Plasma S1P and Sphingosine are not Different Prior to Pre-Eclampsia in Women at High Risk of Developing the Disease

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Abstract Sphingolipids like sphingosine-1-phosphate (S1P) have been implicated in the pathophysiology of pre-eclampsia. We hypothesized that plasma S1P would be increased in women at high risk of developing pre-eclampsia who subsequently develop the disease. Low circulating placental growth factor (PlGF) is known to be associated with development of pre-eclampsia; so further, we hypothesized that increased S1P would be associated with concurrently low PlGF. This was a case-control study using stored maternal blood samples from 14 to 24 weeks of pregnancy, collected from 95 women at increased risk of pre-eclampsia. Pregnancy outcome was classified as uncomplicated, preterm pre-eclampsia (<37 weeks), or term pre-eclampsia. Plasma lipids were extracted and analyzed by ultraperformance liquid chromatography coupled to electrospray ionization MS/MS to determine concentrations of S1P and sphingosine. Median plasma S1P was 0.339 nmol/ml, and median sphingosine was 6.77 nmol/l. There were no differences in the plasma concentrations of S1P or sphingosine in women who subsequently developed pre-eclampsia, no effect of gestational age, fetal sex, ethnicity, or the 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 PlGF. Previous studies have suggested that plasma S1P may be a biomarker of pre-eclampsia. In our larger study, we failed to demonstrate there are 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 pre-eclampsia later in pregnancy.

Supplementary key words S1P • sphingosine • pregnancy • preeclampsia • PlGF

Sphingolipids affect a diverse array of cellular processes and functions in multiple biological systems. Deranged signaling 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 because of its potent effects on cell motility (2). Circulating S1P levels are tightly controlled by sphingosine kinases 1 and 2 and S1P phosphatases (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 (S1P receptors 1–5) and in vitro has been shown to affect the migratory cells of the placenta, extravillous trophoblast (EVT), by interaction with S1P receptor 2 (4). Sphingosine kinase-deficient mice demonstrate impaired placental development (5). Impaired placental development and impaired EVT migration have been implicated in the pathophysiology of pre-eclampsia, 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 pre-eclampsia 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 nonpregnant healthy adult populations of men and women have demonstrated plasma S1P levels of 0.75 ± 0.16 nmol/ml (9) to 1 nmol/ml (10). Concentrations increase in inflamma-

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tory 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 pre-eclampsia 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 pre-eclampsia 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 analyzed samples taken nearer to diagnosis of pre-eclampsia. A larger study of 57 women with a less well-defined pre-eclampsia phenotype suggested an increase in plasma S1P in women with diagnosed pre-eclampsia ([14]).

Circulating levels of sphingosine are 8.0 nmol/l in nonpregnant subjects ([8]). To our knowledge, there is only one previous study examining plasma sphingosine concentration in pre-eclampsia, and this suggested that the plasma concentrations were significantly higher than in the nonpregnant 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 pre-eclampsia. All women included were at increased risk of pre-eclampsia either because of a history of a hypertensive pregnancy disorder and/or because of chronic prepregnancy hypertension. Based on the observed inhibitory effects of S1P on EVT migration in vitro ([4]) we hypothesized that S1P concentrations would be increased in women who went on to develop pre-eclampsia. We investigated the relationship between measurements of S1P, sphingosine, and the pre-eclampsia biomarker, placental growth factor (PlGF), and compared different phenotypes of pre-eclampsia (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 to 24 + 6 weeks; for a small number of women, longitudinal samples were available. For women included in this case-control study, pregnancy outcomes were categorized as uncomplicated (birth ≥37 weeks, birthweight centile ≥10th with no hypertensive complications) or pre-eclampsia (defined using the International Society for the Study of Hypertension in Pregnancy ([16]) guidelines). Women with pre-eclampsia were divided into preterm pre-eclampsia (women who developed the disease before 37 weeks and who were delivered by this gestation) and term pre-eclampsia (women who developed pre-eclampsia after 37 weeks and were delivered after this gestation). In women with prepregnancy 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 pre-eclampsia. There were 127 measurements in total in 95 women; 65 women had a single measurement and 30 women had two measurements.

Sphingolipid extraction and ultraperformance liquid chromatography-MS/MS analysis

Lipid extractions were carried out as previously described ([17]) using a single-phase system to maximize 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-*d*7; Avanti Polar Lipids, Alabaster, AL) and S1P (S1P-*d*7; 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-*d*7 and S1P-*d*7 were 87.7% and 90.1%, respectively. Sphingosine and S1P were analyzed by ultraperformance liquid chromatography coupled to electrospray ionization MS/MS, as previously described ([19]), using an Acquity ultraperformance liquid chromatography system (Waters Corporation, Wilmslow, UK) paired with a triple quadrupole mass spectrometer (Xevo TQ-S; Waters Corporation); data acquisition was carried out using MassLynx software (Waters Corporation). Separation was performed using a reverse-phase Acquity BEH C8 column (1.7 µm 2.1 × 100 mm) 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 ionization was performed in positive-ion mode using the following settings: capillary voltage, 3.5 kV; source temperature, 100°C; and desolvation gas temperature, 450°C. Analytes were quantitated using multiple reaction monitoring: S1P *m/z* 380.249 > 264.278; S1P-*d*7 *m/z* 387.292 > 271.25; and sphingosine *m/z* 300.332 > 282.205; sphingosine-*d*7 *m/z* 307.300 > 289.300.

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

PlGF assay

PlGF levels were obtained using the Roche Elecsys automated platform as previously described ([20]). Published centile distribution reference ranges were used to determine less than fifth centile within our dataset.

Data processing and statistics

Data analysis was performed using GraphPad Prism, version 8 for Windows (GraphPad Software, La Jolla, CA) and

STATA, version 13 (StataCorp, College Station, TX). Distribution of data was checked for normality and logged where appropriate. Single-group comparisons were made using nonparametric tests (Mann-Whitney test or Kruskal-Wallis test). 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 pre-eclampsia 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 prepregnancy hypertension. There were slightly more male infants in the preterm pre-eclampsia group and women with preterm pre-eclampsia 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 (supplemental Fig. S1), between male and female fetuses (supplemental Fig. S2) and women with and without pre-existing hypertension (supplemental Fig. S3). 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 (supplemental Fig. S4). There were no differences between plasma S1P or sphingosine concentrations between uncomplicated outcome, preterm pre-eclampsia, or term pre-eclampsia groups at either 14 + 0 to 17 +

6 weeks or 18 + 0 to 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 with the population average. Removal of outliers outside 95% CI did not affect the absence of relationship between uncomplicated and women with pre-eclampsia outcome.

We examined the relationship between S1P and sphingosine to determine if outliers were the result of correlate S1P and sphingosine (supplemental Fig. S5). One value of sphingosine (35.66 ng/ml) was >10× 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 18.08 ng/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 to 17 + 6 weeks or 18+) to 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 pre-eclampsia) 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 pre-eclampsia, 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 nonpregnant populations (reported mean plasma levels 0.75 ± 0.16 nmol/ml (9) to 1 nmol/ml) but similar to other published studies in pregnant women (9, 13, 14). Lower plasma S1P

TABLE 1. Demographics of women included in the study

Demographic characteristics	Uncomplicated pregnancy outcome ($n = 52$ [%])	Preterm pre-eclampsia ($n = 27$ [%])	Term pre-eclampsia ($n = 16$ [%])	P
Maternal age (median, IQR)	34.6, 5.5	32.4, 8.4	33.3, 5.5	0.054
Ethnicity (white)	25 (48)	15 (55)	9 (56)	0.753
Gestational age at delivery days (median, IQR)	269 (266–274.5)	232 (214–245)	265 (263.5–267.5)	<0.001
Sampling gestation 14 + 0 to 17 + 6 weeks (mean, SD)	112 (8)	114 (7)	114 (7)	0.51
Sampling gestation 18 + 0 to 24 + 6 weeks (mean, SD)	156 (13)	153 (11)	154 (12)	0.73
Birthweight (mean, SD)	3,189, 339.1	1779.7, 687.2	2991.1, 347.5	<0.001
Parity (median, IQR)	1, 2	1, 1	1, 1	0.56
Fetal sex male	22 (42)	9 (56)	12 (43)	0.049
BMI (median, IQR)	25.1, 3.4	25.2, 3.8	25.7, 5.3	0.49
Chronic hypertension	47 (90)	22 (81)	13 (81)	0.447

IQR, interquartile range.
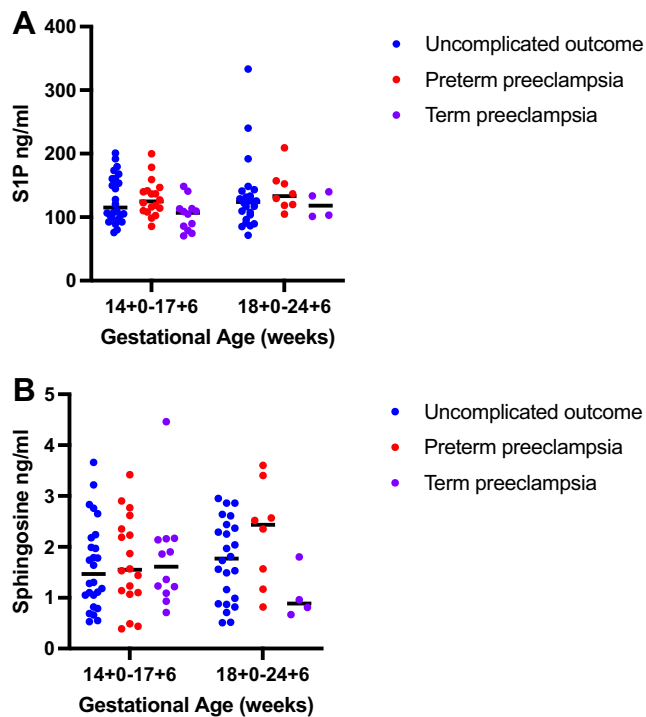
Fig. 1. S1P (A) and sphingosine (B) concentrations at two different time points in women with uncomplicated outcome, preterm pre-eclampsia, and term pre-eclampsia. S1P 14 + 0 to 17 + 6 (*n* = 61), 18 + 0 to 24 + 6 (*n* = 67); sphingosine 14 = 0 to 17 + 6 (*n* = 61) 18 + 0 to 24 + 6 (*n* = 65).

concentrations contrast with pregnancy effects on cholesterol, triglycerides, LDL, and HDL, all of which 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 with published nonpregnant
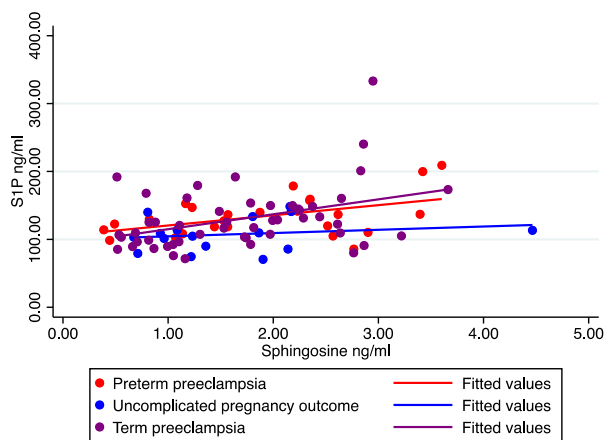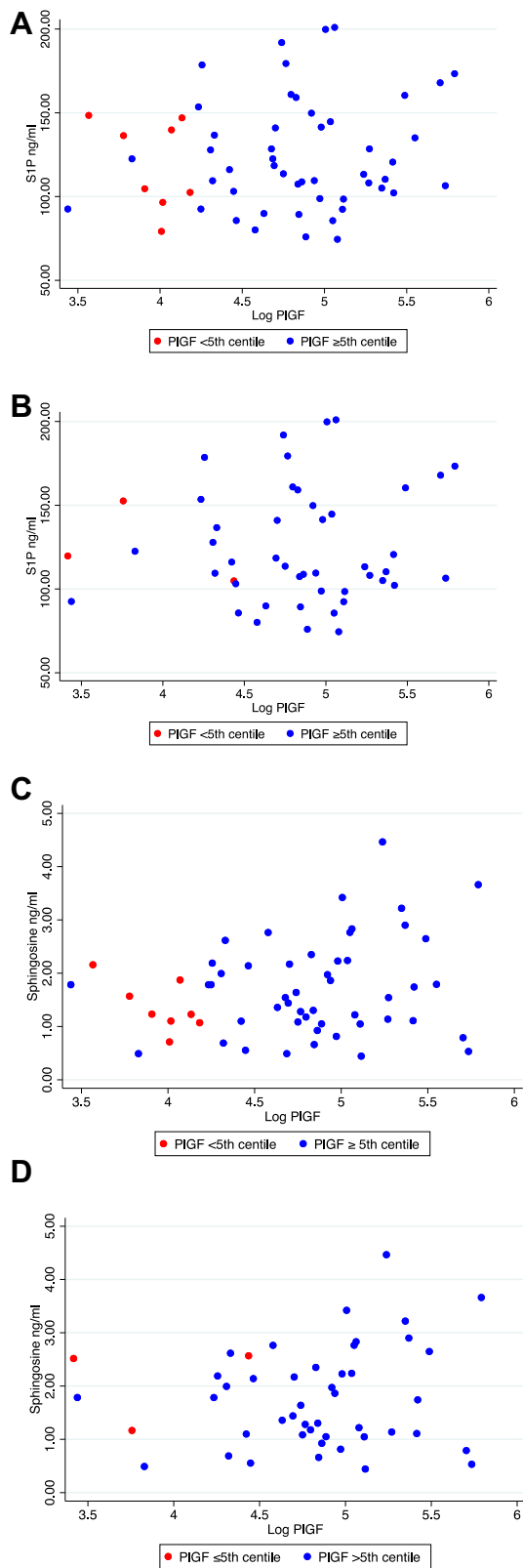

Fig. 2. Relationship between sphingosine and S1P in pregnancies with uncomplicated pregnancies (blue dots), preterm pre-eclampsia (red dots), and term pre-eclampsia (purple dots). Lines demonstrate fitted values and are in same color for each outcome; only earliest gestation sample for each participant was included. Relationship between sphingosine and S1P not significantly different between pregnancy outcome groups (interaction term *P* > 0.05).

cohorts is unlikely to be responsible for the lower concentrations. Differences in sample handling between centers 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 hematocrit than the general population, and S1P has been shown to correlate with hematocrit (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 nonpregnant population. SIP 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 pre-eclampsia 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 0.263 nmol/ml vs. control 0.461 nmol/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.* (14) also demonstrated that in women who had been diagnosed with pre-eclampsia, S1P levels were significantly higher when compared with normal pregnant women. Similarly, this was a relatively small study, but S1P concentrations (0.177 nmol/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 nonpregnancy 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) 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 nonpregnant population (8, 28) but significantly lower than the levels found by

Fig. 3. Relationship between log PlGF and S1P (A/B) and sphingosine (C/D) concentrations. PlGF concentrations less than fifth centile (red dots) and equal to or greater than fifth centile (blue dots). Panels A and C include measurements taken between 14 + 0 to 17 + 6 weeks, and panels B and D include measurements taken between 18 + 0 to 24 + 6 weeks; for women with longitudinal samples, only the earliest sample is included.

Charkiewicz *et al.* (14) in normal and pre-eclampsia pregnancies. It should be noted that the Charkiewicz measurements were from women who had developed clinical pre-eclampsia; 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 because of 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 pre-eclampsia

Our study's main strengths are the cohort size and the inclusion of women who had uncomplicated outcomes, preterm pre-eclampsia, or term pre-eclampsia, which allowed us to test the potential use of S1P and sphingosine as early biomarkers of different phenotypes of the disease. Although our cohort is large, a limitation of our study is that it did not include any women who were at low risk at the outset of pregnancy, and it is therefore possible that the relationship between S1P and sphingosine and pre-eclampsia differs 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 pre-eclampsia, such as PlGF. PlGF is a member of the vascular endothelial growth factor family and, when maternal plasma concentrations are low, is associated with increased chances of pre-eclampsia/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 pre-eclampsia (30). We therefore examined the relationship between S1P and sphingosine and PlGF and were unable to demonstrate any overlap either continuously or using very low levels of PlGF 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 pre-eclampsia were not

present in our well-characterized cohort of term and preterm pre-eclampsia. Circulating S1P or sphingosine concentrations in early pregnancy are not associated with later onset of pre-eclampsia in women at high prepregnancy risk of developing the disease.

Data Availability

Because of the identifiable nature of human data within this article, it is not available for secondary analyses. ⬛

Author Contributions

E. D. J., M. W., and M. D. conceptualization; A. C. K. and A. N. methodology; E. D. J., J. R. W., and J. E. M. investigation; J. E. M. resources; E. D. J. writing–original draft; M. W., M. D., A. N., and J. E. M. writing–review & editing.

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Abbreviations

EVT, extravillous trophoblast; PlGF, placental growth factor; S1P, sphingosine-1-phosphate.

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