Author's response to reviews

Title: Tubular reabsorption and local production govern urine hepcidin-25 excretion

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Author's response to reviews: see over
Dear Mr. H. Henderson,

Thank you for considering the above manuscript for publication and for the comments made by the reviewers. We adjusted the manuscript accordingly and enclose a point by point reply.

The findings reported by Haase-Fielitz et al. indicating that urinary hepcidin may protect against AKI by attenuating heme-mediated injury, triggered us to consider the renal handling of hepcidin. More precisely; we were interested in whether urinary hepcidin excretion is determined by proximal tubular reabsorption and/or local production. We therefore performed this study, which should be regarded as a pilot study. Our results demonstrated that urinary excretion of hepcidin is indeed dependent on tubular reabsorption, since fractional excretion of hepcidin is highly correlated to fractional excretion of beta-2 microglobulin - a low molecular protein, which is normally almost completely reabsorbed by the renal tubules. Yet, 12-24 hours after cardiac surgery, urinary hepcidin excretion increased and did no longer correlate with beta-2 microglobulin, indicating that the increased levels of urine hepcidin are explained by local production. In this study urine samples were collected within 1-2 hours after surgery (at a time point the patient was admitted and stable at the ICU) and 12-24 hours after surgery (morning urine collected the day after the procedure). We agree that the time interval thus varied. However, since hepcidin and beta 2-microglobulin were measured in the same urine sample, this variation does not affect our conclusions. Certainly, now that our data suggest local production of hepcidin, more extensive studies are necessary to evaluate the exact kinetics and location of hepcidin production, and to identify possible factors influencing this process. Since increased urinary levels of hepcidin may attenuate post-surgical AKI, increasing local production may serve as a strategy to reduce the development of AKI.

The results presented in this paper have not been published previously in whole or part, except in abstract format. All authors have approved the final version of the article. There are no conflicts of interest to declare.

We thank the reviewers for their constructive comments and hope that you find our revised manuscript suitable for publication in BMC Nephrology.

Yours sincerely, on behalf of the co-authors,

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Response to comments

Reviewer: Michael MH Haase

Reviewer’s comments:

1. What was the rationale/objective of the study? Please add this information under “Introduction”.

   The objective of this pilot study was to evaluate the renal handling of hepcidin; more precisely, to study whether urinary hepcidin was dependent tubular reabsorption or whether there was evidence of local production. Urinary beta 2-microglobulin was used as a measure of tubular reabsorptive capacity. We describe our hypothesis more clearly in the introduction of our revised manuscript; ‘The objective of this study was to study the role of tubular reabsorption in kidney hepcidin handling.’

2. “urine beta2-MG was only measured in urine with a urinary pH>6.0”. Please provide the information in how many patients beta2-MG was measured in the urine as for example in other large cardiac surgery cohorts >35% of patients had a preoperative urine pH of # 5.5 (Haase et al. Crit Care Med 2009).

   24 patients were initially enrolled, but 5 patients were excluded because of a urinary pH <6 at both time intervals, thus precluding reliable measurement of β2m. In the remaining 19 patients, β2m could be measured in 8 patients 1-2 hours after CAB and in 13 patients 12-24 hours after CABG. We added this to the results section.

3. Also, please, acknowledge the limitation that beta2-MG is not stable under acidic conditions and why alpha1-MG was not chosen.

   We acknowledge the limitation that beta-2 microglobulin is not stable in acidic urine in the ‘Subjects and Methods’, subparagraph ‘Laboratory measurements’. ‘Urine β2m was only measured in urine with a urinary pH >6.0, since degradation of β2m may occur below this pH.’

   In addition, we have added a sentence to the discussion to clarify that alfa-1 microglobulin was not chosen since this protein is bound to other plasma proteins thus precluding the calculation of the fractional excretion of alfa-1 microglobulin; ‘Alfa-1 microglobulin is a more stable marker of proximal tubular reabsorption and can reliably be measured in acidic urine, but is bound to plasma proteins which hampers calculation of the fractional excretion.’

4. The number of patients being operated was very small. Were patients enrolled in a consecutive manner? What were the inclusion and exclusion criteria? How can the authors exclude selection bias in this regard?

   Patients were enrolled consecutively, and all patients undergoing CABG were included. We added this sentence to the methods section.

5. Why did the authors choose to measure urine and serum markers in 8 patients 1-2 hours after CABG and in 13 patients 12-24 hours after CABG? In how many patients, markers were measured at both time points? What was the rationale not to use the same, predefined sample time-point in all 19 cardiac surgery patients?

   This was a pilot study to evaluate urinary hepcidin excretion and the role of tubular reabsorption in patients after CABG. The correlation defined in the patients with kidney disease served as control. The data of the patients after CABG were compared to these controls values. Our hypothesis was that in the CABG patients the ratio FE hepcidin and FE beta-2 microglobulin would be comparable to the ratio in controls. Consecutive samples in CABG patients are not necessary for comparison of a ratio. pH was >6 in approximately 50%
of measurements performed in these 19 patients, allowing reliable quantification of β2m in 8 patients at day 1 and in 13 patients at day 2. As such, the collected samples were largely independent samples. In these samples both hepcidin and beta-2 microglobulin were measured. Of note, two patients were represented in both groups, and measurements were performed at both time points. In patient 1, the fractional excretion (FE) of beta-2 microglobulin and hepcidin were 1.6 and 3.6% on day 1 and 3.1 and 45.5% at day 2. In patient 2, the fractional excretion (FE) of beta-2 microglobulin and hepcidin were 15.1 and 18.3% on day 1 and 3.1 and 49.8% at day 2. Thus, measurements within these subjects at both time intervals are similar to measurements performed in different subjects at both time intervals. We added a sentence in the results section; '24 patients were initially enrolled, but 5 patients were excluded because of a urinary pH <6 at both time intervals, thus precluding reliable measurement of β2m. All remaining 19 patients were operated on pump, except for one patient who had pre-existing impairment of renal function. Two patients developed acute kidney injury after cardiac surgery, with AKI defined as a baseline-to-peak decrease in eGFR by 50% or more during the first five days post-operatively. pH was >6 in approximately 50% of measurements performed in these 19 patients, allowing serum and urine β2m and hepcidin quantification in eight patients 1-2 hours after surgery, and in 13 patients 12-24 hours after surgery. In only 2 patients measurements of β2m and hepcidin could be performed at both time intervals.’

In addition we changed the discussion: ‘This study is the first to document local production of hepcidin in patients after CABG. It has several limitations. First, we included a limited number of patients. Secondly, due to a pH <6.0 β2m could not reliably be measured in all samples. Although alfa-1 microglobulin is a more stable marker of proximal tubular reabsorption and can reliably be measured in acidic urine, it is protein-bound and therefore it is impossible to calculate the fractional excretion.’

6. Please also be more specific in the information on the timing: “CABG, 1-2 hours after surgery”. Does this correspond with a) 1-2 hours after end or start of cardiopulmonary bypass (CPB) or b) 1-2 hours after end of surgery? This corresponds to 1-2 hours after the end of surgery. We changed our manuscript accordingly.

7. Were all 19 CABG patients operated “on pump”? Please add duration of CPB in Table 1.
All patients, but one, were operated on pump, this is added to the methods section; ‘Patients were enrolled in a consecutive manner, all patients undergoing CABG were included.’ And in the results section: ‘All patients were operated on pump, except for one patient who had pre-existing impairment of renal function.’ Duration of CPB is added to Table 1 of the revised manuscript.

8. How many patients developed acute kidney injury after cardiac surgery?
Two patients developed acute kidney injury after cardiac surgery, with AKI defined as a baseline-to-peak decrease in eGFR by 50% or more during the first five days post-operatively. The median increase in serum creatinine from day 0 to 1 was 4%, and from dag 1 to 2 1%. We added this information to the results section of the revised manuscript.

9. Please add a section in the discussion on strength and limitations as well as on potential clinical implications.
The discussion was altered accordingly; ‘This study is the first to document local production of hepcidin in patients after CABG. It has several limitations. First, we included a limited number of patients. Secondly, due to a pH <6.0 β2m could not reliably be measured in all samples. Although alfa-1 microglobulin is a
more stable marker of proximal tubular reabsorption and can reliably be measured in acidic urine, it is protein-bound and therefore it is not suitable to calculate the fractional excretion. Third, this study is a pilot study, and our findings were not corroborated by histopathological data showing extensive proximal tubular uptake in apical endocytic vesicles, nor by data on hepcidin expression or mRNA content in the kidney or macrophages. More extensive studies are necessary to evaluate the exact kinetics and location of hepcidin production, and to identify possible factors influencing this process. Since increased urinary levels of hepcidin are associated with a decreased risk for post-surgical AKI, increasing local production may serve as a strategy to reduce the development of AKI.’

10. The article would further benefit from a stronger discussion section as currently the majority of the discussion deals with results from other studies. How did your findings add to the literature? How did you interpret your findings? Did you consider evidence against the potential local production and tubular reabsorption of hepcidin?

Unfortunately, there is only limited data in literature on tubular reabsorption and local production of hepcidin. We changed the discussion in view of the comments made by the reviewer.

Abstract:

• “Following cardiac surgery, FE of hepcidin-25 increased despite a decline in FE of beta2MG……” Please add e.g. “potentially indicating local production at 12-24 hours”

The text was changed accordingly; ‘Following cardiac surgery, FE of hepcidin-25 increased despite a decline in FE of β2m, potentially indicating local production at 12-24 hours.’

• Conclusions: “…increased urine hepcidin-25 level may reflect tubular dysfunction.”

Consider to be more precise and change “tubular dysfunction” into “reduction in tubular hepcidin uptake”.

The text was changed accordingly; ‘Hepcidin-25 is reabsorbed by the renal tubules and increased urine hepcidin-25 levels may reflect a reduction in tubular uptake.’

Results:

• Please add Spearman correlation coefficients in Figure 3.

Spearman correlation coefficients were added.

• Please be more precise “…there was no evidence of tubular threshold (Figure 3)”. How did you analyze the potential existence of such a threshold? Please, add this information to the statistical analysis section.

Theoretically, high hepcidin-25 serum levels resulting in high tubular delivery of hepcidin-25 could indeed give rise to an increased urinary excretion of hepcidin-25 through megalin saturation. If there is indeed a threshold above which excretion of hepcidin-25 increases than a curve similar to the figure below depicting renal glucose handling would be expected.
Instead of adding this information to the statistical analysis section, we inserted a theoretical threshold in figure 3.

Minor point:
Animal experiments
- “In order investigate...” Please add “to”.
  This was corrected.
Reviewer: Paolo Calzavacca
Reviewer's comments:

Major revision:

Results paragraph:

1. It is not clear to me whether the 8 patients that had samples taken at the end of surgery were the same as the 13 who had samples taken after 12-24 hours. Why are not the same number of patients? I am not sure whether the baseline can be compared to the values at 24 hours given this study design.

   This was a pilot study to evaluate urinary hepcidin excretion and the role of tubular reabsorption in patients after CABG. The correlation defined in the patients with kidney disease served as control. The data of the patients after CABG were compared to these controls values. Our hypothesis was that in the CABG patients the ratio FE hepcidin and FE beta-2 microglobulin would be comparable to the relationship in the controls. Therefore, consecutive samples are not necessary for comparison of a ratio. pH was >6 in approximately 50% of measurements performed in these 19 patients, allowing reliable quantification of β2m in 8 patients at day 1 and in 13 patients at day 2. As such, the collected samples were largely independent samples. In these samples both hepcidin and beta-2 microglobulin were measured. Of note, two patients were represented in both groups, and measurements were performed at both time points. In patient 1, the fractional excretion (FE) of beta-2 microglobulin and hepcidin were 1.6 and 3.6% on day 1 and 3.1 and 45.5% at day 2. In patient 2, the fractional excretion (FE) of beta-2 microglobulin and hepcidin were 15.1 and 18.3% on day 1 and 3.1 and 49.8% at day 2. Thus, measurements within these subjects at both time intervals are similar to measurements performed in different subjects at both time intervals.

   We added a sentence in the results section; ‘24 patients were initially enrolled, but 5 patients were excluded because of a urinary pH <6 at both time intervals, thus precluding reliable measurement of β2m. All remaining 19 patients were operated on pump, except for one patient who had pre-existing impairment of renal function. Two patients developed acute kidney injury after cardiac surgery, with AKI defined as a baseline-to-peak decrease in eGFR by 50% or more during the first five days post-operatively. pH was >6 in approximately 50% of measurements performed in these 19 patients, allowing serum and urine β2m and hepcidin quantification in eight patients 1-2 hours after surgery, and in 13 patients 12-24 hours after surgery. In only 2 patients measurements of β2m and hepcidin could be performed at both time intervals.’

   In addition we changed the discussion: ‘This study is the first to document local production of hepcidin in patients after CABG. It has several limitations. First, we included a limited number of patients. Secondly, due to a pH <6.0 β2m could not reliably be measured in all samples. Although alfa-1 microglobulin is a more stable marker of proximal tubular reabsorption and can reliably be measured in acidic urine, it is protein-bound and therefore it is impossible to calculate the fractional excretion.’

Minor revisions:

Two questions about the protocol:

2. Why a range of 12 to 24 hours for blood and serum collection after cardiac surgery (paragraph: human studies)

   This range can be ascribed to logistical procedures at the ICU. In this study urine samples were collected within 1-2 hours after surgery (at a time point the patient was admitted and stable at the ICU) and 12-24 hours after surgery (morning urine collected the day after the procedure). This is now explained in the methods section of the revised manuscript.

3. when was the 1 ml of fluid replacement given to mice? Was it a single bolus or
repeated bolus (it is not clear and it should be clarified in test. Paragraph: animal experiments)

Prior to 24 h housing in metabolic cages, 2×0.5 ml salt solution was administered subcutaneously to prevent dehydration. To prevent hypothermia, room temperature was raised to 24 °C with a relative humidity of 53-68%. This sentence was added to the methods section.