Author’s response to reviews

Title: Renal kallikrein excretion and epigenetics in human acute kidney injury: Expression, mechanisms and consequences

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Author’s response to reviews: see over
Date: May 17, 2011

To: Lin Lee, Ph.D. <bmcmedicineeditorial@biomedcentral.com>.

Dear Dr. Lee:

Thank you for your decision letter, in which you pointed out that, while this manuscript was not acceptable for publication in BioMed Central Medicine in its present form, that we could attempt further revisions in response to the 4 reviewers.

Since the critiques were amenable to changes in the text, we submit this revised version.

On the attached sheets, we detail our responses to the 4 reviewers. The original critiques are indicated in bold type, while the responses are in regular/plain type. In the revised manuscript, any changes are indicated in yellow highlights.

We believe that the presentation has been strengthened, and look forward to your re-evaluation.

Thank you for your help.

Sincerely,

Daniel T. O’Connor, M.D.
Comments from the 4 reviewers.

Reviewer 1.
Reviewer: Jagdish Sharma.
Comments:
The authors evaluated where the serine protease kallikrein excretion is altered in human acute kidney injury (AKI), and if so what could be the mechanisms. They also examined patients with AKI for urinary expression of kallikrein enzymatic activity.

Major comments:
1. The authors failed to state the race, age and sex of the subject in methods section. It is well known that there is a significance differences in the kallikrein levels between blacks and white Americans.
   Race, age, and sex information are now listed in Tables 1 and 2. Cases and controls did not differ in biogeographic ancestry (p>0.05). In the Results section, we now state: “Since black and white subjects differ in reported KLK1 excretion [5, 15], we evaluated the role of ethnicity in our samples (Table 1). Although cases and controls each included several biogeographic ancestries, KLK1 excretion did not differ significantly in black versus white cases (p=0.26) or black versus white controls (p=0.69), perhaps reflecting the relatively small sample sizes. Disease analyses were adjusted for biogeographic ancestry as a covariate.”

2. They selected control as outpatients: this could change the values of the kallikrein since it is known that the diet (potassium, calcium, salt intake) can effects the kallikrein levels as compared with inpatients where diet can be controlled by the dietician. Whereas, the patients understudy were all inpatients.
   For controls (Table 1), we studied both unhospitalized subjects (outpatients), and inpatients admitted to the hospital intensive care unit (ICU controls), since we wanted controls to be matched as closely as possible to cases.
   In Table 2, we report urinary Na+ excretion for cases and controls; the results did not differ (p=0.347).

3. The p values should be cited on the figures so reader can evaluate the changes.
   P-values have now been embedded in each data figure, including Figure 4.

Reviewer 2.
Reviewer: Masao Kakoki.
Reviewer’s report:
In the manuscript titled “Renal kallikrein (KLK1) activity and epigenetics in human acute kidney injury (AKI): Expression, mechanisms and consequences,” the authors found an unexpected increase in urinary KLK1 excretion in patients with established AKI. Since urinary excretion of epinephrine is high in the AKI patients, and previous studies indicate that of epinephrine increases KLK1 expression, epinephrine may play a promotive role in the elevated urinary kallikrein excretion in AKI patients. The changes in the methylation profile in the promoter of the KLK1 gene may also contribute to the increases KLK1 expression in AKI patients.

Major comments: This clinical study is an interesting study depicting the unexpectedly high urinary excretion of kallikrein in patients with severe AKI. Although it is unclear how the epigenetic changes in the 5’ region of the KLK1 gene occur and how they affect the transcription of KLK1, the findings obtained in the current study are still intriguing.
Discretionary Revisions:

(1) In the title, the description with parentheses ((KLK1) and (AKI)) could be omitted. These parentheses and abbreviations have now been deleted.

(2) In the conclusion of the abstract, the sentence “Unexpectedly, increased KLK1 excretion in AKI was likely a consequence of increments in adrenergic tone during BP depression” can be split into two sentences like “Unexpectedly, increased KLK1 excretion in AKI patients was found. This increase is likely to be due in part to increments in adrenergic tone during BP depression.” We made the suggested change in sentence structure.

(3) In the conclusion of the abstract, the sentence “Furthermore, higher kallikrein excretion predicted an increased frequency of non-recovery from AKI.” could be changed into “Furthermore, the high kallikrein excretion is associated with poor prognosis in AKI.” As a result of additional analyses suggested by Reviewer #4, we have now deleted this sentence altogether (see critique on analysis methods below).

(4) In Figure 6, the exogenous part may be omitted, because it is not the mechanism whereby AKI by itself increases renal KLK1 expression. I understand this point. However, the exogenous factors (pressor drugs, diuretics) are known to trigger renal kallikrein excretion, and a subset of our AKI subjects were exposed to these two drug categories (Table 1), so these are plausible (if partial) explanations for the KLK1 findings in our cases. In the Figure legend, we now state: “Finally, exogenous factors such as adrenergic pressor infusions or diuretic treatment can also increase renal kallikrein production; indeed, since a subset of our AKI cases received such treatments (Table 1), we cannot exclude this possibility.”

Reviewer 3.
Reviewer: Francois Alhenc-Gelas
Reviewer's report:
This study assesses urinary kallikrein activity through S2266 hydrolysis in acute kidney injury patients and also includes a kallikrein (KLK1) gene promoter methylation study in blood and urine of these patients. Reduced S2266 hydrolyzing activity was found in incipient acute kidney injury and, more surprisingly, increased activity was observed in patients with established kidney injury. Testing the value of K1 as a prognosis biomarker in renal injury, even retrospectively, is certainly of potential clinical interest. However there are questions related to data generation and interpretation that need to be addressed for better assessing the potential clinical impact of these observations.

Major points.
1-Kallikrein is followed by measuring hydrolysis of a tri-peptide commercial substrate [D] Val-Leu-Argpara-nitroanilide (S2266). This method has been only validated versus a reference kinin forming assay in normal urine, and may not be specific for kallikrein in pathological situations when other proteases are present (for example trypsin readily hydrolyzes S 2266). In established AKI presumably plasma proteins are excreted in urine and activity of excreted plasma proteases may influence the S2266 data and contribute to high level of activity observed. Aprotinin used for making a blank is a poly-specific protease inhibitor and not a specific kallikrein inhibitor. For example aprotinin inhibits plasmin. Conversely protease inhibitors may also be present in urine in AKI and influence data. The authors should consider validate their measurement by using a kinin forming assay, at least in a representative subset of samples. Also information on urinary protein excretion should be included.
I agree with the reviewer’s comments on assay specificity. In the Methods section, we now state: “Specificity of the S-2266 amidolytic assay for glandular (KLK1, including renal, pancreas, and salivary) kallikrein activity in urine arises from two features: first, the substrate S-2266 is cleaved only by a particular subset of serine proteases, including KLK1; and second, the inclusion of aprotinin in the assay blank, which specifically inhibits serine proteases including KLK1; nonetheless, only an immunoassay specific for lysyl-bradykinin (the kinin product of KLK1) generation could provide absolute assurance of specificity for KLK1.”

I agree that measurement of urine protein is a good idea, and we have now taken the opportunity to evaluate these measurements. In the Results section, we now state: “Urine albumin excretion. Quantitative urine albumin excretion was evaluated in established AKI cases and healthy controls. Albumin values in cases ranged from 2.0-4490 mg/gm creatinine (mean, 1090 mg/gm), but kallikrein and albumin excretions did not correlate (Pearson r=0.006, p=0.54), rendering it unlikely that elevated kallikrein activity in AKI arose simply from pathological excretion of plasma proteases. In healthy controls, albumin excretion was 6.27±0.39 mg/gm creatinine.”

2-The KLK1 gene promoter methylation study is rather descriptive. The discussion around epigenetic data is very speculative. The DNA sources, blood and urine, are both heterogeneous with regard to cell content making it difficult to draw conclusions as to consequences of epigenetic alteration in this clinical setting.

I agree with your caveats. In the Discussion section, we now state: “It should be noted that the sources of DNA for these epigenetic studies in blood and urine are likely to be heterogeneous – blood DNA could emerge from any leukocyte subpopulation, while DNA in urine can emerge from cell types other than renal. Nor have we established whether the promoter CpG methylation events we observed have functional consequences for transcription.”

Minor point
-In the scheme presented in figure 6 almost each pathway proposed as controlling urinary kallikrein activity, including KLK1 promoter methylation, is speculative.

I agree with your caveats. Thus, we have made two changes in the Figure legend. First, we clearly indicate the hypothetical nature of the diagram. We title the Figure: “Hypothetical schema integrating experimental findings in this study of KLK1 in AKI.” Second, we indicate that the diagram is not intended as an illustration of fact, but rather to generate hypotheses. In the Figure legend, we now state: “This diagram is presented not as established fact, but rather to generate hypotheses for further investigation.”

Reviewer 4.
Title: Renal kallikrein (KLK1) activity and epigenetics in human acute kidney injury (AKI): Expression, mechanisms and consequences.
Reviewer: Qin Zhang
Reviewer’s report:
This is a potentially very important research article. As the title suggests, this manuscript has tried to cover a wide range of information, including the expression, mechanisms and consequences of KLK1 activity in human acute kidney injury. However, not all of these aspects can be addressed thoroughly within the scope of this article. Researchers from various fields may ask for more data from their perspectives. Nevertheless, I think the “consequence” part should be strengthened due to its potential clinical implications.

• Major Compulsory Revisions
1. Based on my understanding, the author seems to treat the primary outcome, recovery of renal function, as binary variable. That is yes or no, yes for recovery, and no for not recovered. If this is the case, to examine predictors of primary outcome, the author ought to
use logistic regression, instead of linear regression. In case the author used the values of eGFR or eGFR recovery percentage at 6-month follow-up, rather than yes or no for the dependent variable, the author could include a plot to demonstrate the linear relations of baseline KLK1 and eGFR recovery.

I agree with your statistical suggestion, and we have now reanalyzed the categorical outcome in AKI (recovery/non-recovery) using binary logistic regression. On that analysis, we do not see an effect of initial KLK1 excretion on categorical outcome. Therefore, we have removed the previous analysis from the Results section.

We have now also plotted and analyzed the relationships between initial KLK1 excretion and either final eGFR or change in eGFR (initial → final). In neither case do we see a significant prediction by initial KLK1 excretion: for initial KLK1 on final eGFR, r=0.09, p=0.67; for initial KLK1 on change in eGFR, r=0.135, p=0.52. Since the correlations were not significant, we have not included the plots in the final version.

2. I would like the author to add more data regarding the primary outcome, such as how many recovered, how many ended up in dialysis dependent, what was average eGFR at follow-up.

In Table 1, we have now included data on several kinds of outcomes in subjects with established or incipient AKI: requirement of dialysis (at any time), recovery from AKI (as defined in the Table legend and below), remaining dialysis dependent at follow-up, final eGFR at follow-up, eGFR change at follow-up, and sCr change at follow-up.

- Minor Essential Revisions

1. I understand this is a pilot study, which didn’t include big sample size. I am not very particular about statistical comparisons; however, there are a few things I would like the author to include as descriptive information. For instance, table 2 lists the cause for AKI, ischemia, nephrotoxins, Septic and multi-factor, what are average KLK1 levels in these categories? Are they close or some of them may look different than others? Do the nephrotoxins damage kidney directly then affect the blood supply so that KLK1 activities respond differently? I hope some of the theories may be reflected from the data.

Mean and standard error values for kallikrein activity excretion corresponding to each of the causal factors (ischemia, nephrotoxins, septic, multifactorial) of AKI are now listed in Table 2 for established AKI. KLK1 excretion did not vary systematically by causal factor (ANOVA p=0.83). In the Results section, we now state: “KLK1 excretion did not vary by AKI causal group (ANOVA p=0.83).”

2. The author defined the primary outcome renal function recovery as “… a return to within 10% of baseline …”. Did the author mean to say as long as eGFR reach more than 90% baseline, we considered renal function recovered? For instance, if baseline eGFR was 100, recovery required eGFR at follow-up was 90 or higher, is this correct?

Yes, that is exactly how we defined recovery. We have now included the definition in both Methods and the Figure 1 legend.