Author’s response to reviews

Title: Oxidative stress and endothelial function in normal pregnancy versus pre-eclampsia, a combined longitudinal and case control study. The Endopreg study.

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"Oxidative stress and endothelial function in normal pregnancy versus pre-eclampsia, a combined longitudinal and case-control study. The Endopreg study."

Dear Editor,

We would like to thank you for the valuables comments and for the opportunity to resubmit our manuscript to the BMC Pregnancy and Childbirth. In order to meet the expectations of the referees, additional information was added and changes were incorporated in the revised version of the manuscript.
Please find point-by-point responses to the comments and questions raised in the decision letter below.

Sincerely yours,

Dominique Mannaerts, on behalf of all co-authors

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Comments Tracey L. Weissgerber (Reviewer 1)

The manuscript describes the protocol for the Endopreg Study, which measures oxidative stress and endothelial function longitudinally during pregnancy, and at the time of delivery and postpartum in women with normal vs. complicated pregnancies (preeclampsia, IUGR, PIH, preterm birth). This study has the potential to provide interesting data on the time course of changes in oxidative stress and endothelial function in normal pregnancies, and at delivery and postpartum in complicated pregnancies. However, there are some points that require clarification.

We thank the reviewer for the appreciation and the interest in our work. We used the constructive criticism to improve our manuscript. In the following, we will address the formulated comments in chronological order.

The description of "angiogenic or placental PE, formerly called early onset" and "non-angiogenic or maternal PE, formerly called late onset" oversimplifies and overstates existing findings. While many women with early onset PE have abnormal concentrations of angiogenic markers, there are also women with preeclampsia at term who have abnormalities in angiogenic markers. These women do not fit into the authors' classification scheme. There are also women who develop early onset preeclampsia AND have significant maternal risk factors, including underlying cardiovascular or renal disease, hypertension, diabetes, etc. The field is currently focused on identifying and learning to recognize different subtypes of preeclampsia (1,2); yet the authors present this as fait accompli. Are the authors confident that if a women with preeclampsia has normal concentrations of sFlt-1 and PIGF, we can conclusively say that the placenta is completely normal? Or that there are only two types of PE? A more balanced approach would be to state that both maternal and placental factors contribute to PE and the relative contributions of
each likely vary from woman to woman. Researchers are currently working to identify different subtypes of preeclampsia, and classifications based on both clinical presentation (i.e. early vs. late onset; mild vs. severe) and pathophysiologic processes (i.e. maternal vs. placental, angiogenic vs. non-angiogenic) have been proposed. Women with early onset PE are more likely to have abnormalities in pro-and anti-angiogenic markers, compared to women with late onset PE.

The reviewer properly appoints that pre-eclampsia cannot be strictly subdivided into two different entities. As the authors share this opinion, patients in the Endopreg study are not strictly included as early onset or late onset pre-eclamptic patients. PE patients are seen as one group and when all data are gathered, statistical analyses will show whether vascular function and oxidative stress are different dependent on their gestational age at diagnosis.

The introduction part of the manuscript is changed as follows: (page 4, line 8)

“In the past, PE has been divided in two different entities; angiogenic or placental PE (formerly called early onset, before 34 weeks) and non-angiogenic or maternal PE (formerly called late onset, after 34 weeks). Impaired placenta tion and endothelial dysfunction have been described in placental PE, whereas preexisting maternal cardiovascular risk factors (essential hypertension, high BMI, diabetes, renal disease,..) usually precede maternal PE. This description however oversimplifies and overstates recent existing findings. Maternal risk factors can precede early onset PE as well as abnormal concentrations of placental angiogenic factors are found in late onset PE. Fetal growth restriction and endothelial dysfunction caused by systemic inflammation are usually described in placental PE, nevertheless they are common in late onset PE. It is therefore more accurate to state that both maternal and placental factors contribute to PE and research should focus on classifications based on pathophysiologic processes, for instance endothelial and vascular dysfunction and amount of systemic inflammation and OS.”

The paragraph on offspring effects of a PE pregnancy seems out of place, as the protocol does not study offspring.

The reviewer correctly noticed that information on the offspring does not add any value to the manuscript. The authors therefore erased this part of the introduction.

For PE, preventive interventions such as aspirin are only recommended for high-risk women. This should be clarified. Furthermore, that protocol rationale depends on the assumption that these interventions will also be effective in women who would not be identified as high risk by current algorithms, but will be at risk based on elevated oxidative stress and endothelial function in the first trimester. This should be explicitly stated. Finally, the authors should consider the possibility that by looking for women who have abnormalities at 12 weeks, they may be targeting a subset of women predisposed to early onset PE or to specific pathophysiologic pathways (i.e. pre-existing maternal vascular dysfunction). The authors should have a plan for identifying/evaluating possible subgroups of PE patients in their analysis.

I could not find an analysis plan for aim 5 (preeclampsia prediction).
We thank the reviewer for her attentive reading. As correctly stated by Dr Prefumo (Reviewer 2) also, the Endopreg study is not adequate to generate a first trimester predictive model for pre-eclampsia. This study will add information on the physiology of oxidative stress and vascular function in healthy pregnancy and compare this with the pathophysiologic mechanism present in pre-eclamptic patients. Within this view, we took out all statements on prediction and prevention of pre-eclampsia and suggestions on preventive therapy for PE. The authors do, however, agree with the reviewer that preventive interventions are currently only proven to be effective in high-risk women.

The study hypotheses appear out of order. For example, "oxidative stress and endothelial function can be measured" follows two aims based on findings RE: endothelial function and oxidative stress. Also, if we don't already have sufficient information to confirm that the measurements that the authors are using are effective, why are these measurements being performed? What gold standard are the authors comparing their results with to confirm that this aim is successful?

We highly agree with the reviewer that the hypothesis section of our manuscript is not correctly stated. Oxidative stress will be measured in this study using a protocol proven to be correct and reproducible in previous research. (Gielis et al. Longitudinal quantification of radical bursts during pulmonary ischaemia and reperfusion. Eur J Cardiothorac Surg. 2015) FMD is the gold standard for assessing endothelial function. Thijssen et al. Assessment of flow-mediated dilation in humans: a methodological and physiological guideline. Am J Physiol Heart Circ Physiol, 2011).

Since the hypothesis section did not correctly represented our views, this section was adjusted as follows: (page 7, line 2)

“Endothelial and vascular function improve during healthy pregnancy to answer the higher hemodynamic demands. Due to the deficient placentation in PE, disturbed production of (anti-)angiogenic and inflammatory factors results in arterial stiffness and endothelial dysfunction. After PE, this vascular dysfunction continues in patients at risk for developing cardiovascular events later in life.

A certain amount of OS is necessary in healthy pregnancy. The deficient placental oxygenation in PE causes excessive local formation of reactive oxygen and nitrogen species (O2•- and •NO respectively). When the balance between pro-oxidant species and the antioxidants is disturbed, OS arises. We hypothesize that in PE, there is a higher amount of O2•- in the maternal circulation and a lower concentration of •NO and eNOS measurable in the placenta.

Endothelial and vascular dysfunction is correlated to the amount of OS present in the circulation.”

There is nothing in the protocol that tests aim 4 (oxidative stress and endothelial function can be improved by interventions…)
The reviewer is correct that the protocol does not contain any tests on improvement of endothelial function or oxidative stress. In the new version of the manuscript the authors rewrote the hypothesis section thoroughly.

Note that the definition of PE as written does not clearly require a sustained elevation in blood pressure. Do the authors require multiple elevated BP measurements?

The reviewer correctly notices that this information is not clearly specified in the manuscript. As stated by the ACOG guideline for hypertension in pregnancy (2013), hypertension is defined as follows:

“Hypertension greater than or equal to 140 mmHg systolic or 90 mmHg diastolic, must be confirmed at least 4 hours apart. Hypertension greater than or equal to 160 mmHg systolic or 110 mmHg diastolic can be confirmed after a short interval (minutes) to facilitate antihypertensive treatment.”

This paragraph is added in methodology section of the manuscript (page 9, line 20).

For early and late PE, please clarify whether <34 weeks refers to the gestational age of PE diagnosis or of delivery.

Since the authors decided to no longer subdivide the pre-eclamptic patients in early and late PE, this information was deleted from the inclusion criteria section. However, when after data-analysis, measurements seem to differ depending on gestational age, early pre-eclampsia will be defined as the diagnosis of pre-eclampsia before the 34th pregnancy-week. (Tranquilli, The definition of severe and early-onset preeclampsia. Statements from the International Society for the Study of Hypertension in Pregnancy (ISSHP) 2012)

Will FMD measurements include assessment of the shear stimulus, as recommended by guidelines? Will LFMC be measured?

We thank the reviewer once again for her attentive reading and extensive knowledge concerning the subject. We highly agree that these measurements are not well documented in the text, although they are regularly performed by our very experienced investigators (IG and TS). Since LFMC is known to add important information concerning the control of arterial tone at rest, therefore complementing (and not overlapping with) the information provided by FMD, it is of utmost importance to include this measurements in our study protocol. Shear stimulus, although not always easy to measure at the time of cuff deflation, is of large additional value to the interpretation of FMD/FMC results.

We therefore added the following text to the methods section of the manuscript (page 10, line 16):

“Low–flow-mediated constriction (LFMC) is calculated as the percent decrease in arterial diameter in the last 30 seconds of cuff occlusion as compared with resting diameter. After 5 minutes of occlusion, the cuff is deflated. Brachial diameter is recorded continuously (eTracking)
from the time point of cuff inflation to 5 minutes after cuff deflation. FMD (in % from baseline value) is expressed as (post-ischemic maximal diastolic diameter change - baseline diastolic diameter)/ baseline diastolic diameter. During the measurements, radial artery blood flows will be measured at 3 different time-points; at rest, 15 seconds before cuff deflation, and immediately upon cuff deflation using pulsed-wave Doppler.”

For all measurements, will the person completing the measurement or be blinded to study group?

We thank the reviewer for this question on an important topic. For the FMD measurements, the person performing the measurements will be blinded since this is an external investigator. The other vascular measurements will be performed by DM or EF, investigators that will also inform the patients on the study and ask for consent, so these measurements are unfortunately not blinded. However, data analysis will be blinded, as are the analyses of oxidative stress on maternal blood and placenta.

The case-control study includes 44 women/group (control, preeclampsia, PIH, pre-term birth, IUGR), where women with complications will be matched to controls for maternal and gestational age, parity, smoking behavior and ethnic group. In order to do this, the authors would have to carefully select women in each pregnancy complication group to ensure matching - this could lead to biased selection of women with complications. For example, the study includes a pre-term birth group; therefore matching for gestational age will require all other women in the study to complete testing prior to term. Women who develop PE, PIH or IUGR at term could not be enrolled. Even matching each group separately to the control group would be impossible, given that the control group includes the same number of women as the other groups. Adjusting for potential confounding variables in the analysis would be a better approach.

We highly agree with the reviewer that this approach is not easily achievable or demands a very large control population. While executing the Endopreg study over the last year, the authors became aware of the difficulties of including such large study populations and performing the large amount of investigations on them. Therefore, the authors decided to reduce the study population to pre-eclamptic and control patients first. For this reason, in the new (adjusted) version of the protocol, only pre-eclamptic patients are included, reducing the control group to 44 patients that have to become matched. In the future, when the Endopreg study for pre-eclamptic patients is completed and more experience is achieved, a larger study with the other populations (IUGR, PTB, PIH) will be started.

The rationale for the study is heavily focused on identifying nulliparous women at risk for pregnancy complications; however the study is not restricted to nulliparous women.

The reviewer is very correct to notice that there is confusion on this subject. As stated before, both reviewers have correctly stated that the Endopreg study is not properly set up for the development of a prediction model for pre-eclampsia. Therefore, we took out all statements concerning prevention/prediction. Since the case-control study should be matched for parity and most but not all pre-eclamptic patients are nulliparous, the control population should contain an equal amount of parous women.
Comments Federico Prefumo (Reviewer 2)

This manuscript describes the protocol of an interesting study investigating oxidative stress and maternal endothelial function in normal and complicated pregnancies.

We thank the reviewer his interesting concerns and suggestions. We used the constructive criticism to improve our manuscript. In the following, we will address the formulated comments in chronological order.

I have two major issues to raise:

There is a paucity of details on pre-analytical biological sample handling. How will blood and placental samples be collected and stored? How will placental samples be treated before assays? Just homogenized or submitted to other treatments?

We thank the reviewer for his attentive reading and agree that this information was missing in our manuscript. We therefore expanded the methods section with detailed information on sample handling and storage.

We must clarify to the reviewer that eNOS/iNOS determination in placental tissue using western blotting did not appear to be reproducible enough in our laboratory, for this reason the authors decided to perform immunohistochemical staining to examine expression of eNOS in placental tissue.

The following paragraphs were added to the manuscript: (page 13, line 1)

“Placental concentration of •NO

Placental tissue will be obtained within two minutes after (vaginal or caesarean) delivery. At a standardized central location, a viable sample of 1cm³ placental tissue will be taken avoiding placental infarcts. The sample will be rinsed with saline (NaCl) and immediately added to the spin trap 750µl FeSO4+750 µl DETC (iron (II)diethylidithiocarbamate solution). After one hour of incubation at 37°C, the sample will be snapfrozen and stored in -80°C until analysis with EPR.

Maternal blood concentration of O2•-

Maternal blood will be obtained at 12 weeks and 24-28 weeks in a heparin tube (BD vacutainer) and transported on ice. After 15 minutes, 30µl of spin trap CMH (1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine) will be added to 30µl blood. The whole will be incubated on ice and transferred into a capillary after 5 minutes. The sample will then be snapfrozen and stored in -80°C until analysis.

Immunohistochemical staining for eNOS and iNOS
Placental tissue will be obtained shortly after delivery and stained for eNOS and iNOS as previously described by Du et al."

Although this is not listed among the goals of the study in the Methods section, both in the abstract and discussion the authors emphasize the fact that their results will allow to identify pregnancies at risk for pre-eclampsia in the first trimester of pregnancy. Neither the study design, nor the sample size, are adequate to generate a first trimester predictive model for pre-eclampsia. I suggest therefore that they remove completely such claims.

We highly agree with the reviewer that this is a very indistinct part of the manuscript and that indeed the Endopreg study is not designed for the prediction of PE, but rather will add information on the physiology of oxidative stress and vascular function in healthy pregnancy and compare this with the pathophysiologic mechanism present in pre-eclamptic patients. Within this view, we took out all statements on prediction and prevention of pre-eclampsia.

Minor points:

The background section is very long and unfocused. I suggest this might be shortened.

We thank the reviewer for this pertinent remark. We rewrote the background section fundamentally. (page 3, line 1)

“Pre-eclampsia (PE) is a potentially life-threatening pregnancy related vasculopathy characterized by hypertension and proteinuria. PE results in high morbidity-mortality for both mother and her unborn child. Between five and ten percent of pregnancies are complicated by hypertensive disorders and worldwide the incidence of PE has increased by 25% in the past two decades.

In normal pregnancy vascular remodeling of the maternal uterine spiral arteries occurs. Trophoblast cells invade the spiral arterioles within the first 12 weeks of pregnancy and replace the muscular wall of the vessels converting them into wide bore, low resistance, large capacity vessels, a process normally completed by 20 weeks gestation. The free radical nitric oxide (•NO) is an important mediator of the placentation process. •NO is an endogenous endothelium-derived relaxing factor influencing endothelial function. Under physiologic conditions, endothelial release of •NO in the placental circulation dilates the fetal placental vascular bed, ensuring feto-maternal exchange. •NO is formed out of L-Arginine by NOS (Nitric Oxide Synthase). This reaction is regulated by VEGF (Vascular endothelial growth factor), an endothelial mitogen that has an important function in the proliferation of endothelial cells and in angiogenesis. VEGF stimulates eNOS (endothelial NOS) and induces therefore •NO production. In an oxidative environment, the lack of NOS-stabilizing factors results in NOS-uncoupling. NOS-coupling causes a shift from •NO production to superoxide (O2•-) production which maintains an oxidative setting.

The pathogenesis of generalized endothelial dysfunction is well known in PE and is subdivided into two phases. The first phase exists of a poor trophoblast invasion of the spiral arteries during
the placentation process, causing failure to transform the placental bed arteries from high to low resistance vessels. This results in local ischemia, reperfusion damage and oxidative stress (OS). The local damage activates the second phase where disturbed production of angiogenic and antiangiogenic factors (placental growth factor (PIGF) and soluble fms-like tyrosine kinase 1 (sFlt-1), respectively) results in systemic inflammation, endothelial activation, systemic OS and altered endothelial \( \cdot \text{NO} \) production. When this vascular endothelial activation and dysfunction occurs at the level of liver, kidney, brain and placenta, the clinical presentation of PE arises.

In the past, PE has been divided in two different entities; angiogenic or placental PE (formerly called early onset, before 34 weeks) and non-angiogenic or maternal PE (formerly called late onset, after 34 weeks). Impaired placentation and endothelial dysfunction have been described in placental PE, whereas preexisting maternal cardiovascular risk factors (essential hypertension, high BMI, diabetes, renal disease,...) usually precede maternal PE. This description however oversimplifies and overstates recent existing findings. Maternal risk factors can precede early onset PE as well as abnormal concentrations of placental angiogenic factors are found in late onset PE. [6] Fetal growth restriction and endothelial dysfunction caused by systemic inflammation are usually described in placental PE, nevertheless they are common in late onset PE. It is therefore more accurate to state that both maternal and placental factors contribute to PE and research should focus on classifications based on pathophysiologic processes, for instance endothelial and vascular dysfunction and amount of systemic inflammation and OS.

In normal pregnancy, placental OS is present during all three trimesters and is necessary to obtain normal cell function, including activation of redox-sensitive transcription factors and activation of protein kinases. Although OS is a common necessary feature of normal pregnancy, persistent OS gives rise to different disease-states, such as PE. Although considerable research has been devoted to OS in PE, less attention has been paid to the evolution of OS during the course of normal pregnancy. Little research has described an increase in \( \cdot \text{NO} \) concentration with gestational age, suggesting an important role for \( \cdot \text{NO} \) in the cardiovascular changes of normal pregnancy.

Recent literature has elucidated that PE is an important risk factor for cardiovascular disease in later life. Bellamy et al. and McDonald et al. describe a 3-fold risk for hypertension and a 2-fold risk of ischemic heart disease and stroke in women with a history of PE. Women with hypertensive disorders during pregnancy also have a greater risk of chronic kidney disease and end-stage renal disease. With a view to detecting those women at risk, objectifying endothelial function and vascular function after healthy pregnancy and PE can help to establish reference values for disturbed post-pregnancy vascular function.”

Page 9, line 18: investigators IG and TS do not seem to be listed among manuscript authors.

The reviewer correctly notices that Inge Goovaerts and Tibor Stoop are not listed in the author list. However, since they will perform the FMD measurements during the study, they are mentioned in the acknowledgements section.

Is the Mindray VS 900 monitor validated for use in pregnancy?
We thank the reviewer for this important remark. The Mindray VS 900 monitor has been validated previously over the range 0–300 mmHg against a mercury manometer in a pregnant population.

Technical comments

Editor Comments:

Please re-format your Declarations section as detailed in our submission guidelines. Please also ensure that all authors are listed under Authors contributions and their contributions detailed: at the moment three of the authors are not mentioned in this section.

We thank the editor for the appreciation and the interest in our work and adjusted these comments in the declarations section of our manuscript. (page 17, line 1)

DECLARATIONS

Ethics approval and consent to participate

The Research and Ethics committee of the Antwerp University Hospital approved the study protocol (Belgian number: B300201524783), and written informed consent was obtained from all subjects.

Consent for publication

Not applicable

Availability of data and material

Not applicable

Competing interests

None of the authors have any conflicts of interest to declare.

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Authors’ contributions

DM and YJ designed the study, with input from JG, PC, EVC, MS, WG, JC. Study amendments were completed by DM. DM and EF performed the measurements. All authors contributed to critical revision of the manuscript and approved the final manuscript.

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