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# Second and third trimester serum levels of HtrA1 in pregnancies affected by pre-eclampsia

Sasha Skinner [a,*], Daniel L. Rolnik [a], Yao Wang [b], Guiying Nie [b,c], Argyro Syngelaki [d], Kypros H. Nicolaides [d], Fabricio da Silva Costa [a,e]

[a] Department of Obstetrics and Gynaecology, Monash University, Clayton, Victoria, Australia
[b] Centre for Reproductive Health, Hudson Institute of Medical Research, Clayton, Victoria, Australia
[c] School of Health and Biomedical Sciences, RMIT University, Melbourne, Australia
[d] Fetal Medicine Research Institute, King's College Hospital, London, UK
[e] Department of Gynecology and Obstetrics, Ribeirão Preto Medical School, University of São Paulo, Ribeirão Preto, São Paulo, Brazil

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ABSTRACT

*Introduction:* Altered placental expression of high temperature requirement factor A1 (HtrA1) is implicated in abnormal trophoblastic invasion and endothelial dysfunction in pre-eclampsia (PE). Serum levels of HtrA1 have been proposed as a novel biomarker to improve the prediction of PE. This study assesses serum HtrA1 levels in prospectively collected samples of women who developed PE compared to normotensive pregnancies.
*Methods:* This was a case-control study of serum HtrA1 levels in second and third trimester samples in women who later developed preterm or term PE compared to controls. Overall, 300 serum samples were drawn from a prospective observational study of adverse pregnancy outcomes in three different gestational age windows (19–24, 30-34 and 35–37 weeks) at the Fetal Medicine Research Institute, King's College Hospital, London. Serum HtrA1 levels were determined by enzyme-linked immunosorbent assay (ELISA) by a blinded laboratory professional. Median HtrA1 MoM values, adjusted for gestational age and maternal characteristics, were compared between cases and controls at each gestational age group.
*Results:* Women who later developed PE, compared to controls, had significantly higher maternal weight and more frequently had chronic hypertension or a history of PE in a previous pregnancy. In normotensive pregnancies, serum HtrA1 increased with increasing gestational age, whereas, in PE pregnancies HtrA1 levels remained stable, but were not significantly different from control pregnancies at any gestational age.
*Discussion:* Serum HtrA1 levels are not significantly different in women who develop PE compared to controls.

## 1. Introduction

Pre-eclampsia (PE) affects 2–8% of pregnancies worldwide, and is characterized by hypertension, widespread endothelial dysfunction and/or fetal growth restriction with significant association with morbidity and mortality for mother and child [1–3]. Preterm PE, with delivery <37 weeks' gestation, and especially early PE, with delivery <34 weeks is strongly associated with abnormal early placentation, resulting in poor placental perfusion. Conversely, features of abnormal placentation are less commonly found in pregnancies that develop term PE, with delivery ≥37 weeks [4,5].

Prediction of PE in the first trimester allows commencement of aspirin prior to 16 weeks' gestation, which reduces rates of preterm PE by 62% and very early severe disease by about 90% [6]. The current best first trimester prediction model identifed up to 90% of early PE by combining maternal factors, mean arterial pressure (MAP), uterine artery pulse index (UtA-PI) and placental growth factor (PLGF), but are less accurate in predicting term disease. As term PE is more prevalent, only 50% of all PE is detected using first trimester screening models for a screen positive rate of 10% [7]. Term PE prediction can be improved to 70% by third trimester screening, using a combination of maternal factors, MAP, PLGF, and soluble fms-like tyrosine kinase-1 (sFlt-1) which is elevated closer to disease onset [8]. Mid and late trimester prediction of PE enables individualization of antenatal care and fetal growth surveillance [9].

The use of additional placental biomarkers is proposed to further improve PE prediction [10]. One such group of biomarkers are high

* Corresponding author.
*E-mail address:* sasha.m.skinner@gmail.com (S. Skinner).

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**Abbreviations**

| | |
|---|---|
| ANOVA | Analysis of variance |
| ELISA | Enzyme-linked immunosorbent assay |
| HtrA1 | High temperature requirement factor A1 |
| MAP | Mean arterial pressure |
| MoM | Multiples of the normal median |
| PE | Pre-eclampsia |
| PLGF | Placental growth factor |
| sFlt-1 | Soluble fms-like tyrosine kinase-1 |
| UtA-PI | Uterine artery pulse index |

temperature requirement factors A 1–4 (HtrA1-4), a group of proteases involved in cell-signaling, mitochondrial homeostasis and protein quality control [11]. HtrA proteases are implicated in multiple disease processes related to abnormal angiogenesis, including oncogeneses, cerebral small vessel disease, arthritic disease, Alzheimer's and age-related macular degeneration [11–15]. HtrA1 is expressed in the placenta and altered expression is thought to play a role in abnormal trophoblastic invasion and placental development [16–21].

Studies of women with PE demonstrate abnormal levels of HtrA1 in placental tissue and maternal serum, which appear to differ between early- and late-onset disease [16,21–24]. However, previous studies showed conflicting results, some of which may be explained by inconsistencies in differentiating early- and late-onset PE and small study numbers. It is unclear if serum levels of HtrA1 could be used in predicting PE prior to clinical symptoms. The aim of this study was to compare second and third trimester serum levels of HtrA1 between women who went on to develop PE and those who did not.

## 2. Methods

### 2.1. Study population

This was a nested case–control study drawn from a prospective cohort study of adverse pregnancy outcomes in women attending for their routine second and third trimester hospital visits in pregnancy at the Fetal Medicine Research Institute, King's College Hospital, London, between July 2011 and January 2016.

Patient characteristics including maternal age, racial origin (Caucasian, Afro-Caribbean, South Asian, East Asian and mixed), method of conception (spontaneous or assisted conception requiring the use of ovulation drugs or *in vitro* fertilization), cigarette smoking during pregnancy (yes or no), history of chronic hypertension (yes or no), systemic lupus erythematosus (yes or no), antiphospholipid syndrome (yes or no), pre-gestational diabetes (yes – type 1 or type 2 – or no), family history of PE in the mother of the patient (yes or no) and obstetric history including parity (parous or nulliparous if no previous pregnancies at or after 24 weeks' gestation), previous pregnancy with PE (yes or no) and maternal weight and height were also recorded. No women included in the study were prescribed aspirin, as use of aspirin for pre-eclampsia prevention was not widely endorsed clinically until 2018.

PE was defined according to the criteria established by the International Society for the Study of Hypertension in Pregnancy [25], as systolic blood pressure of 140 mmHg or more and/or diastolic blood pressure 90 mmHg or more developing after 20 weeks' gestation together with significant proteinuria or other signs of maternal organ dysfunction in a previously normotensive woman. Significant proteinuria is defined by 300 mg or more in 24 h or two readings of at least ++ on dipstick analysis of midstream or catheter urine specimens if no 24-h collection is available. In PE superimposed on chronic hypertension, significant proteinuria (as defined earlier or worsening proteinuria compared to baseline levels) should develop after 20 weeks' gestation in women with known chronic hypertension (history of hypertension before conception or the presence of hypertension at the booking visit before 20 weeks' gestation in the absence of trophoblastic disease).

### 2.2. Blood collection and analysis

Written informed consent was obtained from all participants agreeing to take part in the study, which was approved by the UK National Research Ethics Committee (reference number: 02-03-033). Overall, 300 samples were obtained: 120 at 19–24 weeks' gestation (60 controls, 30 cases of preterm PE and 30 cases of term PE), 120 at 30–34 weeks' gestation (60 controls, 30 cases of preterm PE and 30 cases of term PE), and 60 at 35–37 weeks' gestation (40 controls and 20 cases of term PE). These gestational age windows were chosen to coincide with antenatal visit attendance of participants.

Maternal blood was collected, and the serum was stored at −80 °C after centrifugation until analysis. HtrA1 levels were determined using enzyme-linked immunosorbent assay (ELISA) by a laboratory professional who was blinded to the outcomes of the pregnancies. HtrA1 ELISA was performed in half-area high binding 96-well plates (Corning, NY, USA) as previously reported [22], but using a biotinylated detection antibody which shortened the assay. In brief, plates were coated overnight at 4 °C with a sheep polyclonal antibody against human HtrA1 (R&D System, USA) at 1 μg/ml in 0.1 M sodium carbonate/bicarbonate buffer. The plates were then washed with PBS (137 mM NaCl, 2.7 mM KCL, 10 mM $Na_2HPO_4$ and 1.8 mM $KH_2PO_4$) containing 0.05% Tween 20 (PBS-T) and blocked with 1% BSA (Bovogen, Australia) in PBS for 90 min at 37 °C. The plates were again washed with PBS-T and incubated with antigen for 2 h at room temperature with gentle agitation. Next, the wells were washed with PBS-T and incubated with a biotinylated mouse monoclonal antibody (2.5 μg/ml) raised in-house against human HtrA1 for 1 h at room temperature with gentle agitation, which was followed by incubation with streptavidin-HRP conjugate (Dako, Denmark, diluted to 0.78 μg/ml in PBS-T) for 50 min at room temperature with gentle agitation. The plates were then thoroughly washed with PBS-T, and one-step ultra TMB substrate (Thermo Scientific, Australia) was added for color development. The color reaction was stopped with 1 M sulphuric acid and the absorbance at 450 nM was measured immediately on a CLARIOstar microplate reader (BMG Labtech, Ortenberg, Germany). All wash steps were performed using an automated plate washer (ELx50 Washer, Bio-Tek, Winooski, VT, USA). Serum samples were diluted 1/10 in PBS-T. The standard was prepared from recombinant HtrA1 (BioTeZ, Berlin, Germany) and curve fitting used five-parameter logistic (5 PL) nonlinear regression. The sensitivity and specificity of this ELISA were confirmed to be the same as the previously reported assay [22].

### 2.3. Statistical analysis

Categorical variables are presented as absolute numbers and proportions and compared between cases and controls using Chi-squared or Fisher's exact test. Continuous baseline characteristics are summarized as means and standard deviations. The distribution of HtrA1 results was made Gaussian after logarithmic transformation. Multiple linear regression with stepwise backward elimination was then applied on logarithmically transformed HtrA1 control values to evaluate the influence of gestational age at blood sampling and maternal characteristics on biomarker results. HtrA1 values in the study groups are presented in multiples of the normal median (MoM), adjusted for gestational age and maternal characteristics.

The distributions of HtrA1 Log MoM values were then compared between the groups at each gestational age interval with one-way analysis of variance (ANOVA), with Dunnett's post-hoc adjustment for multiple comparisons within each gestational age period and using the control group as the reference category. Between-group comparisons were considered statistically different at a 0.05 significance level.

To investigate a possible association between HtrA1 MoM values and severity of disease given by the gestational age at delivery with PE, Cox proportional-hazards regression was applied using the interval between sampling and delivery as the primary endpoint and censoring deliveries without PE.

Statistical analysis was performed with SPSS Statistics (IBM Corp. Released 2019. IBM SPSS Statistics for Macintosh, Version 26.0. Armonk, NY: IBM Corp).

## 3. Results

Maternal characteristics according to pregnancy outcome are presented in Table 1. Women who later developed PE, compared to healthy controls, had significantly higher maternal weight, had more often a positive history of PE in a previous pregnancy and suffered significantly more from chronic hypertension. In women who later developed PE, UtA-PI and MAP were higher in second and third trimesters, whilst sFlt-1 was elevated and PLGF significantly lower in third trimester serum samples. Neonates of women who developed PE had lower mean birthweight compared to controls.

**Table 1**
Baseline and pregnancy characteristics of the study population.

| Characteristic | Controls (n = 160) | PE (n = 140) |
|---|---|---|
| Maternal age in years, mean ± SD | 32.1 ± 5.9 | 31.8 ± 5.2 |
| Maternal weight in kg, mean ± SD | 69.2 ± 14.6 | 77.7 ± 18.8* |
| Maternal height in cm, mean ± SD | 163.9 ± 6.7 | 164.5 ± 6.0 |
| Maternal BMI in kg/m$^2$, mean ± SD | 28.5 + 5.4 | 31.5 + 6.6* |
| **Racial origin** | | |
| Caucasian, n (%) | 78 (48.8) | 60 (42.9) |
| Afro-Caribbean, n (%) | 59 (36.9) | 68 (48.6) |
| South Asian, n (%) | 11 (6.9) | 9 (6.4) |
| East Asian, n (%) | 7 (4.3) | 1 (0.7) |
| Mixed, n (%) | 5 (3.1) | 2 (1.4) |
| **Parity** | | |
| Nulliparous, n (%) | 70 (43.8) | 79 (56.4) |
| Parous with no previous PE, n (%) | 85 (53.1) | 40 (28.6)* |
| Parous with previous PE, n (%) | 5 (3.1) | 21(15.0)* |
| Cigarette smoker, n (%) | 11 (6.9) | 7 (5.0) |
| Family history of PE, n (%) | 10 (6.3) | 6 (4.2) |
| **Conception** | | |
| Spontaneous, n (%) | 153 (95.6) | 132 (94.3) |
| Assisted, n (%) | 7 (4.4) | 8 (5.7) |
| Chronic hypertension, n (%) | 3 (1.9) | 22 (15.7)* |
| History of SLE/APS | 1 (0.6) | 1 (0.7) |
| History of Diabetes (Type 1 or 2) | 5 (3.1) | 1 (0.7) |
| Neonatal gender female, n (%) | 79 (49.4) | 79 (56.4) |
| Birthweight in g, mean ± SD | 3222 ± 311 | 2597 ± 923* |
| **Screening markers at 20–22 weeks** | | |
| Uterine artery PI, n; mean ± SD | 60; 1.08 ± 0.23 | 60; 1.50 ± 0.57* |
| Mean arterial pressure, n; mean ± SD | 60; 83.9 ± 8.0 | 60; 94.7 ± 13.3* |
| Placental growth factor in pg/ml, n; mean ± SD | 39; 352.1 ± 175.4 | 44; 363.8 ± 293.5 |
| Soluble fms-like tyrosine kinase-1 in pg/ml, n; mean ± SD | 29; 1752.6 ± 1060.9 | 37; 2399.9 ± 2423.6 |
| **Screening markers at 30–32 weeks** | | |
| Uterine artery PI, n; mean ± SD | 60; 0.81 ± 0.24 | 60; 1.05 ± 0.41* |
| Mean arterial pressure, n; mean ± SD | 60; 87.8 ± 7.5 | 60; 104.0 ± 11.6* |
| Placental growth factor in pg/ml, n; mean ± SD | 39; 792.9 ± 603.2 | 36; 417.3 ± 664.5* |
| Soluble fms-like tyrosine kinase-1 in pg/ml, n; mean ± SD | 39; 1862.6 ± 1267.6 | 36; 5221.5 ± 4970.9* |
| **Screening markers at 35–36 weeks** | | |
| Uterine artery PI, n; mean ± SD | 40; 0.73 ± 0.21 | 20; 0.92 ± 0.29* |
| Mean arterial pressure, n; mean ± SD | 40; 91.2 ± 9.0 | 20; 98.7 ± 8.2* |
| Placental growth factor in pg/ml, n; mean ± SD | 13; 331.1 ± 287.8 | 6; 115.9 ± 58.6* |
| Soluble fms-like tyrosine kinase-1 in pg/ml, n; mean ± SD | 13; 3869.8 ± 2963.3 | 6; 6969.3 ± 2760.3* |

PE: pre-eclampsia; SD: standard deviation; SLE: Systemic Lupus Erythematosus; APS: antiphospholipid syndrome; PI: pulsatility index * $p < 0.05$.

HtrA1 was detectable at 19–24 weeks' gestation in all samples except two pregnancies (0.7% of the total, one control and one pregnancy later affected by PE), and detectable in all cases and controls at 30–34 and 35–37 weeks' gestation. The range of HtrA1 was wide in both cases and controls. In normotensive pregnancies HtrA1 values increased with increasing gestational age, while in pregnancies affected by PE HtrA1 levels were relatively stable throughout gestation. HtrA1 levels were also negatively influenced by maternal height. No other maternal characteristics influenced HtrA1 levels including age, ethnicity, smoking, chronic hypertension, method of conception, obstetric, medical and family history. The equation for the calculation of expected values of HtRA1 is given by:

Expected $\log_{10}$ HtrA1 = 3.067748 + (0.0234937 * Gestational age in weeks) - (0.0120917 * Height in cm).

After adjustment for gestational age at testing and maternal height, HtrA1 MoM values were not significantly different in cases of preterm or term PE when compared to normotensive pregnancies (Table 2 and Fig. 1A). Cox regression analysis demonstrated no statistically significant associations between HtrA1 MoM values and the interval between sampling and delivery with PE at any of the three gestational age windows ($p = 0.41$ at 19–24 weeks, $p = 0.48$ at 30–34 weeks, and $p = 0.33$ at 35–37 weeks) (Fig. 1B).

## 4. Discussion

### 4.1. Main findings

HtrA1 is detectable in maternal serum in the second and third trimesters of both normotensive pregnancies and pregnancies affected by PE. Serum HtrA1 tends to increase with increasing gestational age in normotensive pregnancies, but remains relatively stable throughout gestation in PE affected pregnancies. However, HtrA1 levels do not differ significantly between controls and preterm or term PE at any gestational age window.

### 4.2. Comparison to previous studies

HtrA1 is consistently detected and shown to progressively increase in both the placenta and maternal serum throughout gestation [17,21,24]. Localization of HtrA1 is demonstrated in numerous tissues with high

**Table 2**
Comparison of median HtrA1 values between different outcome groups at 19–24, 30–34 and 35–37 weeks' gestation.

| | HtrA1 (ng/μL), Median (IQR) | P value | HtrA1 MoM, Median (IQR) | P value |
|---|---|---|---|---|
| **19–24 weeks** | | | | |
| Control (n = 60) | 39.4 (28.2–75.9) | – | 1.006 (0.720–1.818) | – |
| Preterm PE (n = 30) | 43.3 (18.2–89.1) | 0.663 | 1.026 (0.436–2.442) | 0.976 |
| Term PE (n = 30) | 48.4 (36.1–123.5) | 0.091 | 1.153 (0.890–2.929) | 0.952 |
| **30–34 weeks** | | | | |
| Control (n = 60) | 68.5 (44.0–118.5) | – | 0.974 (0.570–1.738) | – |
| Preterm PE (n = 30) | 70.7 (51.5–87.2) | 0.140 | 1.081 (0.721–1.342) | 0.767 |
| Term PE (n = 30) | 61.5 (45.2–88.1) | 0.694 | 0.938 (0.728–1.414) | 0.979 |
| **35–37 weeks** | | | | |
| Control (n = 40) | 71.6 (49.5–125.8) | – | 0.758 (0.508–1.532) | – |
| Term PE (n = 20) | 78.1 (60.0–96.4) | 0.550 | 1.018 (0.671–1.416) | 0.930 |

Comparisons between groups were performed with one-way ANOVA on logarithmically transformed values with post-hoc Dunnett's adjustment for multiple comparisons, with the control group used as a reference category.

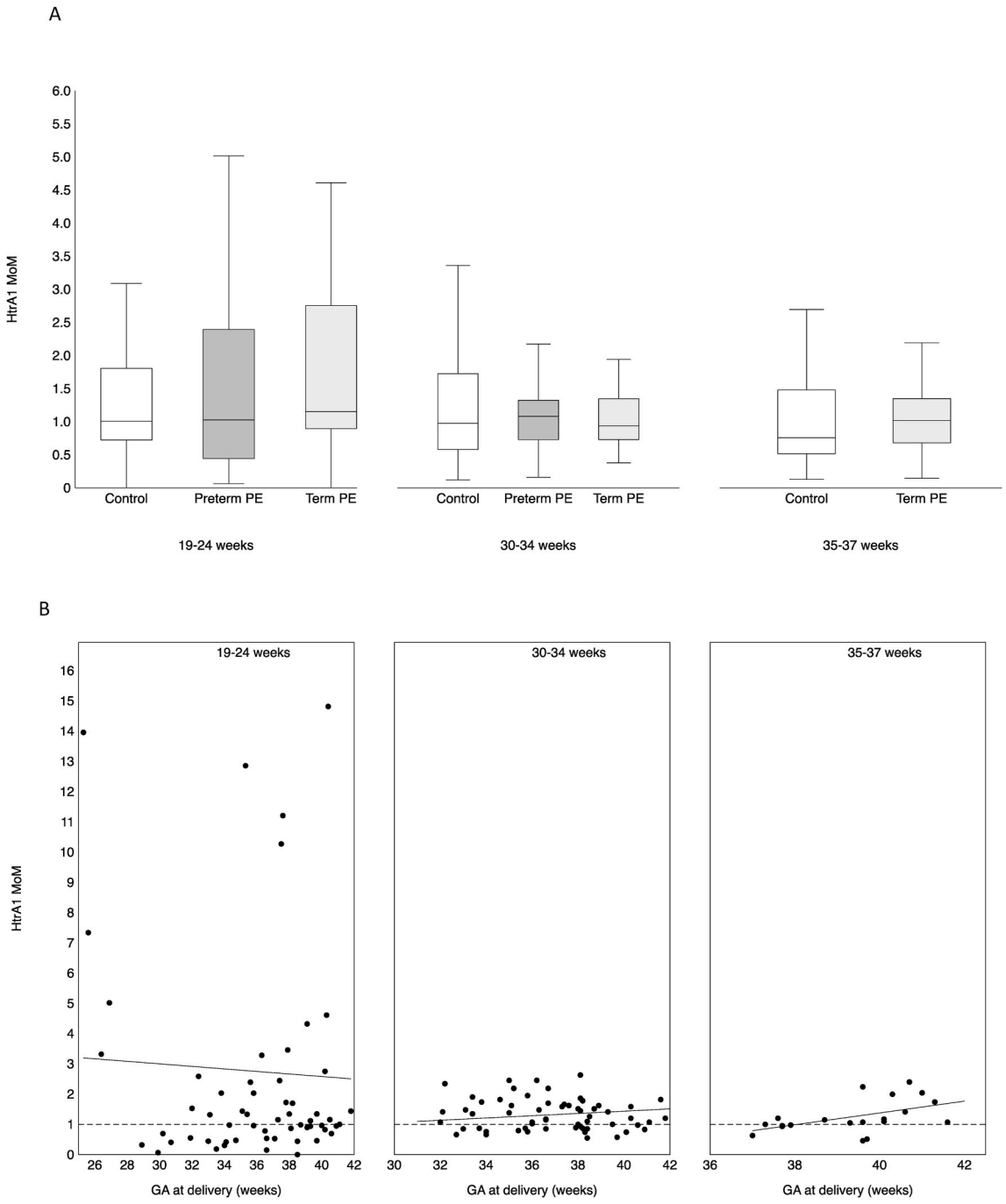


**Fig. 1.** A) Box plots of HtrA1 MoM values in normotensive pregnancies (white boxes), in preterm and term pre-eclampsia; B) Scatter plots and lines of best fit of HtrA1 MoM values according to gestational age at delivery in pregnancies affected by pre-eclampsia. The dashed lines represent the expected value of 1.0 MoM in normotensive pregnancies.

levels in the placenta in normotensive pregnancies [11,17,20]. HtrA1 is most notably expressed in the cytoplasm of syncytiotrophoblasts and cytotrophoblasts in placental villi, suggesting a role in placental development [18–21].

Inconsistent results on placental expression of HtrA1 in pregnancies affected by PE have been reported. While some studies demonstrated downregulation of placental HtrA1 expression in PE with associated fetal growth restriction [19,21], others demonstrated upregulation of HtrA1 expression in PE placentas, particularly in early-onset or severe PE at disease presentation [16,26].

Reports on maternal serum levels of HtrA1 in PE have also been conflicting. Studies have demonstrated elevation of HtrA1 levels in women diagnosed with PE only at certain gestational ages [21,23,27], elevated levels at disease presentation only in early-onset PE with reduced levels in late-onset PE [22], or no significant change compared to controls [28]. Importantly, previous studies are limited by not differentiating between preterm and term PE, not adjusting for gestational age or other factors that are shown to influence HtrA1 levels, and by small sample sizes. Additionally, most studies used serum samples collected either at the time of diagnosis or at the time of delivery.

Of studies where serum samples for HtrA1 were collected prospectively, Zong et al. [23] collected serum samples every four weeks from 5 weeks' gestation, reporting that HtrA1 levels were elevated at 13–16 weeks and decreased at 21–24 weeks in women who later developed PE.

Gesuita et al. [27] also demonstrated elevated HtrA1 levels in prospectively collected first trimester serum samples of 14 women who later developed pre-eclampsia compared to controls. However, neither of these studies adjusted for other factors that may influence HtrA1 levels or differentiated between early and late onset pre-eclampsia. Our study did not assess HtrA1 levels in the first trimester, we found no difference in HtrA1 levels at 19–24 weeks. Our results are consistent with those of a recent study by Teoh et al. [29] which assessed serum HtrA1 at 15 and 20 weeks' gestation in prospectively collected samples, and found no difference in HtrA1 between controls and early- or late-onset PE at either gestational age.

### 4.3. Study limitations

The case-control design of our study enables assessment of associated factors in rare outcomes such as preterm PE, however it also introduces potential for recall and sampling bias. In our study, the risk of recall bias is reduced by using prospectively collected pregnancy data and maternal serum samples. Additionally, laboratory professionals were blinded to pregnancy outcome during serum analysis, thus reducing observer bias. Our study is the largest to prospectively evaluate serum HtrA1 levels at different gestation ages in women who go on to develop preterm and term PE compared to controls. Nonetheless, due to the small number of PE cases included, the study may have been underpowered to detect small differences in HtrA1 levels between cases and controls. However, our findings demonstrate large variabilities of HtrA1 serum levels in both normotensive and PE pregnancies, suggesting that HtrA1 is unlikely to be a useful predictive marker of PE in the second and third trimesters.

Our study is limited in that it did not assess the association of HtrA1 serum levels and placental histopathology, of which previous studies have reported conflicting outcomes. Additionally, our study did not assess levels of HtrA1 in the first trimester, at which time a previous study has demonstrated elevated levels in women who develop pre-eclampsia [27]. It is possible that serum HtrA1 levels in women who later develop pre-eclampsia are only altered in early pregnancy in association with altered placentation. However, further larger studies are required that account for other factors that may influence HtrA1 serum levels and correlate with placental findings.

### 4.4. Clinical implications

Our study did not show a significant difference in second and third trimester HtrA1 serum levels between normotensive and PE pregnancies. Additionally, there was a large variability in HtrA1 levels in women with both normotensive and PE pregnancies. This is consistent with large variations in findings from the available literature. Collectively, this likely precludes smaller clinically significant differences that were not seen due to power and suggests HtrA1 is unlikely to be beneficial even in combination with other biomarkers.

In future studies, it is important to evaluate new biomarkers in the context of currently accepted biomarkers, such as MAP, sFlt-1 and PLGF. To be of additional clinical benefit, future biomarkers would need to provide independent predictive capacity on larger cohorts to increase detection rates of previously developed predictive models.

In conclusion, serum HtrA1 levels are not significantly different in women who developed PE compared to controls and are not predictive of preterm and term pre-eclampsia when measured in the second or the third trimester.

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## Declaration of competing interest

None.

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