Author’s response to reviews

Title: NGAL as a possible biomarker for sepsis-induced kidney failure: evaluating changes in NGAL expression in a rat model of acute kidney injury

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Author’s response to reviews: see over
Dear editor,

We would like to submit the manuscript entitled "Renal neutrophil gelatinase associated lipocalin expression in lipopolysaccharide-induced acute kidney injury in the rat" to BMC Nephrology as a regular article. This is an original work of our institute and has not been published, and it will not be submitted or published elsewhere. Please let me introduce some elementary knowledge of our study.

Neutrophil gelatinase associated lipocalin (NGAL) has been demonstrated to be a highly predictive biomarker of acute kidney injury (AKI). Based on the available evidence, the use of humoral NGAL as a marker of septic AKI is promising but requires further investigation. In particular, the relationship between humoral NGAL, NGAL expression and renal injury requires additional clarification. However, few studies have specifically investigated the expression of NGAL in renal tissues during sepsis. Thus, in this study we investigate the pattern of renal NGAL expression, and to explore the relationship between renal inflammation mediators such as TNFα and IL-6, and plasma/urinary NGAL in a rat model of sepsis-induced kidney injury. We first expound the temporal changes and biology sources of NGAL gene expression in the tubular epithelia occurs following LPS-treatment, and this finding is coupled with the observation of renal TNFα and injury. Only uNGAL levels accurately reflect the changes of NGAL mRNA.

Thank you very much for your consideration. If you have any questions about my submission, please contact me.

Sincerely yours,
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Response to Frank Therened’s report

Major compulsory Revisions:
1. Figure 2 and legend to Figure 2 do not match. Figure 2 B does not show southern blots as described in the legend. Figure 2C does not describe ISH signal time-course but RT-PCR time-course.

   Reply: We apologize because we incorrectly explained Figure 2. The correct explanation is as follows: “A, In situ hybridization signal time-course. A strong hybridization signal was observed in the cortical tubular epithelia in samples in the 3-, 6- and 12-hour LPS groups compared with a weak signal in the 1- and 24-hour LPS groups, and there was no signal in the controls. B, Relative expression of NGAL mRNA (relative to the controls) is shown. C, Agarose gel electrophoresis imprinting expresses the intensity of NGAL mRNA in the LPS-treated rats.” Figure 2 and its legend have been corrected in the manuscript, and we have added densitometry results of the PCR marker. The NGAL primer is shown in Table 2, and its PCR product was 163 bp, which is shown in Figure 2C.

2. Figure 4B does not show a southern blot but RT-PCR.

   Reply: We apologize for incorrectly explaining Figure 4. Figure 4B shows an image of 2% agarose gel electrophoresis imprinting to verify the PCR products. The fourth band in the blot is iNOS, which was observed in the rat model in our preliminary experiment, and then abandoned. TNFα is the third band. The primers and PCR products are shown in the Table and image below. The literature source that we used for the TNFα primer is presented as well below. Figure 4B and its legend have been corrected in the manuscript, and we have added the densitometry results of the PCR marker.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers</th>
<th>Length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNFα:</td>
<td>5’- TACTCCAGGTTCTCTTCAGG -3’</td>
<td>103</td>
</tr>
<tr>
<td></td>
<td>5’- GGAGGTGACTTTCTCTGGA -3’</td>
<td></td>
</tr>
<tr>
<td>IL-6:</td>
<td>5’- TCCTTGAAACTCTACAAGGCC -3’</td>
<td>190</td>
</tr>
<tr>
<td></td>
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</tr>
<tr>
<td>iNOS</td>
<td>5’- AGCATCCCAAGTACGGAG -3’</td>
<td>402</td>
</tr>
<tr>
<td></td>
<td>5’- GGACGCCAAATCCAGT -3’</td>
<td></td>
</tr>
<tr>
<td>GAPDH:</td>
<td>5’- GGCATGGAGCTGTCATGA -3’</td>
<td>244</td>
</tr>
<tr>
<td></td>
<td>5’- TTCACCACCAGGGAAGGC -3’</td>
<td></td>
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</tbody>
</table>
3. The authors need to discuss the work of Paragas N et al. (Nat Med. 2011 Feb;17(2):216-22) in the context of their study.

Reply: As suggested by the reviewer, we have carefully read the literature and discussed the work of Paragas et al. in Discussion of the revised manuscript. We found that some of the observations in their study are similar to our study. We made the following observations: 1) NGAL was induced in both mouse and rat kidneys by lipid A or LPS i.p.; 2) kidney NGAL and uNGAL were closely correlated, both
temporally and in the intensity of their response; 3) Paragas et al suggested that NF-κB plays an important role in NGAL regulation, and in our study, we found NGAL mRNA was related to TNFα mRNA, which is an important activated factor of NF-κB. We also found that some of the results were different between the two studies as follows: 1) Paragas et al demonstrated that renal NGAL and uNGAL were more sensitive measures of ischemia-reperfusion (I/R) AKI than were sCr and uCr, confirming earlier work in mice (ischemic renal injury) and humans (renal injury after cardiac surgery). In our study, sCr was the same sensitive as renal NGAL mRNA and uNGAL in LPS-induced AKI. We believe that the reasons for this result are increased sCr production by fever, asitia and decreased output by transient anuria after LPS i.p.. 2) Paragas et al demonstrated that NGAL mRNA was expressed by the thick ascending limbs of Henle, the macula densa, and intercalated cells of the collecting ducts 24 h after I/R AKI or treatment with lipid A (15 mg/kg). No NGAL expression was found in the proximal tubules. These data were confirmed by NGAL-Luciferase 2 quantitative analysis of cortical and medullary cells treated with lipid A for 24 hours. In our study, we observed weak NGAL mRNA expression in the distal tubular epithelium and medullary collecting ducts of the control kidneys; however, NGAL mRNA expression was not detected by qRT-PCR. In contrast, 24.3-49.6% of the tubular cells, including the proximal tubules, were NGAL mRNA positive in the kidneys of Groups 2 to 5 (1, 3, 6 and 12 hours post-LPS treatment, respectively), but NGAL mRNA expression was significantly decreased in the samples from Group 6 (24 hour post-LPS treatment). This finding was also confirmed by qRT-PCR. These data described above suggest the following. 1) Different parts of the kidney have different responses to factors causing injury, including endotoxin, but overall, the injured epithelia are the source of NGAL. 2) Epithelia in proximal tubules might be sensitive to endotoxin, and they can upregulate the NGAL gene even though the LPS concentration is low, but the reactive period is short. Thus, NGAL expression can be detected in the early stage after LPS administration, and then decrease to baseline levels. 4) The cells in the medullary tubules might be insensitive, and they will not become active until they face a high concentration of LPS. However, they can increase NGAL expression longer than the cells in proximal tubules.

4. The authors should emphasize what is specific/novel about their study compared to the human studies or the work of Paragas et al.?

Reply: We have added information to the Discussion to highlight our new findings as follows:
1) “Moreover, the relationship between NGAL in renal injury and humoral NGAL needs to be clarified. Paragas and colleagues [45] verified that uNGAL originates in the kidney from results of cross-transplants between NGAL knockout and wild-type mice, followed by renal artery clamping. They found that NGAL is present in the kidney, liver, spleen, lung and trachea after lipid A treatment, which indicates that NGAL in the blood is not a good marker of septic AKI [45]. Our study revealed, for the
first time, the pattern of renal NGAL expression during the early stage of endotoxemia. We found that renal NGAL could be a useful biomarker of renal epithelia injury. In particular, uNGAL exactly reflected the change in renal NGAL expression, whereas pNGAL was not as accurate in septic AKI. Therefore, based on the findings of these studies, it appears that uNGAL levels are related to NGAL gene expression in the kidney and uNGAL has the ability to act as a marker for the diagnosis and monitoring of AKI in patients with sepsis. This could lead to renoprotective therapies and avoidance of renal injury. However, pNGAL or sNGAL levels may be misleading in the diagnosis of septic AKI. Misdiagnosis may result in conservative strategies and the optimal therapeutic time in critically ill patients may be missed.”

2) “Furthermore, for the first time, we showed that NGAL mRNA was related to TNFα mRNA levels in the injured kidney, and that NGAL mRNA upregulation closely followed TNFα mRNA, similar to a down-regulation cytokine in the inflammation cascade. In previous studies [17, 45], it has been found that NGAL is suppressed by an NF-κB inhibitor in primary kidney cells after lipid A administration and that the NGAL:siderophore:Fe complex can protect proximal tubule N-cadherin, and it inhibits cell death. Therefore, is it possible that TNFα/NF-κB/NGAL is an important mechanism for regulating tubular cells through increasing proliferation and/or inducing/suppressing apoptosis during sepsis. This possibility should be investigated in future studies.”

Minor Essential Revisions:
5. p. 4 define ISH
Reply: We have spelled out ISH as ‘in situ hybridization” instead.

6. p. 4 2nd paragraph …established a suitable animal model…,
not …suitable for animal…
Reply: We have corrected this error in the manuscript.
Response to Mehryar Habibi Habibi Roudkenar’s report

1, the title is not appropriate and does not reflect the finding of this study
Reply: The title of our study has been changed to “Renal neutrophil gelatinase associated lipocalin expression in lipopolysaccharide-induced acute kidney injury in the rat”. We based this title on the fact that we detected dynamic changes in NGAL expression in LPS-induced renal injury. Moreover, we also examined the relationship between renal NGAL mRNA, inflammatory mediators and humoral NGAL.

2, Purpose and goal of the study have not been highlighted in introduction. Importance of NGAL as a early biomarker and brief description of NGAL should be mentioned in the introduction part. This has been mentioned in the discussion, it would be better transfer this to introduction.

Reply: We have added some information in the Background to illustrate the importance of NGAL as a early biomarker as follows: “Recently, genomic, transcriptomic, and proteomic techniques have identified neutrophil gelatinase associated lipocalin (NGAL) as an early marker of AKI [5, 6]. NGAL has been investigated in a range of different clinical settings, such as contrast-induced nephropathy, AKI after cardiac surgery or kidney transplantation and AKI in the critical care setting. Overall, the sensitivity for NGAL to predict AKI is 0.815 (95% confidence interval, 0.732-0.892) and it is a promising biomarker for AKI, similar to troponin for acute myocardial infarction [7].” We further defined the purpose of our study. To accurately investigate NGAL in critically ill patients, the role of NGAL in impaired renal tissue needs to be determined. To alert the clinician to a potentially reversible stage of the illness, the relationship between NGAL and early inflammation mechanisms needs to be investigated, in particular the relationship to TNFα in renal during sepsis. We have added the following text to the last paragraph in the Introduction: “Therefore, the purpose of this study was to investigate the pattern and localization of renal NGAL expression, and to explore the relationship between renal inflammation mediators, such as TNFα and IL-6, and humoral NGAL in a rat model of lipopolysaccharide (LPS)-induced kidney injury.”

3- the reasons to measure IL6 and TNF# in parallel to NGAL should be mentioned in the end part of introduction

Reply: We have added information in the Background giving the reasons for measuring IL-6 and TNFα, as follows: “Furthermore, a biomarker should temporally reflect the pathophysiology initiated by a stimulus leading to injury so as to alert the
 clinician to a potentially reversible stage of the illness. In the case of septic AKI, the relationship between NGAL and early inflammation mechanisms in the kidney will ought to be clearly established. However, the mechanisms of septic AKI remain undefined. Increasing evidence suggests that intrarenal inflammatory mediators play an important role in the pathogenesis of kidney injury[11-14]. Specially, tumor necrosis factor α (TNFα) is a pivotal proinflammatory mediator and interleukin (IL)-6 is an accessory factor [15, 16]. However, it remains unknown whether NGAL expression is related to changes in renal IL-6 and TNFα during the early stage of septic AKI.”

4- One of the main and new finding of this study was positive correlation between NGAL and TNFα. Therefore this should be highlighted throughout the manuscript especially in discussion part.

Reply: We appreciate the reviewer’s comment. We have added information to the Discussion to highlight the new findings as follows: “Sepsis is associated with the production of many inflammatory mediators, including TNFα [51, 52]. TNFα is released first when sepsis occurs and leads to cleavage of the nuclear factor κB (NF-κB) inhibitor. Once this inhibitor is removed, NF-κB is able to initiate the production of mRNA, which induces the production of other proinflammatory cytokines, chemokines, and adhesion molecules [53]. For instance, Knotek et al demonstrated that TNFα is a critical mediator of endotoxin-induced sepsis, and that TNFα inhibition prevents physiological changes and morbidities associated with LPS administration in wild-type and inducible nitric oxide synthase knockout mice [54]. Therefore, TNFα is a key mediator of LPS-induced acute renal failure, acting through its receptor, TNFR1 [55]. Early changes in renal TNFα mRNA levels after endotoxemia have not been reported. In our study, we found that TNFα mRNA was induced by 24-fold within 3 hours after LPS treatment. The result of our study also confirm the findings of Wang and colleagues, they showed that LPS induced TNF-α protein by 24-fold in the kidney 16 hours after administration[14]. Furthermore, for the first time, we showed that NGAL mRNA was related to TNFα mRNA levels in the injured kidney, and that NGAL mRNA upregulation closely followed TNFα mRNA increase, similar to a down-regulation cytokine in the inflammation cascade. Previous studies [22, 50] have found that NGAL is suppressed by the NF-κB inhibitor in primary kidney cells after lipid A administration, and that the NGAL:siderophore:Fe complex preserves proximal tubule N-cadherin and inhibits cell death. Therefore, is it possible that TNFα/NF-κB/NGAL is an important mechanism for regulating tubular cells by increasing proliferation and/or inducing/suppressing apoptosis during sepsis? This possibility should be investigated in future studies”.

5- The concentration of NGAL, measured by ELISA, should be checked whether pg/ml is correct? I think they should be ng/ml since they were compared with standard ranging from 1-200 ng/ml as described in the methods
Reply: We appreciate the reviewer pointing this out and have corrected our error, and changed the units to “ng/ml”.

6- Quantification of mRNAs expression are not clear. The authors should describe how did they calculated relative expression? Real time PCR or densitometry of the PCR bands? In figure 2, there is no data to address southern blot analysis. The result of this figure is confusing and not convincing.

Reply: We have added several sentences to the Methods section to describe how we calculated the relative expression of mRNA: “The fold change in mRNA levels of each gene was calculated using the △△CT method. The mRNA levels were normalized by using GAPDH as a housekeeping gene, and they were compared with the control group. The authenticity and size of the PCR products were confirmed by 2% agarose gel electrophoresis imprinting. The primers used for amplification are listed in Table 2.”

The explanation for Figure 2 is not correct. We apologize for this error. Figure 2C shows an image of 2% agarose gel electrophoresis imprinting to verify the NGAL PCR product. We have added the densitometry results of the PCR bands. The NGAL primer is shown in the table and its PCR product was 163 bp, which is shown in the image below. Figure 2 and its legend have been corrected in the manuscript, and we have added the densitometry of the PCR marker.

NGAL
Forward primer: GATGTTGTATCCTTGAGGCCC
Reverse primer: CACTGACTACGACCAGTTTGCC
PCR product size: 163 (bp), Rattus norvegicus

7- figure 4, the size of bands should be indicated, why the PCR size of TNF# is large? This figure in my view again is not convincing.

Reply: The explanation for Figure 4 is not correct. We apologize for this error. Figure 4B shows an image of 2% agarose gel electrophoresis imprinting to verify the PCR products. The fourth band is iNOS, which was observed in the rat model in our preliminary experiment, and then abandoned. TNFα is the third band. The primers and PCR products are shown in the table and image below. The literature source of
the TNFα primer is also presented. Figure 4 and its legend have been corrected in the revised manuscript, and we have added the densitometry results of the PCR marker.

Table. Primers used for qRT-PCR

<table>
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<th>Primers</th>
<th>Length (bp)</th>
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<tr>
<td>TNFα:</td>
<td>5’- TACTCCCAGGTCTCTTCAAGG -3’ 5’- GGAGGTGACTTTTCCTGGTA -3’</td>
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<td>5’- TCCCTTGAACTCTACAAGGACC -3’ 5’- GTATCCACCATATGCCAGGC -3’</td>
<td>190</td>
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<td>iNOS</td>
<td>5’- AGCATCCCAAGTGACGTGAGT-3’ 5’- GGACCAGCAATCCAGT-3’</td>
<td>402</td>
</tr>
<tr>
<td>GAPDH:</td>
<td>5’- GGCATGGACTGTGCTGA -3’ 5’- TTCACCACCATGGA GAAGGC -3’</td>
<td>244</td>
</tr>
</tbody>
</table>

Fig. 1: Products of qRT-PCR shown by agarose gel electrophoresis imprinting.
<table>
<thead>
<tr>
<th>Gene (rat)</th>
<th>Forward</th>
<th>Reverse</th>
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<tbody>
<tr>
<td>vWF (Von Willebrand factor)</td>
<td>CCCACCGGATGGCTAGGTATT</td>
<td>GAGCCGGATCTGTGTTGAGGTT</td>
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<tr>
<td>CD31 (PECAM-1)</td>
<td>CTCTTAAGAGCAAAAGAGCAACTTC</td>
<td>TACACTGTATTCCATGTCTCTGG</td>
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<tr>
<td>VE-cadherin</td>
<td>ACCAGTGACAGGGCCAACTT</td>
<td>GGCCTCCACAGTCAAGGGTTATAC</td>
</tr>
<tr>
<td>VEGFR2 (Flk-1)</td>
<td>ATCTACACTCCAGTGGT</td>
<td>GAAATGGTGACCTGTGATCTTGA</td>
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<td>VEGFR1 (Flt-1)</td>
<td>GACAGAGCAACAGGAGGC</td>
<td>AGCCAGGAGTGAGAAATCA</td>
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<td>TACE (ADAM17)</td>
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<td>GAPDH</td>
<td>AGAACATCATCCCTGATCC</td>
<td>CACATTGGGGGTAGGAACAC</td>
</tr>
</tbody>
</table>

*Cell Transplant. Author manuscript; available in PMC 2010 April 7.*

Fig. 2: Literature source of the TNFα primer.