Author's response to reviews

Title: Abnormal Urinary Excretion of NKCC2 and AQP2 in Response to Hypertonic Saline in Chronic Kidney Disease. An Intervention Study in Patients with Chronic Kidney Disease and Healthy Controls

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Version: 4
Date: 30 April 2014

Author's response to reviews: see over
RESPONSES TO REVIEWERS

Response to comments raised by reviewer Ewout J Hoorn regarding:

MS: 1908766592122614
Title: Abnormal Urinary Excretion of NKCC2 and AQP2 in Response to Hypertonic Saline in Chronic Kidney Disease. An Intervention Study in Patients with Chronic Kidney Disease and Healthy Controls

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Our response to the referee’s comments are in red font.

Version: 2
Date: 8 April 2014
Reviewer: Ewout J Hoorn

Reviewer’s report:
Jensen et al. analyzed distal tubular sodium and water handling in human CKD. To do so, they infused hypertonic saline in patients with CKD and healthy volunteers and subsequently analyzed the response in plasma and urinary parameters, including vasoactive substances and immunoassays of urinary sodium and water transporters. After hypertonic saline, they observe an attenuated decrease in urinary output and free water clearance in patients with CKD. This response is accompanied by a (greater) increase in urinary AQP2 and NKCC2. They interpret this as a defective tubular response to volume expansion (abstract) and as an inability to concentrate urine (conclusions in discussion section).

This is an interesting study into human physiology and pathophysiology during CKD by a group with a track record in this type of studies. However, I have three major comments that I would like the authors to address in a revised version:

1. I disagree with the main conclusion, namely that the CKD patients have an inability to concentrate urine. What the authors show is that the CKD patients excrete the sodium in the hypertonic saline normally (or at least without significant differences from controls). However, they have a problem to excrete the water load present in the hypertonic saline. This appears due to an exaggerated response in u-NKCC2 and u-AQP2, and therefore it seems the other way round: they concentrate their urine too well.

This may have important implications for the tendency of CKD patients to develop hyponatremia (see emerging literature on this topic by for example Kovesdy et al., Circulation 2012 and Combs & Berl, AJKD 2014). Please comment and discuss.

>> A) We acknowledge your arguments, but disagree. It is correct that CKD patients excrete the relative same amount of sodium in response to 3 % saline. But they do NOT have a problem excreting their water – in contrast the have an attenuated decrease in CH2O compared to healthy controls in response to 3 % saline (figure 3C). Thus, CKD patients reabsorb less water than controls. This is also shown in figure 3B, where it is clearly demonstrated that CKD patients are not able to increase u-osmolality to the same degree. However, you are correct, that u-AQP2 increased more in CKD patients. - i.e. they activate a higher amount of AQP2 water channels. Although more AQP2 channels
were activated, patients still cannot reabsorb enough water through the remnant nephrons. Therefore we must conclude that patients do have an inability to concentrate their urine in response to 3% hypertonic saline. This was not clear in the former manuscript, but has been corrected in revised manuscript (see discussion p. 20 line 1-13).

B) Regarding the tendency to hyponatremia in CKD patients, we point out that in this study, p-sodium increased relatively more in CKD patients compared to healthy controls. We thank you for the reference to Berl et al’s paper: “dysnatremias in patients with kidney disease” that discuss patients with CKD’s concentration and diluting abilities. We would like to address that Berl et al reports that: “hyponatremia is relatively common in ESRD”......“it was more likely due to interdialytic excessive water intake, as patients with oliguric kidney failure cannot regulate their water excretion in response to AVP and rely on sodium and water removal during renal replacement”
We did not study CKD patients with eGFR below 15 ml/min. Thus, our study-population, of patients with mild to moderate CKD, have some degree of concentration ability although less than healthy subjects.

I also think the NKCC2 findings should be interpreted more in the context of vasopressin and concentrating mechanism rather than sodium handling.
>> The discussion section has been rewritten. We hope you find that NKCC2 has been interpreted more in context to AVP in revised manuscript.

I would also suggest changing the “novelty” of this study (Page 17, Discussion), because mentioning that this group is the first to conduct such a study is not a merit by itself if the main findings are not emphasized.
>> We have stressed the importance of NKCC2 further in revised manuscript.

2. Urinary transporter immunoassays: A paragraph should be added to discuss what the authors believed is picked up as a signal from the immunoassays.
>> Added in revised manuscript (see page 17 line 20-22)

What do the authors believe to be the source of the transporters? Are these exosomes, as alluded to once in the study? If so, do the authors believe the differences in transporters are due to higher protein abundance on the same number of vesicles or are more vesicles excreted?
>> Paragraph regarding this subject have been added in revised manuscript (see page 17 line 22 - page 18, line 5)

Any concern that non-specific signals are picked up because no (ultra)centrifugation was applied?
>> We cannot exclude that an increased non-specific binding in non-ultracentrifuged vs. ultracentrifuged may have occurred. However, we measure non-specific binding in each assay and it was 6-7 %, which is acceptable in RIA.
3. The presentation of the data is poor: The manuscript is much too long (> 7000 words) and the Methods section is almost a paper by itself (> 2000 words). Because the study’s main conclusions are limited, I think it should be possible to report these results aiming at half the words (~3500 words). Currently the lengthy manuscript distracts the reader from easily grasping the main message.

>> We agree. Regarding the method section, we describe a new method of measuring u-NKCC2 by RIA. Therefore we have not made this section shorter. The rest of the revised manuscript has been shortened by approximately 800 words.

There are also too many tables and figures. Table 1: many parameters are not relevant, including p-cholesterol, liver enzymes, and HbA1c. Table 1-2 may be combined if only relevant parameters are shown. Table 2: the baseline values of the transporters do not add in my opinion and may also be summarized as “no differences” in text. Table 2: instead of u-Na/K and FENa/K, I think UNa/K * V is a better measure of 24h urine and I suggest replacing this. Tables 3-5: difficult to digest the data, better to represent significant results graphically, u-NKCC2 is both in Table 3 and Figure 4. Table 6: can be removed, this is not really adding new information, because it was already shown that the CKD patients had an attenuated decrease in cH2O. Figure 1: may be converted to supplemental material. Figures 3, 4, and 6 may be combined to have one figure for all transporter assays, panel A could show AQP2, y-ENaC, and NKCC2 for CKD vs patients, and panel B the distribution between CKD3 and 4. I suggest deleting U-NKCC2exr because this parameter is also not reported for the other transporters.

>> Revisions have been made:
Table 1+2 have been combined to table 1. We have kept FENa and FEK, as we find it important parameters to show the differences in tubular function.
Table 3 has been converted to figure 3 and combined with former fig. 2
Table 4 is converted to fig. 4
Table 5+6 have been deleted.
Figure 1 remains
Figure 2 has been combined to fig. 3
Figure 3 has been combined with former fig. 4 + 6 to figure 2 (A-F)
Figure 5 has been deleted
Figure 7 now equals fig 5.

Discussion: I would prefer a more focused review with an integrated discussion rather than different subsections, which increase length and result in unnecessary textbook-like discussions.

>> We have rewritten the discussion section, to decrease length and to focus on fewer relevant subjects.

Response to comments raised by Reviewer Thomas Larsen regarding:

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Title: Abnormal Urinary Excretion of NKCC2 and AQP2 in Response to Hypertonic Saline in Chronic Kidney Disease. An Intervention Study in Patients with Chronic Kidney Disease and Healthy Controls
Our responses to the referee’s comments are in red font

Version:2 Date:14 April 2014. Reviewer:Thomas Larsen

Reviewer’s report:
Jensen et al report results of an interventional case-control study investigating the renal tubular response to hypertonic saline infusion in CKD stage III-IV compared with healthy controls. The trial was well-designed with utilization of a standardized diet prior to the experimental examination adding additional strength to the study. By means of quantifying the urinary excretion of the NKCC2, ENaC# and AQP2 membrane transport proteins, the authors document pathophysiologic changes in renal sodium and water handling. The paper highlights important alterations in renal tubular function, and illustrates the kidney’s inability to effectively compensate for acute volume expansion in later stages of CKD.

- Major Compulsory Revisions
1. Regarding analysis of FENa, NKCC2, AQP2 and ENaC, the authors performed a post hoc sub-analysis dividing the CKD population into two groups consisting of those with stage III and IV disease, resp. These results offer important contributions to the overall conclusion of the study. However, it should be clearly stated in the discussion whether this was a pre-planned analysis.
   >> A statement has been included under “strengths and limitations” (see p. 21 line 23 – page 22 line 3)

- Minor Essential Revisions
1. Regarding “inclusion criteria” on page 3, paragraph 3: Please correct “stage II-IV” to “stage III-IV”.
   >> corrected

2. Three CKD patients and three healthy controls were excluded from urine analyses due to incomplete voiding. Were these subjects reasonably matched regarding gender and age, or could this represent a potential cause of confounding in the study?
   >>The subjects, excluded from urine analysis due to incomplete voiding, were not matched by age or gender in the two groups. The subjects excluded consisted of: 2 female CKD patients age 70 (eGFR 22 ml/min/m²) and age 68 (eGFR 33 ml/min/m²) and 1 male CKD patient age 68 years (eGFR 48 ml/min/m²). 2 male healthy subjects age 67 and 45 years, and 1 female healthy subject age 55 years. All though the subjects excluded from the patient group, were of older age compared to the healthy group, there were no statistical difference in mean age between the remaining females and males in the two groups.

3. The abbreviations NKCC2 and NK2CC are used interchangeably on pages 10 and 11. I would suggest using only NKCC2.
4. On page 14, paragraph 3 “Figure 2B” should read “Figure 3B”
5. Labels for figure “A” and “B” does not appear on figures 2 and 3.
6. The label of the Y-axis in figure 4B should read “ng/mmol” instead of “ng/min”.
7. Please revise grammar in the legend for Figure 4.
>> Figures have been revised. Any errors should have been corrected during this process.

8. On page 12, paragraph 2 it states that 11 patients were on loop diuretics. However, on page 13, paragraph 2 and on page 19, paragraph 3, it appears that 8 patients were treated with furosemide. Were 3 patients then on a non-furosemide loop diuretic? If so, it would be reasonable to include those in the analysis of u-NKCC2 in “furosemide vs no-furosemide” users. If not, please correct accordingly.
>> We regret the misinformation about furosemide treatment in CKD patients. 11 CKD patients were treated with furosemide. However the 3 CKD patients excluded from urine analysis, were among these 11, and was not a part of the data analysis regarding uNKCC2 and furosemide. The two paragraphs mentioned, are deleted in the revised manuscript. Furosemide treatment is only mentioned briefly in results (p 13 line 7-10)

- Discretionary Revisions
1. How did the authors ensure that none of the patients included in the study had chronic heart failure?
>> We excluded patients with Chronic Heart Failure by studying medical records at pre screening, conducting a thorough medical history and clinical examination inclusive ECG. We have kept the exclusion criteria as in the previous version.

2. On page 19, the authors write in paragraph 2 “The quantity of excreted NKCC2 in urine reflects the activity of sodium transport via NKCC2, just as…”. Is that an assumption or is the statement supported by previous studies? If so, please ad a reference.
>> Regarding AQP2 and ENaC studies have been performed that supports this fact (se references at page 17 line 19-20). As this is the first study to measure u-NKCC2 we have no other references to support this. A correction has been made in manuscript (p 17 line 18)

Without this clarification the statement in paragraph 4 on the same page that reads “Thus, the lack of change in urine [NKCC2] does not always reflect a lack of change within the plasma membrane of kidney epithelial cells”, may appear contradictory.
>> It has been clarified in revised manuscript (see p. 18 line 16-20)

Level of interest:An article of importance in its field
Quality of written English:Acceptable
Statistical review:No, the manuscript does not need to be seen by a statistician.
Declaration of competing interests:
From 2009-2012, I was employed at the institution where the current trial was conducted. Although having worked with Dr. Jensen and some of the co-authors in the past, I have not had any involvement in the current trial. Additionally, I have
no financial competing interests in relation to this paper.