OBSTETRICS & GYNECOLOGY

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- Comments from the reviewers and editors (email to author requesting revisions)
- Response from the author (cover letter submitted with revised manuscript)*
- Email correspondence between the editorial office and the authors*

The corresponding author has opted to make this information publicly available.

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Questions about these materials may be directed to the *Obstetrics & Gynecology* editorial office: obgyn@greenjournal.org.

Date: Aug 17, 2018

To: "Richard Michael Burwick" ▮▮▮▮▮▮▮▮▮▮▮▮▮▮▮▮

From: "The Green Journal" em@greenjournal.org

Subject: Your Submission ONG-18-1367

RE: Manuscript Number ONG-18-1367

Terminal Complement Activation in Preeclampsia (COPA), a Multicenter Study

Dear Dr. Burwick:

Your manuscript has been reviewed by the Editorial Board and by special expert referees. Although it is judged not acceptable for publication in Obstetrics & Gynecology in its present form, we would be willing to give further consideration to a revised version.

If you wish to consider revising your manuscript, you will first need to study carefully the enclosed reports submitted by the referees and editors. Each point raised requires a response, by either revising your manuscript or making a clear and convincing argument as to why no revision is needed. To facilitate our review, we prefer that the cover letter include the comments made by the reviewers and the editor followed by your response. The revised manuscript should indicate the position of all changes made. We suggest that you use the "track changes" feature in your word processing software to do so (rather than strikethrough or underline formatting).

Your paper will be maintained in active status for 21 days from the date of this letter. If we have not heard from you by Sep 06, 2018, we will assume you wish to withdraw the manuscript from further consideration.

REVIEWER COMMENTS:

Reviewer #1:

This cross-sectional multicentre case control study stratified for gestational age examined plasma and urinary levels of complement protein C5b-9 in women with preeclampsia, gestational hypertension and essential hypertension compared to normotensive pregnant women. It provides further interesting data regarding the state of complement activation in various hypertensive disorders of pregnancy.

The article would be improved by presenting the multivariate analysis to support the premise of absence of association between urinary C5b-9 levels and proteinuria as this finding is inconsistent with previous research findings (Morita et al, 2000, J Am Soc Nephrol). In addition it would be of great interest to readers to know which variables contributed most to the weakening of the association between the complement levels and odds of having preeclampsia with severe features after multivariate analysis.

The authors' comments in line 299 need to be specified as relating to urinary levels of C5b-9 and a speculation offered as to how the lack of difference in plasma levels of C5b-9 in all groups of hypertensive patients (except early preeclampsia) fits into their conclusions regarding the role of complement activation in the pathogenesis of preeclampsia. In addition the possible reasons for the lack of difference in urinary levels of C5b-9 between women with early and late preeclampsia should merit a comment.

This is a substantial contribution to the literature regarding the state of complement activation in normal and pathologic pregnancy.

Reviewer #2:

MANUSCRIPT NUMBER: 18-1367

TITLE: Terminal complement activation in preeclampsia

Overall: This is a research report of complement levels in women in with hypertension in pregnancy. the authors sought to measure complement in blood and urine in women who met diagnostic criteria of the full spectrum of hypertension in pregnancy.
 A major weakness is that this is a focused pathophysiology paper that may not be relevant to everyday practice.

IRB/Ethics approval was obtained

ABSTRACT Introduction:
1.   Methods: Please include a primary outcome measure in the methods and the measure of effect the project was aiming to identify.
INTRODUCTION:
2.   Line 131 - the hypothesis as written in somewhat vague. Please include specific numbers/levels or differences that the authors sought to find in this lab based study.

MATERIALS AND METHODS:
3.   An overall statement of the type of study should be made at the beginning of the Materials and Methods section - cross-sectional? Retrospective or prospective cohort?
4.   The population and setting needs to be more clearly stated.
5.   Clearly state the eligibility criteria for entry into the study
6.   Please clearly state the primary outcome of the study - as the way the paper is currently written there are mulitple complement levels that were of interest (Lines 182)
7.   If the intended audience of the paper is clinicians in practice the authors will need to provide more complete information on complement levels and their relationship to preeclampsia.
RESULTS:
8.   A participant flow chart should be included - this will provide readers with an understanding of the final analysis population, and this is also a crucial component of reporting according to STROBE.
9.   Table 1 - by enrollment group or by comparison group? Or by type of hypertension.
10.   Appendices 1 and 2 seem either unnecessary or they should be more informative and included in tables.
11.   Please include a statement of how many women were approached/screened/enrolled in the opening of the results.
12.   The purpose of the tables to describe the results are unclear - the tables need more complete titles.
DISCUSSION:
13.   Please discuss how will this paper fit in with existing literature? If this paper should be of interest to general obgyns in practice then please address this in the discussion.

Reviewer #3:

This study undertook a novel approach to distinguishing between preeclampsia with severe features and controls that included normotensive patients and patients with chronic hypertension, gestational hypertension, and preeclampsia without severe features. Based on the hypothesis that preeclampsia is a complement-mediated disorder, the authors examined terminal complement effector C5b-9 (membrane attack complex) which they believed to be a key mediator in its development. They found a urine level of C5b-9 that clearly distinguished preeclampsia with severe features from other hypertensive complications of pregnancy and conclude that it may prove to be a useful marker for future studies of disease evolution as well as for developing strategies for prospective disease intervention.

This study that is well designed, conducted, and analyzed with a clearly stated hypothesis that is relevant to the condition under examination. My only comment would be that the discussion section is too long and repeats data in the results section that have already been presented. Their discussion of complement activation in the disease process has been covered in the introduction as well.

Reviewer #4:

Complement factor C5 split products increases indicating excessive activation have been reported in relation to preeclampsia for about 30 years. It has also been suggested that the association between preeclampsia and complement activation is more pronounced for early-onset preeclampsia, which also tends to be a more severe form. The study reported by Burwick et al. was designed to test the hypothesis that terminal complement activation, as measured by C5b-9 in maternal blood and urine is increased specifically in preeclampsia with severe features. The hypothesis was not verified when levels of C5b-9 were compared in plasma of cases vs controls, however, in the urine, levels were most specifically increased in early-onset preeclampsia with severe features. The findings are consistent with previous reports. The difference between plasma and urine determinations is considered by the authors to be the consequence of a more profound complement activation with renal involvement in more severe cases.

On page 8, as part of the background, it is stated that activation of C5 exceeds regulatory capacity in preeclampsia. It may be warranted to include that other investigators have not found the same overwhelmed regulatory capacity beyond C3a. See Banadakoppa M, Vidaeff AC, Yallampally U, et al. Disease Markers 2015, and also your reference #10 (Buurma et al). In the latter report, placental mRNA expression of the complement regulatory proteins was measured in preeclampsia close to delivery, noting in fact a significant upregulation of CD55 and CD59 mRNA expression.

Among the limitations of the study, briefly discussed on page 16, I would consider:
-    Limitation to only one split product determination, with no evaluation of the alternative pathway initiation complex,

considered by some to be stronger related to preeclampsia (your references #19 and #35 and also Gilbert JS et al. Hypertension 2012)
- C5b-9 measured at a single point after the clinical diagnosis. Complement activation detected in the clinical stage may be just the consequence of the systemic and local inflammatory reactions that characterize the final clinical stage in the development of preeclampsia, not allowing causal inferences.
- Also inherent to a case-control approach is the potential for bias and the inability to draw definitive conclusions regarding causal relationships.
I would also temper a little bit the discussion of the ROC analysis because a result of 0.74 is only fair and cannot be considered a good discriminator.
Other observations:
- Line 159: serum creatinine range of 0.5 to 1.1 mg/dl is not the normal range for pregnancy.
In contrast to many other "dead-end" associations studied in relation to the development of preeclampsia, there is some indication that therapeutic manipulations of the complement system may be feasible in pregnancy and the authors appropriately discuss this in the last paragraph of the manuscript.

STATISTICAL EDITOR COMMENTS:

The Statistical Editor makes the following points that need to be addressed:

Table 1: Unless there were no missing values, should cite entries as n(%), not just as %. Were the distributions normal for continuous variables? If not, then should cite as median (range or IQR) and test non-parametrically. It appears that urine protein/creatinine has skewed distribution, for example.

lines 176-178: For the purposes of model analysis, what value was entered into the model for the undetectable levels, since zero is an undefined value for logistic models?

lines 195-197 and Figures 2a, 2b, 3a, 3b: The data appear highly skewed and the authors rightly used non-parametric stat testing. However, if multiple groups were compared, should have used Kruskal-Wallis, rather than Wilcoxon. Need to clarify was the p < .001 specific pairwise testing with each of the other groups vs "all other groups". Should provide (could be on-line supplement) the data in Table format corresponding to the graphs.

lines 248-256: Need to provide a Table for the univariate and multivariable analyses of OR and aORs and justify the number of adjustors used relative to the counts of subsets, such as C5b-9 ≥ 22 ng/ml vs 7 adjustors. (count = 42/104 vs 7 variables is an unfavorable ratio and likely an over fitted model.)

Fig 4: Need to provide specificity, sensitivity (each with CIs). The PPV is not relevant and not replicable, since this was a highly specialized sample of women with various states of HTN, as well as healthy controls, so cannot be generalized to other groups with other rates of HTN prevalence.

EDITOR COMMENTS:

1. Thank you for your submission to Obstetrics & Gynecology. In addition to the comments from the reviewers above, you are being sent a notated PDF that contains the Editor's specific comments. Please review and consider the comments in this file prior to submitting your revised manuscript. These comments should be included in your point-by-point response cover letter.

The notated PDF is uploaded to this submission's record in Editorial Manager. If you cannot locate the file, contact Katie McDermott and she will send it by email – kmcdermott@greenjournal.org.

2. The Editors of Obstetrics & Gynecology are seeking to increase transparency around its peer-review process, in line with efforts to do so in international biomedical peer review publishing. If your article is accepted, we will be posting this revision letter as supplemental digital content to the published article online. Additionally, unless you choose to opt out, we will also be including your point-by-point response to the revision letter, as well as subsequent author queries. If you opt out of including your response, only the revision letter will be posted. Please reply to this letter with one of two responses:
    1. OPT-IN: Yes, please publish my response letter and subsequent email correspondence related to author queries.
    2. OPT-OUT: No, please do not publish my response letter and subsequent email correspondence related to author queries.

3. In order for an administrative database study to be considered for publication in Obstetrics & Gynecology, the database used must be shown to be reliable and validated. In your response, please tell us who entered the data and how the accuracy of the database was validated. This same information should be included in the Materials and Methods section of the manuscript.

4. Standard obstetric and gynecology data definitions have been developed through the reVITALize initiative, which was convened by the American College of Obstetricians and Gynecologists and the members of the Women's Health Registry Alliance. Obstetrics & Gynecology will be transitioning as much as possible to use of the reVITALize definitions, and we encourage authors to familiarize themselves with them. The obstetric data definitions are available at http://links.lww.com /AOG/A515, and the gynecology data definitions are available at http://links.lww.com/AOG/A935.

5. Because of space limitations, it is important that your revised manuscript adhere to the following length restrictions by manuscript type: Original Research reports should not exceed 22 typed, double-spaced pages (5,500 words). Stated page limits include all numbered pages in a manuscript (i.e., title page, précis, abstract, text, references, tables, boxes, figure legends, and appendixes).

Please limit your Introduction to 250 words and your Discussion to 750 words.

6. Specific rules govern the use of acknowledgments in the journal. Please edit your acknowledgments or provide more information in accordance with the following guidelines:

* All financial support of the study must be acknowledged.
* Any and all manuscript preparation assistance, including but not limited to topic development, data collection, analysis, writing, or editorial assistance, must be disclosed in the acknowledgments. Such acknowledgments must identify the entities that provided and paid for this assistance, whether directly or indirectly.
* All persons who contributed to the work reported in the manuscript, but not sufficiently to be authors, must be acknowledged. Written permission must be obtained from all individuals named in the acknowledgments, as readers may infer their endorsement of the data and conclusions. Please note that your signature on the journal's author agreement form verifies that permission has been obtained from all named persons.
* If all or part of the paper was presented at the Annual Clinical and Scientific Meeting of the American College of Obstetricians and Gynecologists or at any other organizational meeting, that presentation should be noted (include the exact dates and location of the meeting).

7. The most common deficiency in revised manuscripts involves the abstract. Be sure there are no inconsistencies between the Abstract and the manuscript, and that the Abstract has a clear conclusion statement based on the results found in the paper. Make sure that the abstract does not contain information that does not appear in the body text. If you submit a revision, please check the abstract carefully.

In addition, the abstract length should follow journal guidelines. The word limits for different article types are as follows: Original Research articles, 300 words. Please provide a word count.

8. Only standard abbreviations and acronyms are allowed. A selected list is available online at http://edmgr.ovid.com /ong/accounts/abbreviations.pdf. Abbreviations and acronyms cannot be used in the title or précis. Abbreviations and acronyms must be spelled out the first time they are used in the abstract and again in the body of the manuscript.

9. The journal does not use the virgule symbol (/) in sentences with words. Please rephrase your text to avoid using "and/or," or similar constructions throughout the text. You may retain this symbol if you are using it to express data or a measurement.

10. We discourage claims of first reports since they are often difficult to prove. How do you know this is the first report? If this is based on a systematic search of the literature, that search should be described in the text (search engine, search terms, date range of search, and languages encompassed by the search). If on the other hand, it is not based on a systematic search but only on your level of awareness, it is not a claim we permit.

11. Please review the journal's Table Checklist to make sure that your tables conform to journal style. The Table Checklist is available online here: http://edmgr.ovid.com/ong/accounts/table_checklist.pdf.

12. The American College of Obstetricians and Gynecologists' (College) documents are frequently updated. These documents may be withdrawn and replaced with newer, revised versions. If you cite College documents in your manuscript, be sure the reference you are citing is still current and available. If the reference you are citing has been updated (ie, replaced by a newer version), please ensure that the new version supports whatever statement you are making in your manuscript and then update your reference list accordingly. If the reference you are citing has been withdrawn with no clear replacement, please contact the editorial office for assistance (obgyn@greenjournal.org). In most

cases, if a College document has been withdrawn, it should not be referenced in your manuscript (exceptions could include manuscripts that address items of historical interest). All College documents (eg, Committee Opinions and Practice Bulletins) may be found via the Resources and Publications page at http://www.acog.org/Resources-And-Publications.

13. Figure 1: Please confirm that this is original to this manuscript.

When you submit your revision, art saved in a digital format should accompany it. If your figure was created in Microsoft Word, Microsoft Excel, or Microsoft PowerPoint formats, please submit your original source file. Image files should not be copied and pasted into Microsoft Word or Microsoft PowerPoint.

When you submit your revision, art saved in a digital format should accompany it. Please upload each figure as a separate file to Editorial Manager (do not embed the figure in your manuscript file).

If the figures were created using a statistical program (eg, STATA, SPSS, SAS), please submit PDF or EPS files generated directly from the statistical program.

Figures should be saved as high-resolution TIFF files. The minimum requirements for resolution are 300 dpi for color or black and white photographs, and 600 dpi for images containing a photograph with text labeling or thin lines.

Figures should be no smaller than the journal column size of 3 1/4 inches. Art that is low resolution, digitized, adapted from slides, or downloaded from the Internet may not reproduce. Refer to the journal printer's web site (http://cjs.cadmus.com/da/index.asp) for more direction on digital art preparation.

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If you choose to revise your manuscript, please submit your revision via Editorial Manager for Obstetrics & Gynecology at http://ong.editorialmanager.com. It is essential that your cover letter list point-by-point the changes made in response to each criticism. Also, please save and submit your manuscript in a word processing format such as Microsoft Word.

If you submit a revision, we will assume that it has been developed in consultation with your co-authors, that each author has given approval to the final form of the revision, and that the agreement form signed by each author and submitted with the initial version remains valid.

Again, your paper will be maintained in active status for 21 days from the date of this letter. If we have not heard from you by Sep 06, 2018, we will assume you wish to withdraw the manuscript from further consideration.

Sincerely,

Nancy C. Chescheir
Editor in Chief of Obstetrics & Gynecology

2017 IMPACT FACTOR: 4.982
2017 IMPACT FACTOR RANKING: 5th out of 82 ob/gyn journals

September 8, 2018

Attn: Editorial Board

        Obstetrics and Gynecology

Re: ONG-18-1367- Manuscript Revision 1

Dear Editorial Board,

Thank you for the opportunity to revise manuscript ONG-18-1367, "Terminal Complement

Activation in Preeclampsia (COPA), a Multicenter Study".

We appreciate the thoughtful comments from the Editor and Reviewers, and we have provided a

detailed reply in the following pages.   We included all modifications in our revised manuscript

using the Track Changes feature.   All authors have read and approved the revision of the

manuscript and we look forward to sharing this work with your readers.

Thank you for your time and consideration.

Sincerely,

Richard M. Burwick, MD, MPH
Cedars-Sinai Medical Center
8635 W. 3rd St., MOT, Suite 160W
Los Angeles, CA 90048
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Reply to Reviewers:

I. Reply to comments by editor (NCC)

*OPT-IN: Yes, please publish my response letter and subsequent email correspondence related to author queries.

*Word count: We made numerous edits to the introduction and discussion to meet the word counts (250 and 750, respectively).

1. The objective for the abstract should be a simple "to" statement without background.

We revised objective statement:

"To determine if C5b-9 levels in blood and urine are increased in preeclampsia with severe features."

2. You did not, I"m sure, design it at 6 centers. Was it completed at 6 centers? Performed at 6 centers?

We revised statement:
 "…performed at 6 centers…"

3. **This sentence is not clear.   Is it 2:1 cases to controls or 2:1 controls to cases? Perhaps you could write it something like "The Complement and Preeclampsia in the Americas (COPA) study is a prospective, multi-center case control study performed at 6 centers in Columbia from November 2015 to July 2016. Cases were women with preeclampsia with severe features while controls could be healthy, or have chronic hypertension, gestational hypertension, or preeclampsia without severe features. Controls were enrolled in a 2:1 ratio with cases. " Also, sentence starting on line 83 is not complete.**

We revised statement as recommended.

4. Line 81: Delete. THen you can say" Soluble C5b=9 levels were measured by enzyme linked immunosorbent assays (BD....) in urine and blood.

We revised text as recommended.

5. **Do not begin a sentence with a numeral. Either reorganize your sentence to not start with a number OR write out the number in words.**

We revised text to write out 352.

6. **Line 86-87: shouldn't you be only comparing cases and controls?**

We modified the abstract and results sections to first present the results of our primary aim (cases vs. controls) and then the secondary aim (cases vs. control sub-groups).

7. What is the range in values of the urine levels?

- Range of urine values provided (0-158.4 ng/ml)

8. **what do you mean by "Complement associated disease"? Your thesis is that complement is a key mediator in preeclampsia. When is preeclampsia NOT mediated by complement? isn't it urine C5b-9 only?**

- We meant to emphasize that in some cases of severe preeclampsia there is significant complement-associated disease. In other cases, complement involvement is less pronounced. However, we recognize the potential confusion and we removed the term "complement-associated disease" from the abstract and precis statement. In addition, we placed greater emphasis on urinary C5b-9.

9. please clarify what you mean by systemic inflammation....not usually what i see as a descriptor of the defining character of preeclampsia.

- Since this may not be a common descriptor of preeclampsia, we modified the introductory statement as follows:

"Preeclampsia, defined by hypertension with proteinuria or end-organ injury, impacts 2-4% of pregnancies. It may arise from placental inflammation or ischemia, with systemic activation of leukocytes and endothelial cells."

10. **Can you clearly state your primary and any secondary outcomes? Did you have a hypothesis?**

- In our submission we provided hypothesis in lines 149-152. However, we now recognize that the statement may have been too vague. Therefore, we updated our aims and hypothesis as follows:

Abstract- "The primary outcome was C5b-9 levels in cases versus controls, and the secondary outcome was C5b-9 levels in cases versus individual control sub-groups."

Introduction- "Thus, our primary aim is to compare blood and urine levels of C5b-9 in preeclampsia with severe features, to controls with either healthy pregnancy, chronic

hypertension, gestational hypertension, or preeclampsia without severe features. We hypothesize that C5b-9 levels are increased in preeclampsia with severe features."

Methods: The primary outcome was C5b-9 levels in cases versus controls, and the secondary outcome was C5b-9 levels in cases versus individual control sub-groups.

11**. delete subheadings**

- subheadings removed

12. **was this a convenience sample or were patients enrolled sequentially (ie, all eligible patients approached)**

- eligible patients were enrolled sequentially, during periods in which research coordinators were available. We updated this wording in the Methods section.

13. same blank for both urine and plasma?

- In the methods section we provided the lower limit of detection for urine because many urine C5b-9 values were below this level. However, plasma C5b-9 levels never fell below the limit of detection. We added the following text for clarity:

"Plasma C5b-9 values were above the limit of detection in all subjects"

14. **please describe these the same way: 50% difference or 2 fold difference. Why did you use different cut off**

- The standard deviation of C5b-9 levels in plasma is much smaller than the deviation of C5b-9 levels in urine. Thus, we would have required a larger sample size to detect a 50% difference in urine values (rather than 200% difference). To avoid confusion, we have modified the text:

"Based on prior findings[12], we determined that 100 cases and 200 controls (50 per group) were required to demonstrate a 50% difference in plasma C5b-9 levels and a 200% difference in urinary C5b-9 levels between cases and controls, with alpha =0.05 and power =0.80. We anticipated that a smaller difference in plasma C5b-9 levels could be detected between groups, due to lower standard deviation of C5b-9 levels in plasma as compared to urine."

15. **did you give them target #s for the different gestational ages?**

Yes, and we modified text in the Methods section as follows:

"Cases were women with preeclampsia with severe features while controls could be healthy pregnancies, or those with chronic hypertension, gestational hypertension, or preeclampsia without severe features. Controls were enrolled in a 2:1 ratio with cases. COPA targeted enrollment of 100 cases of preeclampsia with severe features, including 50 cases <34 weeks and 50 cases ≥34 weeks. Individual sites were given a target of 50 subjects to enroll in the study, and

coordinators were instructed to enroll 2 controls for every case (with controls matching the gestational age category of the case, <34 or ≥34 weeks). Diagnoses were made in accordance with the 2013 American College of Obstetricians and Gynecologists' criteria for hypertension in pregnancy (Appendix 1).[3] Teleconferences were held monthly during the study period to gauge study progress and enrollment numbers at each site."

16. **Line 193: are presented**
- text modified

17. **how did you define optimal? Best sensitivity or specificity?**

- We classified the optimal cut-point as the ROC value that correctly classified the most subjects with severe preeclampsia. We added this to the Methods section describing the ROC analysis.

18. **isn't this almost by definition since these are the criteria for severe features?**

- We understand this point, but we'd like to show that complement levels are increased in association with markers of end-organ injury other than proteinuria. We chose severe laboratory features because they are more objective measures of end-organ injury, as compared to symptoms (headache, abdominal pain). It is worth noting that only 40% of subjects with severe preeclampsia in our study had pronounced complement activation (e.g., urine c5b9 >22ng/ml). Thus, the laboratory data is necessary to show that women with severe preeclampsia and pronounced complement activation are more likely to have end-organ injury than those women with severe preeclampsia but a lower degree of complement activation.

- We modified the discussion to clarify this point:

"The kidney is most vulnerable to complement activation, likely due to decreased expression of complement regulators compared to other end-organs such as the brain.[37] However, end-organ effects are not limited to the kidney. Subjects with marked urinary excretion of C5b-9 were also more likely to have hemolysis and thrombocytopenia."

19. **Line 262- This is called a primacy claim (your paper is the first or biggest) and must either be deleted or supported by providing the search terms used, dates, and data bases searched (Medline, Ovid, Pubmed, Google Scholar, etc) in order to substantiate your claim.**

- Primary claim removed and text modified

20. **Line 275- The Journal style does not include the use of the virgule (/) except in numeric expressions. Please edit here and in all instances.**

- Sentence modified to remove use of the virgule (/).

21. **Line 319 - also a primacy claim--see above note**

- Primary claim removed and text modified

22. **Line 324 - all preeclampsia or just severe preeclampsia?**

- Primarily severe forms of preeclampsia; text modified accordingly.

Reviewer #1.

1. This cross-sectional multicentre case control study stratified for gestational age examined plasma and urinary levels of complement protein C5b-9 in women with preeclampsia, gestational hypertension and essential hypertension compared to normotensive pregnant women. It provides further interesting data regarding the state of complement activation in various hypertensive disorders of pregnancy.

- thank you for the positive comment

2. **The article would be improved by presenting the multivariate analysis to support the premise of absence of association between urinary C5b-9 levels and proteinuria as this finding is inconsistent with previous research findings (Morita et al, 2000, J Am Soc Nephrol). In addition it would be of great interest to readers to know which variables contributed most to the weakening of the association between the complement levels and odds of having preeclampsia with severe features after multivariate analysis.**

- We added Table 2 to show the data for univariable analysis (urine C5b-9 and severe preeclampsia), followed by multivariable regression analysis with stepwise adjustment for maternal factors, urine protein and urine creatinine. This will help the reader understand that urine protein is the primary variable that attenuates the association between C5b-9 levels and severe preeclampsia. However, as we have noted in the text, the association between urinary C5b-9 and severe preeclampsia remains significant, independent of proteinuria (OR 10.0, 95% CI 3.5-28.8, p<0.001).

While we did not state that there is a lack of association between urinary C5b-9 levels and proteinuria, we have added the following line to avoid confusion:

"Adjustment for urine protein led to the greatest attenuation in the odds ratio, due to the correlation between urine protein and urine C5b-9 levels (r=0.57, p<0.001)."

3. **The authors' comments in line 299 need to be specified as relating to urinary levels of C5b-9**

- We have modified the text to state "…urinary levels of C5b-9…"

4. And a speculation offered as to how the lack of difference in plasma levels of C5b-9 in all groups of hypertensive patients (except early preeclampsia) fits into their conclusions regarding the role of complement activation in the pathogenesis of preeclampsia

We added text in the discussion to address this point:

"We find that increased urinary excretion of terminal complement effector C5b-9 differentiates preeclampsia with severe features from other hypertensive disorders of pregnancy. This may reflect more profound complement activation with renal involvement. Plasma C5b-9 levels are uniformly increased in hypertensive disorders of pregnancy, possibly due to endothelial dysfunction and systemic inflammation, common to these disorders. "

5. In addition the possible reasons for the lack of difference in urinary levels of C5b-9 between women with early and late preeclampsia should merit a comment.

- We did not specifically comment on this comparison, so we added it to the results section:

"There was no difference in urine C5b-9 levels between subjects with early and late-onset preeclampsia."

- In the discussion, we added a comment.

"Moreover, urinary excretion of C5b-9 occurs in both early and late-onset preeclampsia, suggesting that terminal complement activation is a key feature of disease regardless of gestational age."

6. This is a substantial contribution to the literature regarding the state of complement activation in normal and pathologic pregnancy.

- thank you for the positive comment

Reviewer #2

This is a research report of complement levels in women in with hypertension in pregnancy. the authors sought to measure complement in blood and urine in women who met diagnostic criteria of the full spectrum of hypertension in pregnancy. A major weakness is that this is a focused pathophysiology paper that may not be relevant to everyday practice.

- We recognize that complement proteins may be an obscure topic for many clinicians, but we tried to emphasize the translational aspect of our study by describing the future potential for terminal complement blockade in preeclampsia. Moreover, we described a published case in

which terminal complement blockade was effective in treating severe preeclampsia / HELLP syndrome and prolonged pregnancy 17 days. We hope that readers will find interest in this possibility.

- We added the following line to acknowledge that plasma C5b-9 levels can be measured in clinical setting (but we did not detect a discriminatory level), while urinary C5b-9 is not available at this time:

"Urinary measurement of C5b-9 does not have immediate clinical applicability because it is not validated for use in patient samples. While plasma C5b-9 levels can be measured from patient samples, we did not detect a discriminatory level for clinical use."

1. **Abstract / Methods: Please include a primary outcome measure in the methods and the measure of effect the project was aiming to identify.**

- We added a primary and secondary outcome in the abstract and methods as follows:

"The primary outcome was C5b-9 levels in cases versus controls, and the secondary outcome was C5b-9 levels in cases versus individual control sub-groups."

The measure of effect is listed in the Methods section, but we made a slight modification:

"Based on prior findings[12], we determined that 100 cases and 200 controls (50 per sub-group) were required to demonstrate a 50% difference in plasma C5b-9 levels and a 200% difference in urinary C5b-9 levels between cases and controls, with alpha =0.05 and power =0.80. We anticipated that a smaller difference in plasma C5b-9 levels could be detected between groups, due to lower standard deviation of C5b-9 levels in plasma as compared to urine."

2. **INTRODUCTION: Line 131 - the hypothesis as written in somewhat vague. Please include specific numbers/levels or differences that the authors sought to find in this lab based study.**

We modified the hypothesis statement as follows:

"We hypothesize that C5b-9 levels are increased in preeclampsia with severe features."

Similar to prior question regarding anticipated lab differences:
"Based on prior findings[12], we determined that 100 cases and 200 controls (50 per sub-group) were required to demonstrate a 50% difference in plasma C5b-9 levels and a 200% difference in urinary C5b-9 levels between cases and controls, with alpha =0.05 and power =0.80. We anticipated that a smaller difference in plasma C5b-9 levels could be detected between groups, due to lower standard deviation of C5b-9 levels in plasma as compared to urine."

3. **Materials and Methods. An overall statement of the type of study should be made at the beginning of the Materials and Methods section - cross-sectional? Retrospective or prospective cohort?**

We moved this statement to the beginning of the methods section:

"The Complement and Preeclampsia in the Americas (COPA) study is a prospective, multi-center case-control study performed at 6 centers in Colombia from November 2015 to July 2016."

4. **The population and setting needs to be more clearly stated. Clearly state the eligibility criteria for entry into the study**

We modified the text as follows:

"Eligible subjects were enrolled sequentially by trained research coordinators during available work hours. Cases were women with preeclampsia with severe features while controls could be healthy pregnancies, or those with chronic hypertension, gestational hypertension, or preeclampsia without severe features. Controls were enrolled in a 2:1 ratio with cases. COPA targeted enrollment of 100 cases of preeclampsia with severe features, including 50 cases <34 weeks and 50 cases ≥34 weeks. Individual sites were given a target of 50 subjects to enroll in the study, and coordinators were instructed to enroll 2 controls for every case (with controls matching the gestational age category of the case, <34 or ≥34 weeks). Diagnoses were made in accordance with the 2013 American College of Obstetricians and Gynecologists' criteria for hypertension in pregnancy (Appendix 1).[3] Teleconferences were held monthly during the study period to gauge study progress and enrollment numbers at each site.

Subjects were enrolled from outpatient clinics, labor and delivery floors, antepartum units, and triage or emergency wards. Clinical diagnoses were confirmed within the first 24 hours after enrollment once blood pressure, laboratory values and symptoms were clarified….
…Exclusions were: gestational age <24 weeks, uncertain dates, multifetal gestation (≥2), major chromosomal abnormality, fetal demise at entry, pre-existing diabetes mellitus or insulin-dependent gestational diabetes mellitus, chronic kidney disease, systemic lupus erythematosus, immunodeficiency, untreated bacterial or viral infection (including suspected Zika virus), active use of heparin, eculizumab or immunosuppressive agents, or inability to sign informed consent."

5. **Please clearly state the primary outcome of the study - as the way the paper is currently written there are mulitple complement levels that were of interest (Lines 182)**

We added the following statements:
Abstract- "The primary outcome was C5b-9 levels in cases versus controls, and the secondary outcome was C5b-9 levels in cases versus individual control sub-groups"

Introduction- "Thus, our primary aim is to compare blood and urine levels of C5b-9 in preeclampsia with severe features, to controls with either healthy pregnancy, chronic

hypertension, gestational hypertension, or preeclampsia without severe features. We hypothesize that C5b-9 levels are increased in preeclampsia with severe features."

6.   If the intended audience of the paper is clinicians in practice the authors will need to provide more complete information on complement levels and their relationship to preeclampsia.

- We recognize that complement proteins may be an obscure topic for many clinicians, but we tried to emphasize the translational aspect of our study by describing the future potential for terminal complement blockade in preeclampsia.   Moreover, we described a published case in which terminal complement blockade was effective in treating severe preeclampsia / HELLP syndrome and prolonged pregnancy 17 days. We hope that readers find interest in this possibility, while understanding that complement biomarker C5b-9 is available for measurement in plasma but not urine.

- We made many edits to the introduction and discussion, which we hope add clarity.   We have also maximized the word count for both sections.

RESULTS:
7.   A participant flow chart should be included - this will provide readers with an understanding of the final analysis population, and this is also a crucial component of reporting according to STROBE.

- Unfortunately, we do not have the number of subjects who were approached/screened but not enrolled.   Thus, we listed the number of subjects who were consented and enrolled (n=352) at the beginning of the Results section. However, we added the list of subject enrollment by study site for better clarity.   Since blood and urine samples were collected immediately after enrollment, C5b-9 levels were available on all subjects.   We acknowledge that STROBE says to consider use of a flow diagram, but this was less applicable in our circumstance.

8.   Table 1 - by enrollment group or by comparison group? Or by type of hypertension.

- change made to "enrollment group"

9.   Appendices 1 and 2 seem either unnecessary or they should be more informative and included in tables.

- We removed Appendix 1 since the diagnostic criteria for hypertensive disorders of pregnancy in our study was identical to the 2013 ACOG criteria. Thus, we simply provided the reference to the 2013 ACOG publication.

- We removed Appendix 2 and moved relevant data to the results section

"We enrolled 352 subjects in COPA, with the following distribution by study site: Hospital Universitario San Vicente Fundación (n=85); Clínica Reina Sofía - Sanitas (n=60); Clínica Universitaria Bolivariana (n=58); E.S.E. Clinica de Maternidad Rafael Calvo (n=53); Hospital Universitario San Ignacio (n=49) and; Hospital General de Medellín (n=47)."

10.   Please include a statement of how many women were approached/screened/enrolled in the opening of the results.

Unfortunately, we do not have the number of subjects who were approached/screened but not enrolled. Thus, we listed the number of subjects who were consented and enrolled (n=352) at the beginning of the Results section. Since blood and urine was collected immediately after enrollment, C5b-9 levels were available on all enrolled subjects.

11.   The purpose of the tables to describe the results are unclear - the tables need more complete titles.

- Table 1 provides baseline characteristics of study subjects. We are unsure how to make the title more complete, but we did modify the title of Table 1 to "…comparison group…" as previously suggested. If the reviewer has a specific suggestion for making the title more complete we are agreeable to modify it.

DISCUSSION:
12.   Please discuss how will this paper fit in with existing literature? If this paper should be of interest to general obgyns in practice then please address this in the discussion.

As noted above, we tried to emphasize the translational aspect of our study by describing the future potential for terminal complement blockade in preeclampsia. Moreover, we described a published case in which terminal complement blockade was effective in treating severe preeclampsia / HELLP syndrome and prolonged pregnancy 17 days. We hope that readers find interest in this possibility, while understanding that complement biomarker C5b-9 is available for measurement in plasma but not urine.

To address how this paper fits in with existing literature we modified discussion in numerous places (paragraphs 3-5) and added a few references.

Reviewer #3:

This study undertook a novel approach to distinguishing between preeclampsia with severe features and controls that included normotensive patients and patients with chronic

hypertension, gestational hypertension, and preeclampsia without severe features. Based on the hypothesis that preeclampsia is a complement-mediated disorder, the authors examined terminal complement effector C5b-9 (membrane attack complex) which they believed to be a key mediator in its development. They found a urine level of C5b-9 that clearly distinguished preeclampsia with severe features from other hypertensive complications of pregnancy and conclude that it may prove to be a useful marker for future studies of disease evolution as well as for developing strategies for prospective disease intervention.

1. This study that is well designed, conducted, and analyzed with a clearly stated hypothesis that is relevant to the condition under examination.

- Thank you for the positive comment.

2. My only comment would be that the discussion section is too long and repeats data in the results section that have already been presented.

- We shortened discussion to 750 words and removed repetitive data from results section.

3. Their discussion of complement activation in the disease process has been covered in the introduction as well.

We made many edits/revisions to the introduction and discussion to improve clarity and minimize overlap

Reviewer #4.

Complement factor C5 split products increases indicating excessive activation have been reported in relation to preeclampsia for about 30 years. It has also been suggested that the association between preeclampsia and complement activation is more pronounced for early-onset preeclampsia, which also tends to be a more severe form. The study reported by Burwick et al. was designed to test the hypothesis that terminal complement activation, as measured by C5b-9 in maternal blood and urine is increased specifically in preeclampsia with severe features. The hypothesis was not verified when levels of C5b-9 were compared in plasma of cases vs controls, however, in the urine, levels were most specifically increased in early-onset preeclampsia with severe features. The findings are consistent with previous reports. The difference between plasma and urine determinations is considered by the authors to be the consequence of a more profound complement activation with renal involvement in more severe cases.

1. **On page 8, as part of the background, it is stated that activation of C5 exceeds regulatory capacity in preeclampsia. It may be warranted to include that other investigators have not found the same overwhelmed regulatory capacity beyond C3a. See Banadakoppa M, Vidaeff AC, Yallampally U, et al. Disease Markers 2015, and also your reference #10 (Buurma et al). In the latter report, placental mRNA expression of the complement**

regulatory proteins was measured in preeclampsia close to delivery, noting in fact a significant upregulation of CD55 and CD59 mRNA expression.

- The study by Banadakoppa et al. describes measurement of C3a, C4a, C5a and Bb in the amniotic fluid during second trimester of pregnancy in women who eventually develop preeclampsia.   Banadakoppa et al. report increased levels of C3a and Bb in amniotic fluid of women who develop preeclampsia, which is consistent with our discussion on this topic. We reported that C3a and Bb are increased in blood in women who eventually develop preeclampsia, thus we added the Banadakoppa reference to that area of discussion.   Their finding that C5a is not increased in 2nd trimester is consistent with our statement that upstream factors rather than downstream factors are elevated in early pregnancy.

Text modified as follows:
"While terminal complement effector C5b-9 is associated with active clinical disease, upstream complement pathways are likely strained from early pregnancy.   In women with high blood levels of upstream complement split products C3a or Bb, preeclampsia is 3-4 times more likely,[19,35] and 8-10 times more likely if obesity is present.[27] Levels of C3a and Bb are also increased in amniotic among women who eventually develop preeclampsia.[36]"

- The study by Buurma et al. reports increased placental mRNA expression of CD55 and CD59 in preeclampsia. This is consistent with increased placental expression of complement regulators to combat increased complement activation at the placental interface. We believe that upregulation of CD59 at the placental interface is not inconsistent with increased C5 activation as measured by soluble C5b-9 in plasma and urine.   We added text to this regard in the discussion:

"The placenta may upregulate expression of CD59, a membrane bound inhibitor of C5b-9, to combat terminal complement activation in preeclampsia.[10] Yet, we have shown here and previously, that maternal C5b-9 levels are increased in preeclampsia with severe features.[11] "

2. **Among the limitations of the study, briefly discussed on page 16, I would consider:**
-   Limitation to only one split product determination, with no evaluation of the alternative pathway initiation complex, considered by some to be stronger related to preeclampsia (your references #19 and #35 and also Gilbert JS et al. Hypertension 2012)

- We described in other parts of intro and discussion that our emphasis was the terminal complement pathway (which is shared by alternative and classical pathways). We did not evaluate initiation complexes, or upstream split products, of either the classical or alternative pathway.   We chose to focus on their shared terminal effector C5b-9, which is the primary target for complement blockade in clinical use (e.g., aHUS, PNH).

To address the reviewer's concern, we added the following line in the Limitations section of the discussion:

"We also did not measure other upstream complement split products of classical or alternative complement pathways, but instead focused solely on their shared terminal effector."

3. Limitations: C5b-9 measured at a single point after the clinical diagnosis. Complement activation detected in the clinical stage may be just the consequence of the systemic and local inflammatory reactions that characterize the final clinical stage in the development of preeclampsia, not allowing causal inferences.

We modified text as follows:
"Due to the observational design, we are unable to draw definitive conclusions regarding causal relationships. For example, it remains unknown whether C5b-9 is present in urine before the onset of preeclampsia or if levels rise or fall with progression of disease (e.g., preeclampsia to HELLP syndrome)."

4. Limitations: Also inherent to a case-control approach is the potential for bias and the inability to draw definitive conclusions regarding causal relationships.

- modified text:
"We are unable to draw definitive conclusions regarding causal relationships"

5. Limitations: I would also temper a little bit the discussion of the ROC analysis because a result of 0.74 is only fair and cannot be considered a good discriminator.

We utilized ROC analysis to show that urinary levels of C5b-9 are useful to differentiate preeclampsia with severe features from other hypertensive disorders.   The results were significant from a statistical point of view, and we reported them as such. However, to avoid increased emphasis on the ROC analysis, which was not our intention, we moved the ROC figure to Appendix 2.   In place of the figure in the main manuscript, we provided a multivariable regression table that was desired by multiple reviewers.

6. Other observations: Line 159: serum creatinine range of 0.5 to 1.1 mg/dl is not the normal range for pregnancy.

- thank you, we agree with this observation.   The reference ranges listed in the Methods section are those provided by the central laboratories at study sites.   The central laboratories in our study did not provide pregnancy specific ranges. However, in our analysis, we evaluated urinary C5b-9 levels in those with serum creatinine ≥1.0 mg/dl, as we felt this to be more reflective of abnormal kidney function in pregnancy

7. In contrast to many other "dead-end" associations studied in relation to the development of preeclampsia, there is some indication that therapeutic manipulations of the complement system may be feasible in pregnancy and the authors appropriately discuss

this in the last paragraph of the manuscript.

- thank you for the positive comment

STATISTICAL EDITOR COMMENTS:

1. **Table 1: Unless there were no missing values, should cite entries as n(%), not just as %.**

- We updated the data in Table 1 as suggested since there were a few missing values.

2. **Table 1: Were the distributions normal for continuous variables? If not, then should cite as median (range or IQR) and test non-parametrically. It appears that urine protein/creatinine has skewed distribution, for example.**

- We apologize for the oversight. The urine protein/creatinine data was non-normal and thus we have revised the data in Table 1 to present it as median (IQR). Testing was performed by non-parametric equality of medians test. Other continuous variables were normal in distribution, with testing by *sktest* command in Stata. The *sktest* command presents a test for normality based on skewness and another based on kurtosis and then combines the two tests into an overall test statistic.

- We modified the methods section as follows:
"Differences between study groups were assessed by Chi-square test for dichotomous data, t-test or analysis of variance for normal continuous data, and non-parametric equality of medians test for non-normal continuous data. Data normality was determined based on tests of skewness and kurtosis, with non-normal data displayed as medians (interquartile range, IQR)."

3. **Lines 176-178: For the purposes of model analysis, what value was entered into the model for the undetectable levels, since zero is an undefined value for logistic models?**

- For logistic models, we created a dichotomous variable for urinary C5b-9 levels ($\geq$22 ng/ml vs. <22 ng/ml). Thus, undetectable urinary C5b-9 levels were not an issue in regression analyses.

4. **Lines 195-197 and Figures 2a, 2b, 3a, 3b: The data appear highly skewed and the authors rightly used non-parametric stat testing. However, if multiple groups were compared, should have used Kruskal-Wallis, rather than Wilcoxon. Need to clarify was the p < .001 specific pairwise testing with each of the other groups vs "all other groups". Should provide (could be on-line supplement) the data in Table format corresponding to the graphs.**

We corrected the methods to note that a non-parametric equality of medians test was used rather than Wilcoxon ranksum test. In Stata, the "median" command performs a nonparametric K-sample test on the equality of medians. It tests the null hypothesis that the K samples were drawn from populations with the same median. For two samples, the chi-squared test statistic is computed both with and without a continuity correction.

- We modified the results section to make it clear that we first compared cases to "all other groups" and then compared cases to individual groups.

- We added Appendix to display figure data (2a,2b,3a,3b) in Table format, as requested.
- This is added as Appendix 1 in revised manuscript

5. **lines 248-256: Need to provide a Table for the univariate and multivariable analyses of OR and aORs and justify the number of adjustors used relative to the counts of subsets, such as C5b-9 ≥ 22 ng/ml vs 7 adjustors. (count = 42/104 vs 7 variables is an unfavorable ratio and likely an over fitted model.)**

- Thank you for the suggestion, we have added Table 2 with the regression data. For clarity we showed univariable followed by stepwise multivariable regression.

- Regarding number of adjustors, there appears to be some confusion. The cases in our regression model were women with preeclampsia with severe features, of which there were 104 cases. We evaluated 8 total variables (predictors). Thus, the ratio of cases to predictors in our final regression model was 13:1, which is in line with standard recommendations (>10:1). Others have recommended that N (cases) be > predictors +50, which we met easily. To add clarity, we provided a stepwise regression to show the impact of clinical factors followed by urine protein and urine creatinine.

6. **Fig 4: Need to provide specificity, sensitivity (each with CIs). The PPV is not relevant and not replicable, since this was a highly specialized sample of women with various states of HTN, as well as healthy controls, so cannot be generalized to other groups with other rates of HTN prevalence.**

- There appears to be some confusion since we did not estimate PPV in our manuscript.   We agree that PPV is significantly influenced by disease prevalence and thus we did not use it.   We chose to report the positive likelihood ratio (sensitivity / 1-specificity), which we feel is a clinically useful way to gauge the value of a diagnostic test.

- We have provided specificity and sensitivity with CI's, and we made edits in the text to improve clarity. Specificity 96.8% (93.5-98.4%); Sensitivity 40.4% (31.0-50.5%)

hi Randi,

I am attaching revision with Track changes, to address the concerns noted.   I am also attaching a slight modification to the table in appendix, if that is ok.

thanks!

From: Randi Zung <RZung@greenjournal.org>
Sent: Monday, September 17, 2018 6:42:10 AM
To: Burwick, Richard M.D.
Subject: [External] Your Revised Manuscript 18-1367R1

Dear Dr. Burwick:

Your revised manuscript is being reviewed by the Editors. Before a final decision can be made, we need you to address the following queries. Please make the requested changes to the latest version of your manuscript that is attached to this email. **Please track your changes and leave the ones made by the Editorial Office.** Please also note your responses to the author queries in your email message back to me.

1. General: The Editor has made edits to the manuscript using track changes. Please review them to make sure they are correct.

2. Please submit a completed STROBE checklist.

3. Please provide a completed author agreement form for Dr. Bernal using the latest version of our author agreement form, which can be found at http://edmgr.ovid.com/ong/accounts/agreementform.pdf. Note that **both** the "Authorship" and "Disclosure of Potential Conflicts of Interest" sections need to be completed, along with providing a signature. Please read the form carefully.

4. Line 81 (Abstract and Manuscript): It's redundant to cite AUC, specificity, likelihood ratios and aORs (line 107-109). Maybe they should simply cite the AUC and aORs since that is what they said they would use to assess outcomes in Methods.

I do feel, however, that the conclusion should be more balanced and reflect the original objectives. That is, for the primary comparisons (cases vs controls) urinary concentrations were significantly different, but plasma concentrations were not. Then they should cite the secondary outcomes that were significant (severe pre-eclampsia vs hypertensive controls). They seem to be emphasizing the most significant differences, even those were among the secondary outcomes (lines 91-92).

5. Line 82 (Abstract and Manuscript): Throughout the entire submission, please change "level/levels" to "concentration/concentrations." Please make sure this change is applied everywhere.

6. Abstract-Conclusion: Please note the edits made to this section. Please edit your Discussion so that this sentence also appears there.

To facilitate the review process, we would appreciate receiving a response within 48 hours.

Best,
Randi Zung

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From:
To: Stephanie Casway
Subject: Re: [External] O&G Figure Revision: 18-1367
Date: Wednesday, September 12, 2018 4:29:44 PM

hi Stephanie,

these look great, just one thing on Figures 2 and 3.

On the Y-axis should be C5b-9 (not C5b-g)

thanks!

From: Stephanie Casway <SCasway@greenjournal.org>
Sent: Wednesday, September 12, 2018 12:32:19 PM
To: Burwick, Richard M.D.
Subject: [External] O&G Figure Revision: 18-1367

Good Afternoon Dr. Burwick,

Your figures and legend have been edited, and PDFs of the figures and legend are attached for your review. Please review the figures CAREFULLY for any mistakes.

PLEASE NOTE: Any changes to the figures must be made now. Changes at later stages are expensive and time-consuming and may result in the delay of your article's publication.

To avoid a delay, I would be grateful to receive a reply no later than Friday, 9/14. Thank you for your help.

Best wishes,

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