.1 %, respectively. Sphingosine and S1P were analysed by ultraperformance liquid chromatography couple to electrospray ionisation tandem mass spectrometry (UPLC/ESI-MS/MS), as previously described (19), using an Acquity UPLC system (Waters Corporation, Wilmslow, UK) paired with a triple quadrupole mass spectrometer (Xevo TQ-S; Waters Corporation); data acquisition using MassLynx software (Waters Corporation). Separation was performed using a reverse-phase Acquity BEH C8 column (1.7 µm 2.1 x 100mm) at a flow rate of 0.3 ml/min and a column temperature of 30°C. Separation was performed using a gradient system of mobile phase A (water containing 0.1 % (v/v) formic acid) and mobile phase B (methanol containing 0.1 % (v/v) formic acid). Electrospray ionisation was performed in positive-ion mode using the following settings: capillary voltage, 3.5kV; source temperature, 100°C; desolvation gas temperature, 450°C. Analytes were quantitated using multiple reaction monitoring (MRM):
S1P m/z 380.249>264.278; S1P-d7 m/z 387.292>271.25; sphingosine m/z 300.332>282.205; sphingosine-d7 m/z 307.300>289.300.

Calibration lines were constructed using synthetic standards (Avanti Polar Lipids) to allow accurate quantitation. Results are expressed as ng/mL plasma and have been converted to nmol/ml to aid comparison with previously published results.

**PIGF assay**

PIGF levels were obtained using the Roche Elecsys automated platform as previously described (20). Published centile distribution reference ranges were used to determine <5th centile within our dataset.

**Data processing and statistics**

Data analysis was performed using GraphPad Prism version 8 for Windows (GraphPad Software, La Jolla, CA, USA) and STATA v13 (StataCorp LLC 4905 Lakeway Drive, College Station, Texas). Distribution of data was checked for normality and logged where appropriate. Single group comparisons were made using non-parametric tests (Mann-Whitney or Kruskall-Wallis). Correlation between continuous measurements was assessed using linear regression; the earliest measurement per woman was included in both the regression and between group analyses. The impact of subsequent preeclampsia on the relationship between analytes was assessed using an interaction term. P< 0.05 was considered significant for all analyses.

**Results**

Participant characteristics are shown in Table 1. There were no differences between women with different pregnancy outcomes. Most women included in the study had pre-pregnancy hypertension. There were slightly more male infants in the preterm preeclampsia group and women with preterm preeclampsia delivered earlier and with lower birthweight infants as expected. To determine if there were potential confounding demographic variables within the dataset that were masking any differences between outcome groups, we compared S1P and sphingosine levels between difference ethnicities (supp Fig. 1), between male and
female fetuses (supp Fig.2) and women with and without pre-existing hypertension (supp Fig. 3). There were no differences between any compared groups. Linear regression did not identify a significant relationship between either S1P or sphingosine and birthweight (p=0.987 and 0.827 respectively).

Mean plasma S1P concentrations were similar to previously reported ranges in pregnant women from LC-MS experiments (0.339 (CI 0.308-0.37) nmol/ml) (9). Mean plasma sphingosine was also similar (6.77 (CI 4.94- 8.61) nmol/l) (21). S1P and sphingosine levels were not significantly affected by gestational age at sampling and were not significantly different between sampling points in women with longitudinal measurements (supp Fig. 4). There were no differences between plasma S1P or sphingosine concentrations between uncomplicated outcome, preterm preeclampsia, or term preeclampsia groups at either 14+0-17+6 weeks or 18+0-24+6 weeks (Fig. 1); this did not alter with inclusion of longitudinal measurements from the same woman. There were some unexplained outliers in the dataset with increased S1P or sphingosine compared to the population average. Removal of outliers outside 95% confidence interval did not affect the absence of relationship between uncomplicated and women with preeclampsia outcome.

We examined the relationship between S1P and sphingosine to determine if outliers were the result of correlate S1P and sphingosine (supp Fig. 5). One value of sphingosine (35.66ng/ml) was >10x more than all other sphingosine values and was unrelated to S1P concentrations, as a result it was excluded from further analysis. Using regression, we tested the relationship between S1P and sphingosine which was significant. For every 0.1 ng/ml increase in sphingosine, S1P increased by 18.08ng/ml (95%CI 9.73-26.44, p<0.0001). However, this relationship was unaffected by different pregnancy outcomes (interaction term p>0.05) (Fig. 2).

There was no group difference between the correlation of S1P with PlGF at either 14+0-17+6 weeks or 18+-24+6 weeks (t test logged values; p=0.727 and p=0.789). There was no relationship between sphingosine with PlGF at either 14-17+6 weeks or 18-24 weeks (t test logged values; p=0.513 and 0.527). There was no significant association between S1P or
sphingosine and PlGF (which is associated with later development of preeclampsia) at either 14-18 weeks or 18-24 weeks (Fig. 3).

Discussion

Our study has demonstrated that in a cohort of pregnant women who are at increased risk of developing preeclampsia, S1P and sphingosine levels do not appear to be different before the development of disease.

Mean plasma S1P levels for the whole cohort were lower than in previously reported non-pregnant populations (reported mean plasma levels 0.75 ± 0.16 nmol/mL (9) to 1nmol/ml, but similar to other published studies in pregnant women (9, 13, 14). Lower plasma S1P concentrations contrasts with pregnancy effects on cholesterol, triglycerides, LDLs and HDL which all increase in pregnancy (22, 23). Age, BMI and smoking have not been shown to significantly effect S1P concentrations (24) so the younger age and increased BMI of the cohort compared to published non-pregnant cohorts is unlikely to be responsible for the lower concentrations. Differences in sample handling between centres have been reported to be responsible for some difference in reported concentrations (25), but this would seem unlikely here as our findings and other pregnancy cohorts from geographically distant investigators are similar. Pregnant women have a lower haematocrit than the general population and S1P has been shown to correlate with haematocrit (25), but the strength of relationship is insufficient to explain the significant differences observed and at present it remains unclear why plasma concentrations of S1P in pregnancy seem consistently lower than the non-pregnant population. S1P effects are partially dependent on carrier binding with potentially longer lasting effects when bound to HDL rather than albumin (26), so it should be noted that different S1P concentrations alone do not fully explain its circulating biological effects (27).

Previous studies examining the relationship between plasma S1P and preeclampsia have reported inconsistent findings. In a similar longitudinal study Dobierzewska et al (9)
examined S1P levels in first, second and third trimester women, but failed to demonstrate any significant differences between women with uncomplicated outcomes or those who developed preeclampsia at any gestation which is consistent with our findings. This contrasted with findings from Melland et al. (13) who found lower S1P concentrations (preeclampsia 0.263nmol/ml versus control 0.461nmol/ml). However, this was a small study of only 10 patients and analysis was performed in serum not plasma in which concentrations are higher (24). Charkiewicz et al. also demonstrated that in women who had been diagnosed with preeclampsia, S1P levels were significantly higher when compared to normal pregnant women (14). Similarly, this was a relatively small study, but S1P concentrations (0.177nmol/ml +/-0.014) were also significantly lower than all the other reported pregnancy concentrations. There are no apparent differences in the normal pregnancies between the cohorts, so the levels reported may reflect differences in measurement methodology which has been suggested to be the cause of differences in measured concentrations between different non-pregnancy cohorts (24)(27). It is also possible that our lower concentrations and those of Dobierzewska et al (9) are the result of our patient populations pre-existing risk status being different from other published studies that report a rise in S1P. Melland et al. (13) and Dobierzewska et al (9) and do not include information on prior pregnancy risk status; Charkiewicz et al. (14) excluded women with previous chronic hypertension at time of diagnosis of preeclampsia which clearly differs from our cohort.

As with S1P, mean plasma sphingosine plasma concentrations were marginally lower than the concentrations in published non-pregnant population (8)(28), but significantly lower that the levels found by Charkiewicz et al. (14) in normal and preeclampsia pregnancies. It should be noted that the Charkiewicz measurements were from women who had developed clinical preeclampsia, however this does not explain the very high levels seen in normal outcome pregnancies in that study which is not in keeping with published ranges of sphingosine fractions in plasma (8). It is possible that due to the magnitude of difference, reported concentrations are the result of different measurement techniques, but as with S1P we are unable to fully explain these different results.
In our data we noticed significantly increased levels of S1P and sphingosine in some subjects and on interrogating the data there was a correlation between the two sphingolipids. To our knowledge this continuous relationship is a novel finding that has not been previously observed in plasma in any patient group. As with individual S1P and sphingosine measurements, there was no relationship with combined measurements and pregnancy outcome.

**S1P and sphingosine as predictors of preeclampsia**

Our study’s main strengths are the cohort size and the inclusion of women who had uncomplicated outcomes, preterm preeclampsia or term preeclampsia which allowed us to test the potential use of S1P and sphingosine as early biomarkers of different phenotypes the disease. Although our cohort is large a limitation of our study is that it did not include any women who were low risk at the outset of pregnancy, and it is therefore possible that the relationship between S1P and sphingosine and preeclampsia differ in our higher risk cohort. A further study limitation arises from the fact that the samples were taken early in pregnancy before development of the disease features did not allow for near disease measurement and therefore it remains uncertain if these sphingolipids could be useful when combined with existing biomarkers of preeclampsia, such as PIGF. PIGF is a member of the VEGF family and, when maternal plasma concentrations are low, is associated with increased chances of preeclampsia/fetal growth restriction (29). In later pregnancy low concentrations are predictive of imminent disease, but very low levels between 14 and 24 weeks are known to be associated with an increased risk of preterm preeclampsia (30). We therefore examined the relationship between S1P and sphingosine and PIGF and were unable to demonstrate any overlap either continuously or using very low levels of PIGF as indicative of potentially high-risk pregnancies.

**Conclusion**

Plasma S1P concentrations are lower in pregnancy, but previously reported changes in S1P concentrations prior to the development of preeclampsia were not present in our well characterised cohort of term and preterm preeclampsia. Circulating S1P or sphingosine
concentrations in early pregnancy are not associated with later onset of preeclampsia in women at high pre-pregnancy risk of developing the disease.

Acknowledgements

Women attending MAViS who donated samples.
Riainne Wallworth, technician.
Neil O’Hara for excellent technical support.

Funding support

This study was performed with support from Medical Research Council (UK) MR/M02296X/1

Data availability

Due to the identifiable nature of human data within this manuscript it is not available for secondardy analyses.

References


Figure legends

Figure 1. S1P(A) and sphingosine(B) concentrations at 2 different time points in women with Uncomplicated outcome, Preterm preeclampsia, Term preeclampsia. S1P 14+0-17+6 n=61, 18+0-24+6 n=67; Sphingosine 14=0-17+6 n=61 18+0-24+6 n=65.

Figure 2. Relationship between Sphingosine and S1P in pregnancies with uncomplicated pregnancies (blue dots), preterm preeclampsia (red dots) and term preeclampsia (purple dots). Lines demonstrate fitted values and are in same colour for each outcome; only earliest gestation sample for each participant included. Relationship between Sphingosine and S1P not significantly different between pregnancy outcome groups (interaction term p>0.05).
Figure 3. Relationship between Log PI GF and S1P (A/B) and sphingosine (C/D) concentrations. PI GF concentrations <5\textsuperscript{th} centile (red dots) and ≥5\textsuperscript{th} centile (blue dots). Panels A&C include measurements taken between 14+0-17+6 weeks and panels B&D include measurements between 18+0-24+6 weeks; for women with longitudinal samples only the earliest sample is included.
### Table 1. Demographics of women included in the study.

<table>
<thead>
<tr>
<th></th>
<th>Uncomplicated pregnancy outcome n=52 (%)</th>
<th>Preterm preeclampsia n=27 (%)</th>
<th>Term preeclampsia n=16 (%)</th>
<th>P value</th>
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</thead>
<tbody>
<tr>
<td>Maternal Age (Median, IQR)</td>
<td>34.6, 5.5</td>
<td>32.4, 8.4</td>
<td>33.3, 5.5</td>
<td>0.054</td>
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<tr>
<td>Ethnicity (white)</td>
<td>25 (48)</td>
<td>15 (55)</td>
<td>9 (56)</td>
<td>0.753</td>
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<tr>
<td>Gestational age at delivery days (Median, IQR)</td>
<td>269 (266-274.5)</td>
<td>232 (214-245)</td>
<td>265 (263.5-267.5)</td>
<td>&lt;0.001</td>
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<tr>
<td>Sampling gestation 14+0-17+6 weeks (Mean, SD)</td>
<td>112 (8)</td>
<td>114 (7)</td>
<td>114 (7)</td>
<td>0.51</td>
</tr>
<tr>
<td>Sampling gestation 18+0-24+6 weeks (Mean, SD)</td>
<td>156 (13)</td>
<td>153 (11)</td>
<td>154 (12)</td>
<td>0.73</td>
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<tr>
<td>Birthweight (mean, SD)</td>
<td>3189, 339.1</td>
<td>1779.7, 687.2</td>
<td>2991.1, 347.5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Parity (Median, IQR)</td>
<td>1, 2</td>
<td>1, 1</td>
<td>1, 1</td>
<td>0.56</td>
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<tr>
<td>Fetal sex male</td>
<td>22 (42)</td>
<td>9 (56)</td>
<td>12 (43)</td>
<td>0.049</td>
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<tr>
<td>BMI (Median, IQR)</td>
<td>25.1, 3.4</td>
<td>25.2, 3.8</td>
<td>25.7, 5.3</td>
<td>0.49</td>
</tr>
<tr>
<td>Chronic Hypertension</td>
<td>47 (90)</td>
<td>22 (81)</td>
<td>13 (81)</td>
<td>0.447</td>
</tr>
</tbody>
</table>
Figure 1. S1P (A) and sphingosine (B) concentrations at 2 different time points in women with Uncomplicated outcome, Preterm preeclampsia, Term preeclampsia. S1P 14+0-17+6 n=61, 18+0-24+6 n=67; Sphingosine 14=0-17+6 n=61, 18+0-24+6 n=65.
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D

![Graph showing a scatter plot with Log PIGF on the x-axis and Spriptide on the y-axis. The plot includes points representing PIGF ≤ 5th centile and PIGF > 5th centile.]
Supplemental Figures

Supplemental Figure 1. Relationship between ethnicity (self-reported White, Black, Asian, and undefined) and maternal plasma S1P (A) and sphingosine (B) concentration. Gestational age range presented in weeks + days. n=61 at 14+0-17+6 weeks and n=65 at 18+0-24+6.

A.

B.

Supplemental Figure 2. Relationship between fetal sex and maternal plasma S1P (A) and Sphingosine (B) concentrations. Gestational age range presented in weeks + days, n=27 at 14+0-17+6 weeks and n=39 at 18+0-24+6.

A.

B.
Supplemental Figure 3. S1P and sphingosine concentrations in women with chronic hypertension at booking versus those with a previous history of pregnancy hypertension, n=127.

A.

B.
Supplemental Figure 4. Longitudinal measurements of S1P(A) and Sphingosine(B) between 14+0-17+6 weeks and 18+0-24+6 weeks, n=30 for longitudinal measurements linked by lines, unlinked dot indicate single gestation data points. Gestational age range presented in week+days.

A.

B.
Supplemental Figure 5. Scatter plot of Sphingosine vs S1P demonstrating single outlier value of Sphingosine.
CRediT author statement

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☒ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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