Author's response to reviews

Title: Periodic Assessment of Plasma sFlt-1 and PLGF Concentrations and it's Association with Placental Morphometry in Gestational Hypertension (GH) - A Prospective Follow Study

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Author's response to reviews: see over
Alterations in response to reviewer’s report

TITLE: Periodic Assessment of Plasma sFlt-1 and PLGF Concentrations and it's Association with Placental Morphometry in Gestational Hypertension (GH) - A Prospective Follow Study (MS: 2391833403680320)

AUTHORS: Kamalan Jeevaratnam, Vishna Devi Nadarajah, John Paul Judson, Sivalingam Nalliah and Mohd Farouk Abdullah

In response to associate editor:

Thank you for submitting your manuscript for consideration to be published in BMC Pregnancy and Childbirth. The editors and reviewers have carefully reviewed your manuscript. I am sorry that the paper is not suitable for publication in BMC Pregnancy and Childbirth in it’s present form. Please see the reviewer’s comments and suggestions and make the recommended revisions so that the manuscript can be re-evaluated for publication. The critiques of your paper have been offered in a constructive spirit, and we hope that you will find them helpful. Thank you for giving us the opportunity to review your paper.

We are grateful for this constructive reaction from the reviewers regarding our submitted paper entitled “Periodic Assessment of Plasma sFlt-1 and PLGF Concentrations and it's Association with Placental Morphometry in Gestational Hypertension (GH) - A Prospective Follow Study” and enclose a thoroughly revised version of the paper, in which we have dealt with all the comments made. We have made revisions, and in dealing with these we feel we have emerged with a significantly improved manuscript which we hope will be acceptable for publication.
A. In response to REFEREE 1 (Chloe Zera):

Thank you for the very constructive comments concerning the paper. We hereby resubmit a revised version in which all the points raised by both reviewers are dealt with.

MAJOR POINTS

1. It seems from the introduction that the goal of looking at the angiogenic markers is prediction, while in reading the study I think that the goal was actually to correlate angiogenic markers and placental pathology. This should be more clear, as there isn’t actually predictive data in the manuscript.

We agree with this observation and have thus made the necessary changes in the text.

This is done by:

[1] Reemphasizing the study’s purpose in the Introduction thus replacing the sentence:

From:

This study aims to estimate and compare maternal PlGF and sFlt-1 levels in order to determine correlation between these biomarkers with placental morphometry in mothers with GH.

To:

This study aims to compare maternal PlGF and sFlt-1 levels in GH mothers periodically and later correlate these biomarker levels with placental morphometry. In so doing, the study establishes biomarker values, placental morphometric parameters and their correlation.

[2] Revising the first half of Paragraph 1 in the discussion:

From:

It is increasingly being recognized that a reliable predictive modality is needed for the diagnosis of GH and preeclampsia in a clinical setting. In working towards this objective, this study performs three periodic assessments of both sFlt-1 and PlGF in GH women providing valuable information on how these biomarkers change over time in pregnancy.

To:

Recently, there has been an increase in interest on the role circulating pro and anti-angiogenic factors play in the pathophysiology of GH. However less is known on how these biomarkers vary over the duration of pregnancy and after delivery as well as if they influence placental structural development. This is particularly true in Malaysia where the incidence of GH is relatively high [2, 10] but biomarker screening and follow up studies are limited. The
2. The text references table 1, which I cannot find in the PDF.

We apologize for our oversight in the upload of the table onto the journals website. We have rectified this and the relevant table (previously Table 1 now labeled as Table 2) is included in the revised version’s upload.

<table>
<thead>
<tr>
<th>Group</th>
<th>Villous capillarisation (VC ± SE)</th>
<th>Intervillous Space (IvS ± SE, µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Central (VC-A) Body (VC-B) Periphery (VC-C)</td>
<td>Central (IvS-A) Body (IvS-B) Periphery (IvS-C)</td>
</tr>
<tr>
<td>Normotensive (n=51)</td>
<td>0.12 ± 0.014 0.11 ± 0.007 0.12 ± 0.013</td>
<td>58778.67 ± 1723.64 59015.72 ± 1496.93 61518.28 ± 2479.36</td>
</tr>
<tr>
<td>GH (n=32)</td>
<td>0.15 ± 0.014 0.13 ± 0.015 0.11 ± 0.015</td>
<td>57241.99 ± 1650.84 58380.34 ± 2753.76 55701.89 ± 1303.06</td>
</tr>
</tbody>
</table>

Mean with similar superscript differ significantly with each other at P<0.05

3. I would have liked to see a traditional “table 1” with baseline characteristics of gestational hypertensives vs. the controls, including BMI, race/ethnicity, gestational age at time of sampling, some sort of BP measure (highest SBP/DBP?) as well as birthweight and GA at delivery, given the placental pathology piece.

We agree that it may be valuable to include a ‘traditional table’ describing some patient profile and clinical history that was taken during our study recruitment. We have thus included a new table (previously not provided, now labeled as Table 1) in the revised version which is a clinical and demographic description of the population studied.

<table>
<thead>
<tr>
<th>Average Blood Pressure ± SE (mmHg)</th>
<th>Race</th>
<th>Parity</th>
<th>Maternal Age (years)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antepartum</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Systolic</td>
<td></td>
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<tr>
<td>Diastolic</td>
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<tr>
<td>Intrapartum</td>
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<tr>
<td>Systolic</td>
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<tr>
<td>Diastolic</td>
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<tr>
<td>Postpartum</td>
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<tr>
<td>Systolic</td>
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<tr>
<td>Diastolic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PROTEINURIA</td>
<td>Malay</td>
<td>Chinese</td>
<td>Indian</td>
</tr>
<tr>
<td>Nil</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GH</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AVERAGE BLOOD PRESSURE</td>
<td></td>
<td></td>
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<tr>
<td>Normotensive</td>
<td></td>
<td></td>
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<tr>
<td>112 ± 1</td>
<td>59</td>
<td>31</td>
<td>37</td>
</tr>
<tr>
<td>72 ± 1</td>
<td>21</td>
<td>37</td>
<td>37</td>
</tr>
<tr>
<td>120 ± 2</td>
<td>65</td>
<td>23</td>
<td>23</td>
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<tr>
<td>74 ± 1</td>
<td>1</td>
<td>41</td>
<td>41</td>
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<tr>
<td>107 ± 3</td>
<td>36</td>
<td>36</td>
<td>36</td>
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<tr>
<td>69 ± 2</td>
<td>13</td>
<td>13</td>
<td>13</td>
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<tr>
<td>GH</td>
<td></td>
<td></td>
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<tr>
<td>143 ± 2</td>
<td>30</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>90 ± 2</td>
<td>5</td>
<td>5</td>
<td>5</td>
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<tr>
<td>147 ± 3</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>91 ± 2</td>
<td>18</td>
<td>18</td>
<td>18</td>
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<tr>
<td>123 ± 3</td>
<td>22</td>
<td>22</td>
<td>22</td>
</tr>
<tr>
<td>81 ± 2</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>

SE = Standard Error
mmHg = millimetres of mercury

We further make a reference to Table 1 in the first paragraph of our Result section. This is done by adding the sentences:

Table 1 describes the clinical and demographic information of the study population. Blood pressure during the three study intervals for normotensive women ranged
from 107/69 mmHg to 112/72 mmHg whereas for GH women it ranged from 123/81 mmHg to 147/91 mmHg. Women from the Malay ethnicity represented the most number recruited by ethnicity and women in the 1-4 parity was similarly the highest represented group. Women recruited in the study were mostly between the age groups 25-28 and 29-32. Prior to normotensive and GH comparison, the data was analyzed for confounding effects of ethnicity, parity and maternal age.

4. If the baseline characteristics are similar it helps support the analytic approach that was used (univariate), however without this information readers will not be able to evaluate that piece.

If the goal is to look at longitudinal trends in sFlt/PlGF, then the analysis should be longitudinal (accounting for repeated measures) and not cross-sectional (ANOVA). Also, there was no adjustment for confounders (e.g. severity of disease such as IUGR, maternal BMI, smoking, etc). If this was an intentional choice, then there should be some mention in the statistical analysis section.

In addressing both the above comments, we acknowledge the need to inform readers on the baseline characteristics of the patients studied which has now been provided in Table 1. Furthermore, prior to statistical comparison, we did analyze if there were any variation in ethnicity, parity and gestational age and found none.

[1] We have thus included the following sentence in the first paragraph of the results:

No significant differences were observed for all races, parity and maternal age in the normotensive and GH women for both biomarkers and placental morphometry thus allowing the grouping of the study population by normotensive and GH only.

We agree that maternal BMI, IUGR and smoking are also relevant confounders. With regards to IUGR and smoking, mothers diagnosed with IUGR and those with a history of smoking were excluded from this study.

[1] We have thus revised the sentence in the Methods section:

From:

Patients suffering from essential hypertension, any type of renal disease, diabetes mellitus, heart diseases or infectious disease were excluded from this study.

To:

Patients suffering from essential hypertension, any type of renal disease, diabetes mellitus, heart diseases or infectious disease were excluded from this study. Women with incidence of IUGR and a history of smoking were also excluded.
We however did not check for maternal BMI and have thus included this as a limitation in our study (please see response on ‘limitations’).

5. I think the inverse correlation between sFlt and PlGF is well-established, even in normal pregnancies.

We acknowledge that inverse relationship between these biomarkers have been established before by other researchers. However, we believe that there are several benefits of it in relation to our study:

[1] It specifically focuses the use of these biomarkers on GH only and not preeclampsia. There have been not many studies focusing on GH particularly in our region.

[2] The inverse relationship we show here are a representative of an ethnically diverse population of patients. We have thus included the following sentence in the Discussion.

Furthermore, the inverse relationship between sFlt-1 and PlGF observed in our study is representative of findings from an ethnically diverse population.

[3] By complementing previous findings, it only serves to validate our method of quantification and data analysis for these biomarkers.

6. What is the significance of intrapartum and postpartum angiogenic markers? Any utility to measuring them once the placenta is delivered?

We acknowledge that our justification of measuring these markers at such interval may not have come across clearly in our original manuscript. The study’s was designed to also establish a trend pattern for the production of sFlt-1 particularly, during the course of pregnancy and post-pregnancy. We mentioned in our Discussion that there has been previous studies correlating GH and PIH with later onset of hypertensive diseases (Carpenter, 2007) and also incidences of prolonged hypertension and proteinuria in PIH patients despite placental delivery (Berks et al, 2009). Our study showed that GH mothers have significantly higher sFlt-1 levels intrapartum and 6 weeks post partum compared to normotensive mothers. From these findings we suggest that it may be possible that sFlt-1 may have a role in the development of later onset hypertension and prolonged recovery observed by Carpenter and Berks et al., respectively. We further suggest that prolonged (from intrapartum up to even 6 weeks postpartum) exposure to high levels of sFlt-1 as seen in our GH patients may contribute to future hypertensive disorders and that long term follow ups are necessary in GH women.

[1] We have thus changed the section in the Discussion:

From

The same however, cannot be said of sFlt-1 as it was noted in our study that sFlt-1 remained significantly higher in GH women compared to normotensive women during postpartum period as well. This may be due to the continuous production of sFlt-1 by vascular endothelial cells in our population of GH women, suggesting a prolonged pathophysiological effect. This finding also raises
important questions on the effect of persistently raised postpartum levels of sFlt-1, for example the period of surveillance and even continuation of anti-hypertensive drugs. Cohort studies that have used population-based pregnancy databases consistently identify a clinically significant association of GH and preeclampsia with later hypertensive disorders [12]. This clearly defines a need for long term follow-up for the development of essential hypertension in GH women. A recent study by Berks et al., 2009, on preeclamptic women in the Netherlands indicates that it can take up to 2 years for hypertension and proteinuria to resolve, with resolution being associated to the severity of hypertension during pregnancy [13].

To

As for sFlt-1, our study shows that it remains significantly higher in GH women compared to normotensive women during postpartum period. This finding raises important questions on the effect of persistently raised postpartum levels of sFlt-1, for example on the period of surveillance and even continuation of anti-hypertensive drugs. Cohort studies that have used population-based pregnancy databases consistently identify a clinically significant association of GH and preeclampsia with later hypertensive disorders [12]. This clearly defines a need for long term follow-up for the development of essential hypertension in GH women. A recent study by Berks et al., 2009, on preeclamptic women in the Netherlands indicates that it can take up to 2 years for hypertension and proteinuria to resolve [13]. Findings from these studies suggest that prolonged exposure to high levels of sFlt-1 as seen in our study population may necessitate long term follow ups in GH women.

7. In terms of putting this in context, I think it’s important to directly compare to the Levine paper that showed no difference between GH and normals in cross-sectional analysis.

We thank the reviewer for the suggestion. We have accordingly included the following sentences in Para 2 of the Discussion together with the reference Soluble Endoglin and Other Circulating Antiangiogenic Factors in Preeclampsia by Levine et al., 2006:

We acknowledge that results from this study present a contrasting finding compared to a previous cross sectional study. In the latter study, while no absolute values of the biomarkers were discussed, analysis for sFlt1:PlGF ratios demonstrated no significant differences between GH and normotensive mothers except at 33 through 36 weeks of pregnancy [13]. The contrasting findings at the antepartum period between both studies could be due to variations in the study population (for example: age, parity, ethnicity, smoking status) or in the actual onset of GH versus the diagnosis of GH. Nevertheless, both studies suggest it is worthwhile to examine the levels of these biomarkers at earlier gestational ages in various populations.

8. There was not a clear discussion of limitations.
We appreciate that there exist a need for the discussion of limitations in our study and have accordingly done so by taking the recommendations provided by both reviewers.

[1] We have thus included in the Discussion the following paragraph:

There were several limitations that were present in this study. Shortfalls with regards to follow-up of patients from time of first contact was attributed to the fact that many women use the free government antenatal care services but prefer to deliver at alternative delivery centers which included returning to their parent’s homes or to private medical centers when they were at term gestation. In the initial recruitment, Body Mass Index (BMI) of patients were not obtained and thus we were unable to adjust for its confounding effects. Work done in this study examined two-dimensional evaluations of the placenta and future studies should include three-dimensional morphometric assessment in which more precise and in-depth structural and stereological features can be assessed.

MINOR POINTS

1. There are a few sentences scattered throughout that don’t read clearly—recommend revising.

    Thank you for the detailed observation on this matter. We have re-read the manuscript and have made additional (other than those related to the reviewers comments which are in red) changes to any grammatical or typographical errors. We have also revised sentences that are too long and have attempted to limit the number of words per sentence to provide more clarity where necessary. Such changes have been additionally reflected as blue text throughout the manuscript.
B. In response to REFEREE 2 (Augustine Rajakumar):

We are very grateful for these very constructive comments concerning the paper and resubmit a revised version in which all the points raised by both reviewers are dealt with.

MAJOR POINTS

1. In general the manuscript needs clarity. Many of the statements the authors make are vague. I have only mentioned a few below. It would help if they were a bit more specific.

   We have re-read the manuscript and agree that there are areas that needed improvement. We have made additional (other than those related to the reviewers comments which are in red) changes to any grammatical or typographical errors, revised sentences that are too long and attempted to limit the number of words per sentence to provide more clarity where necessary. Such changes have been additionally reflected as blue text throughout the manuscript.

2. Abstract:
   Line starting with ‘Biochemical markers ……….than the traditional clinical tools used.’
   Expand on the clinical tools.

   Thank you for drawing our attention to this. For clarity purpose we have now revised the sentence:

   From:

   Biochemical markers of abnormal placentation would be more specific in early detection than the traditional clinical tools used.

   To:

   Biochemical markers of abnormal angiogenesis would be more specific in early detection than routine blood pressure and urine dipsticks measurements.

Malaysian setting: Define and expand.

   We agree that the definition for Malaysian setting needs further clarification to allow a firmer understanding of the situation. We have added further justification in the Background section of the manuscript. For the purpose of the abstract we have reworded the following sentence

   From:

   We established periodic values of for sflt-1 and PIGF level for the first time in a Malaysian setting.

   To:

   We established periodic values of for sflt-1 and PIGF level for the first time in an ethnically diverse Malaysian setting.
3. Background:  
Again expand on ‘Malaysian setting’ providing information on ethnic diversity and other factors to justify the current study

As the findings would be of interest to both local and international audience, as per the reviewers request we have provided more details on a Malaysian setting in the Background.

[1] We have thus included the following sentence:

Findings from this study represent an important source of information for a country with an ethnically diverse population and a maternal healthcare system that is undergoing rapid development. This study would be the second only study to quantify these biomarkers in Malaysia and the first to assess the periodic levels and correlate it with placental morphometry.

Sentence that starts with ‘Placenta isolated from early onset ………..late onset PE’. Modify.

The following sentence has been changed:

From:

Placenta isolated from early onset preeclampsia was associated with abnormal morphology compared to late onset preeclampsia [4] suggesting that placental ischemia is an early event related to maternal endothelial dysfunction [5].

To:

Furthermore, placenta isolated from women with early onset preeclampsia was associated with abnormal morphology compared to those with late onset preeclampsia [4] which suggests that placental ischaemia is an early event relating to maternal endothelial dysfunction [5].

Sentence “It remains elusive………..” Modify.

The following sentence has been changed:

From:

It remains elusive whether raised levels of circulating factors like sFlt1 and PlGF cause’s placental structural changes and vasculopathies however these biomarkers are associated more frequently with the presence of the placenta as their levels drop in the absence of the placenta.

To:

The effects circulating factors like sFlt1 and PlGF exert on placental structural changes and vasculopathies remains unclear although raised levels of these biomarkers have frequently been associated with the presence of the placenta.
4. Methods:
Technical details presented in placental studies regarding morphometry are not enough to understand what was carried out. For example, the authors state ‘Three whole thickness placental sections,…….”. What was done to these tissues?

The following sentence has been changed.

From:
Three whole thickness placental sections, measuring about 1 cm$^3$ each were randomly taken from the central (A), body/middle (B) and periphery (C) of the placenta and stained routinely.

To:
Three whole thickness placental sections, measuring about 1 cm$^3$ each were randomly taken from the central (A), body/middle (B) and periphery (C) of the placenta and fixed in formalin. Tissues were subsequently transported to the laboratory for routine tissue processing and paraffin blocking. Tissue blocks were sectioned at 5 μm thickness and stained with Hematoxylin and Eosin.

5. Results:
Authors need to decide on the organization of the study and the presentation. For example the first reference in the Result section refers to Figure 3.

We thank the reviewer for this suggestion. We have made overall revisions necessary to allow for clarity of the manuscript. Such changes have been additionally reflected as red text throughout the manuscript. The first reference in the results does indeed refer to Figure 3 as both Figure 1 and Figure 2 are presented in the Methods section and involve a pictorial guide as to how the morphometric measurements were made.

The authors mention in ‘Morphometric results for villous ……” “As shown in Table 1,…..” I couldn’t find the table and neither the Biomed Central.

We apologize for our oversight in the upload of the table onto the journals website. We have rectified this and the relevant table (previously Table 1 now labeled as Table 2) is included in the revised version’s upload. Table 1 has been included on the request of Reviewer 1 and is a clinical and demographic description of the population studied. Both tables are included below for reference.
### Table 1. Clinical and demographic description of study population

<table>
<thead>
<tr>
<th></th>
<th>Antepartum</th>
<th>Intrapartum</th>
<th>Postpartum</th>
<th>PROTEINURIA</th>
<th>RACE</th>
<th>MATERNAL AGE (years)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Malay</td>
<td>0 1 4 &gt;5</td>
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<tr>
<td></td>
<td>Systolic</td>
<td>Diastolic</td>
<td>Systolic</td>
<td>Diastolic</td>
<td>Chinese</td>
<td>17-24 25-28 29-32 33-36 37-44</td>
</tr>
<tr>
<td>Normotensive</td>
<td>112 ± 1</td>
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<td>120 ± 2</td>
<td>74 ± 1</td>
<td>nil</td>
<td>59 21 37 51 65 1 23 41 36 13 4</td>
</tr>
<tr>
<td>GH</td>
<td>143 ± 2</td>
<td>90 ± 2</td>
<td>147 ± 3</td>
<td>91 ± 2</td>
<td>nil</td>
<td>30 5 10 18 22 5 5 13 11 9 7</td>
</tr>
</tbody>
</table>

SE = Standard Error

mmHg = millimetres of mercury

### Table 2. Morphometric results for placental villous capillarisation and intervillous space

<table>
<thead>
<tr>
<th>Group</th>
<th>Villous capillarisation (VC ± SE)</th>
<th>Intervillous Space (IvS ± SE, µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td>0.13 ± 0.015</td>
</tr>
</tbody>
</table>

Mean with similar superscript differ significantly with each other at P<0.05

6. While there is some useful information in this study, the manuscript limits the enthusiasm for the lack of organization and critical writing.

We thank the reviewer for the constructive criticism. We agree that the manuscript needed some clarity and have thus made revisions as requested by both reviewers. We believe that the new revised version of the manuscript reads better and has developed some clarity due to the feedback and suggestions received from both reviewers.