PEER REVIEW HISTORY

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ARTICLE DETAILS

<table>
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<tr>
<th>TITLE (PROVISIONAL)</th>
<th>Feasibility of nephrinuria as a screening tool for the risk of pre-eclampsia: prospective observational study</th>
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<td>AUTHORS</td>
<td>Zhai, Tianyue; Furuta, Itsuko; Akaishi, Rina; Kawabata, Kosuke; Chiba, Kentaro; Umazume, Takeshi; Ishikawa, Satoshi; Yamada, Takahiro; Morikawa, Mamoru; Minakami, Hisanori</td>
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VERSION 1 - REVIEW

<table>
<thead>
<tr>
<th>REVIEWER</th>
<th>Shigeki Matsubara</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Professor, Obstetrics and Gynecology, Jichi Medical University, Japan</td>
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<tr>
<td></td>
<td>I worked with the last author professor Hisanori Minakami until 2001 at Jichi Medical University. I wrote some papers with him. Except for this, I have no competing interests.</td>
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<td>REVIEW RETURNED</td>
<td>28-Jan-2016</td>
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</table>

GENERAL COMMENTS

The authors showed, 1) urine nephrin-creatinine ratio (NCR) was well correlated with urine protein-creatinine ratio (PCR) in pre-eclamptic women (PE), whereas in non-PE women, NCR did not change, even though PCR increased according to gestation, and 2) PCR predicted later occurrence of PE. Thus, they concluded that NCR may be a useful biochemical marker of prediction of PE. The manuscript is well structured and well written. Data demonstrated here are comprehensive and detailed. Data interpretation is right: clinical application is written modestly and cautiously, which is also right.

From a viewpoint of electron-microscopist (I studied on electronmicroscopy for 19 years), renal morphological and functional changes are expected to occur far before the clinical manifestation of PE: especially podocytes may be vulnerable to various pathological stimuli, and, thus, present data is also pathophysiological explainable, providing insight into PE pathophysiology. For example, podocyte damage may and may not occur in PE and non-PE, respectively, regardless of whether an individual woman showed proteinuria: quite an interesting finding.

This study has two limitations (weakness): 1) this was “partial” longitudinal study, and 2) PE incidence rate is too high. However, the authors clearly stated this in the manuscript and the authors should not be blamed for this weakness.
This paper is addressed at an interesting issue; however, the goal is too ambitious: talking about screening tools is not possible with less than 100 cases, a biased selection, and a consistent overlap of data between "normal" (although one may doubt of such a normality) and PE pregnancies.

Furthermore, the authors should have at least excluded kidney disease, collagen disease, diabetes and obesity, all known to increase the risk for PE: the definition of normal pregnancies on the basis of their outcome is, in my opinion, not acceptable.

the data should be related also to other biomarkers, such as sFlt-1 or PlGF, of growing interest in PE management. Data on placental flows could also be of interest.

I would suggest to re-write the study as a novel approach, underlining much better the limitations and defining in detail the population.

Other points
1. the paper is addressed at a new issue; therefore, the aim should be more balanced: it's a first pilot feasibility study and the goal of assessing nephrinuria as a screening tool is way too far.
2. PCR is a very poor marker of proteinuria; if a new marker of PE has to be assessed, the gold standards (still 24 hour urine collection) should have been used.
3. PE in 14 out of 89 women is an exceedingly high incidence. If these women were at high risk this should be stated. If not there has to be some underlying bias (the worldwide prevalence of PE is around 5%); the authors note this in the last part of their study. however, this is not enough, and they should dissect their population and analyse it better
4. partly longitudinal study doesn't seem a sound definition
PE developed in 14 of the 89 women. The NCR increased with increasing PCR in 14 PE women but not in those with normal pregnancies. Relative risk of later development of PE among asymptomatic 2nd and 3rd trimester women was significantly increased in those with NCR (ng/mg) > 122 (95th percentile value for 75 women with normal pregnancies). The authors conclude that nephrinuria may efficiently differentiate women at higher risk of PE.

I have the following comments:
1. The authors report that 16% women developed PE, which is much higher than any number reported in the literature. Any explanation for that? Should this be mentioned in limitations?
2. Were these women compared with respect to NCR in their 1st pregnancy trimesters? Even if negative, these data would be important to understand the trajectory of NCR.
3. I would not use term "normal" pregnancies, as the "normality" was confirmed based on the lack of hypertension and proteinuria. Still, these women may have some other medical problems and/or obstetric complications. I would prefer the term "normotensive."
4. Similarly, I would not use the term "counterpart characteristics" (abstract) but would stick to preeclamptic pregnancies.
5. The topic should be put in a larger context of podocyte markers in the urine and renal injury in non-pregnant states.

REVIEWER
Huaizhong Hu
West China 2nd University Hospital
Sichuan University
Chengdu, Sichuan
China

REVIEW RETURNED
06-Mar-2016

GENERAL COMMENTS
1. Page 7, second last sentence, "judged" should be changed to "diagnosed".

2. The sample collection is interesting, especially "73 were collected at latent phase with neither SPIP nor hypertension.", as if it was known these women would develop PE. Please clarify why more samples were collected from women that developed PE during the latent phase.

3. Table 1, please use symbols to denote what they are between the brackets.

4. Page 10, last sentence before discussion, differentiated women with higher and lower risk of PE. Please change to "differentiated........ from........".

5. In multiple places in the manuscript, "increasing gestation" is used. For better clarity, I suggest this to be changed to increasing gestation time or increasing gestation weeks.

6. In discussion, this sentence needs to rephrased for better clarity: All of these results suggested that a significant increase in nephrinuria was unlikely to occur in the physiological range of proteinuria in pregnancy, but nephrinuria occurred in parallel with proteinuria beyond a certain degree of proteinuria in PE pregnancy, i.e., a significant increase in nephrinuria preceded pathological proteinuria in PE pregnancy.
7. In the last paragraph, it quoted work by Wang et al.[12] reporting nephrinuria in pregnancy for the first time in 2012, mean NCR (ng/mg) was 107 vs. 1894 and mean nephrin concentration was 86 vs. 1226 ng/mL for women with normal pregnancy vs. women with PE pregnancy having mean proteinuria of 2579 mg/day, respectively. Please note the units are not the same, ng/mg and mg/day. This makes it difficult to compare. Please check the original article for the accuracy.

VERSION 1 – AUTHOR RESPONSE

Reviewer: 1
Reviewer Name: Shigeki Matsubara
Institution and Country: Profesor, Obstetrics and Gynecology, Jichi Medical University, Japan
Competing Interests: I worked with the last author professor Hisanori Minakami until 2001 at Jichi Medical University. I wrote some papers with him. Except for this, I have no competing interests.

The authors showed, 1) urine nephrin-creatinine ratio (NCR) was well correlated with urine protein-creatinine ratio (PCR) in pre-eclamptic women (PE), whereas in non-PE women, NCR did not change, even though PCR increased according to gestation, and 2) PCR predicted later occurrence of PE. Thus, they concluded that NCR may be a useful biochemical marker of prediction of PE.

The manuscript is well structured and well written. Data demonstrated here are comprehensive and detailed. Data interpretation is right: clinical application is written modestly and cautiously, which is also right.

From a viewpoint of electron-microscopist (I studied on electronmicroscopy for 19 years), renal morphological and functional changes are expected to occur far before the clinical manifestation of PE: especially podocytes may be vulnerable to various pathological stimuli, and, thus, present data is also pathophysiologically explainable, providing insight into PE pathophysiology. For example, podocyte damage may and may not occur in PE and non-PE, respectively, regardless of whether an individual woman showed proteinuria: quite an interesting finding.

This study has two limitations (weakness): 1) this was “partial” longitudinal study, and 2) PE incidence rate is too high. However, the authors clearly stated this in the manuscript and the authors should not be blamed for this weakness.

Reviewer: 2
Reviewer Name: Giorgina Barbara Piccoli
Institution and Country: University of Torino, Italy; CH du Mans, Le Mans France.
Competing Interests: none

This paper is addressed at an interesting issue; however, the goal is too ambitious: talking about screening tools is not possible with less than 100 cases, a biased selection, and a consistent overlap of data between “normal” (although one may doubt of such a normality) and PE pregnancies.

Our response: We did not use “normal” in the revised manuscript. One of our novel findings was that nephrinuria did not change in normotensive pregnancies (although proteinurina increased with advancing gestational week even in normotensive pregnancies), while in PE pregnancies, nephrinuria increased with increasing proteinuria and its level was already greater at pre-clinical stage of PE compared to that in normotensive pregnancies. This dissociation between behaviors of nephrinuria and proteinuria raised a possibility of nephrinuria as a screening tool for women at higher risk of pregnancy. Therefore, we also considered this study was a pilot feasibility study including an only small subjects as you mentioned.
Furthermore, the authors should have at least excluded kidney disease, collagen disease, diabetes and obesity, all known to increase the risk for PE: the definition of normal pregnancies on the basis of their outcome is, in my opinion, not acceptable.

Our responses: As the prevalence rate of PE was very high in our population, we investigated detailed backgrounds of participants and added this information to Table 1. We think that we have no reason to exclude some women with various complications included in this study. All participants were normotensive and no SPIP at the enrolment, but 14 of them later developed both hypertension and SPIP and the remaining 75 did neither SPIP nor hypertension. We used a term “normotensive pregnancy” instead of “normal pregnancy” because majority of participants had complications as shown in the revised Table 1.

the data should be related also to other biomarkers, such as sFlt-1 or PlGF, of growing interest in PE management. Data on placental flows could also be of interest.

Our responses: This study aimed to characterize better changes in nephrinuria in normotensive and PE pregnancies. Therefore, most samples were longitudinally collected. We think that it is another problem which biomarkers are better predictors of PE than other biomarkers. As you suggested, this is the pilot study regarding a possibility of nephrinuria as a screening tool of later development of PE.

I would suggest to re-write the study as a novel approach, underlining much better the limitations and defining in detail the population.

Our response: We characterized better the nature of this study, changing the title to a current one. We investigated backgrounds of all participants to know why PE was much prevalent in our population and results were added to the revised Table 1.

Other points
1. the paper is addressed at a new issue; therefore, the aim should be more balanced: it’s a first pilot feasibility study and the goal of assessing nephrinuria as a screening tool is way too far.

Our response: We changed the title reflecting your suggestion.

2. PCR is a very poor marker of proteinuria; if a new marker of PE has to be assessed, the gold standards (still 24 hour urine collection) should have been used.

Our response: We do not think that the 24-h urine collection is superior to PCR. Although the 24-h urine collection has been a traditional gold standard to define SPIP, it has at least two inherent drawbacks; 1) we are always not confident that women collected truly 24-h urines, and 2) as a false positive test result is frequent on dipstick testing, but it is usually very difficult/inconvenient for pregnant women to repeat the 24-h urine collection. In addition, recent guidelines allow the use of PCR instead of the 24-h urine collection.

3. PE in 14 out of 89 women is an exceedingly high incidence. If these women were at high risk this should be state. If not there has to be some underlining bias (the worldwide prevalence of PE is around 5%); the authors note this in the last part of their study. however, this is not enough, and they should dissect their population and analyse it better

Our response: We analyzed reasons why as many as 16 % of women developed PE in the revised version.

4. partly longitudinal study doesn't seem a sound definition

Our response: We deleted “partly longitudinal” from the 2nd Strengths and Limitations of this study in the Article summary.

Reviewer: 3
Reviewer Name: Vesna D. Garovic, MD
In this prospective observational study, eighty-nine pregnant women in whom neither hypertension nor proteinuria was present at enrolment, were recruited to investigate the possibility of nephrinuria as a screening tool for the risk of pre-eclampsia (PE).

Urinary nephrin/creatinine ratio (NCR, ng/mg) and protein/creatinine ratio (PCR, mg/mg) in pregnancy were measured longitudinally during pregnancy. PE was confirmed in women with both SPIP and hypertension.

PE developed in 14 of the 89 women. The NCR increased with increasing PCR in 14 PE women but not in those with normal pregnancies. Relative risk of later development of PE among asymptomatic 2nd and 3rd trimester women was significantly increased in those with NCR (ng/mg) > 122 (95th percentile value for 75 women with normal pregnancies). The authors conclude that nephrinuria may efficiently differentiate women at higher risk of PE.

I have the following comments:
1. The authors report that 16% women developed PE, which is much higher than any number reported in the literature. Any explanation for that? Should this be mentioned in limitations?
   Our response: Results of detailed investigation on reasons why as many as 16% of participants developed PE in our population were added to Table 1. We discussed limitation of this study in the revised version.

2. Were these women compared with respect to NCR in their 1st pregnancy trimesters? Even if negative, these data would be important to understand the trajectory of NCR.
   Our response: Result of the comparison was described in the legend for Fig. 4.

3. I would not use term "normal" pregnancies, as the "normality" was confirmed based on the lack of hypertension and proteinuria. Still, these women may have some other medical problems and/or obstetric complications. I would prefer the term "normotensive."
   Our response: We used "normotensive" instead of "normal" in the revised version.

4. Similarly, I would not use the term "counterpart characteristics" (abstract) but would stick to preeclamptic pregnancies.
   Our response: We reworded to specify characteristics.

5. The topic should be put in a larger context of podocyte markers in the urine and renal injury in non-pregnant states
   Our response: We chose “Renal medicine” as a primary subject heading.

Reviewer: 4
Reviewer Name: Huaizhong Hu
Institution and Country: West China 2nd University Hospital, Sichuan University, Chengdu, Sichuan, China
Competing Interests: No competing interests

1. Page 7, second last sentence, "judged" should be changed to “diagnosed".  
   Our response: We did it.
2. The sample collection is interesting, especially "73 were collected at latent phase with neither SPIP nor hypertension.", as if it was known these women would develop PE. Please clarify why more samples were collected from women that developed PE during the latent phase.
Our response: We did not know at all which women would develop PE at the time of enrolment. We encouraged all women who were asymptomatic with respect to PE to participate in this study and urines of participants were collected longitudinally in most women. We now knew why as many as 16% of women developed PE after the review of backgrounds of all participants.

3. Table 1, please use symbols to denote what they are between the brackets.
Our response: We used asterisks "**".

4. Page 10, last sentence before discussion, differentiated women with higher and lower risk of PE. Please change to "differentiated....... from......".
Our response: We did it.

5. In multiple places in the manuscript, "increasing gestation" is used. For better clarity, I suggest this to be changed to increasing gestation time or increasing gestation weeks.
Our response: We used “gestational week” instead of “gestation” in the revised manuscript.

5. In discussion, this sentence needs to rephrased for better clarity: All of these results suggested that a significant increase in nephrinuria was unlikely to occur in the physiological range of proteinuria in pregnancy, but nephrinuria occurred in parallel with proteinuria beyond a certain degree of proteinuria in PE pregnancy, i.e., a significant increase in nephrinuria preceded pathological proteinuria in PE pregnancy.
Our response: This sentence was deleted in the revised version. Instead, we added following sentence “The nephrinuria in pre-clinical stage of PE pregnancies (before onset of SPIP) was already greater than that in normal pregnancy.”

6. In the last paragraph, it quoted work by Wang et al.[12] reporting nephrinuria in pregnancy for the first time in 2012, mean NCR (ng/mg) was 107 vs. 1894 and mean nephrin concentration was 86 vs. 1226 ng/mL for women with normal pregnancy vs. women with PE pregnancy having mean proteinuria of 2579 mg/day, respectively. Please note the units are not the same, ng/mg and mg/day. This makes it difficult to compare. Please check the original article for the accuracy.
Our response: We checked again the report by Wang et al., and confirmed a description about their study in this manuscript was correct.
**GENERAL COMMENTS**

This is a much improved paper by Zhai et al. that investigates the feasibility of nephrinuria as a screening tool for the risk of preeclampsia. The authors have partly addressed my previous concern as to the population studied given a high percentage of women with PE. They now identify a variety of co-morbidities, with some of them being recognized as major risks for PE development. Given a relatively low incidence of this disease in general population, this reviewer agrees with approach that enriches for PE patients by studying high-risk women. However, additional clarification is needed as to their previous renal disease. In Methods, and under Participants, it is stated that no participants had significant proteinuria. If this is the case, this needs to be mentioned in Table 1, as nephrinuria may not be specific for preeclamptic proteinuria, but rather can accompany any proteinuric disease, such as diabetic nephropathy, or renal disease associated with autoimmune connective tissue disorders. If some women indeed had proteinuria at the time of enrollment, a sensitivity analysis (without these women) should be performed to ascertain the presence of difference after exclusion.

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**VERSION 2 – AUTHOR RESPONSE**

Reviewer: 3  
Reviewer Name: Vesna D. Garovic, MD  
Institution and Country: Mayo Clinic, Rochester, MN, USA  
Competing Interests: Dr. Vesna D. Garovic is the inventor of podocyturia technology for diagnosis and prediction of preeclampsia; the technology has been licensed to a commercial entity; Dr. Garovic and Mayo Clinic have contractual rights to receive royalties from the licensing of this technology.

This is a much improved paper by Zhai et al. that investigates the feasibility of nephrinuria as a screening tool for the risk of preeclampsia.

The authors have partly addressed my previous concern as to the population studied given a high percentage of women with PE. They now identify a variety of co-morbidities, with some of them being recognized as major risks for PE development. Given a relatively low incidence of this disease in general population, this reviewer agrees with approach that enriches for PE patients by studying high-risk women. However, additional clarification is needed as to their previous renal disease. In Methods, and under Participants, it is stated that no participants had significant proteinuria. If this is the case, this needs to be mentioned in Table 1, as nephrinuria may not be specific for preeclamptic proteinuria, but rather can accompany any proteinuric disease, such as diabetic nephropathy, or renal disease associated with autoimmune connective tissue disorders. If some women indeed had proteinuria at the time of enrollment, a sensitivity analysis (without these women) should be performed to ascertain the presence of difference after exclusion.

Reply: We confirmed that all of the 56 and 8 women with complications had not SPIP at enrolment. We added following information to the footnote for Table 1: These 56 women with complications showed a PCR level (mg/mg, median [range]) of 0.026 (0.001 – 0.111) at enrolment. These 8 women with complications showed a PCR level of 0.047 (0.002 – 0.065) at enrolment.

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**VERSION 3 - REVIEW**

| REVIEWER | Vesna D. Garovic  
|----------|------------------|
|          | Mayo Clinic, Rochester, MN USA  

Dr. Vesna D. Garovic is the inventor of podocyturia technology for
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<th>REVIEW RETURNED</th>
<th>15-May-2016</th>
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| GENERAL COMMENTS       | My concerns have been addressed appropriately. |
Feasibility of nephrinuria as a screening tool for the risk of pre-eclampsia: prospective observational study

Tianyue Zhai, Itsuko Furuta, Rina Akaishi, Kosuke Kawabata, Kentaro Chiba, Takeshi Umazume, Satoshi Ishikawa, Takahiro Yamada, Mamoru Morikawa and Hisanori Minakami

BMJ Open 2016 6:
doi: 10.1136/bmjopen-2016-011229

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