Author's response to reviews

Title: Citrate prevents heparin induced complement activation and neutrophil degranulation, when used for anticoagulation during continuous venovenous haemofiltration in critically ill patients.

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Author's response to reviews: see over
Dear Dr Henderson and reviewers,

Thank you very much for the comments regarding our manuscript “Citrate prevents heparin induced complement activation and neutrophil degranulation, when used for anticoagulation during continuous venovenous haemofiltration in critically ill patients.”

In this letter we provide a detailed point-by-point response to each of the referees concerns. We have highlighted all changes made in the manuscript.

On behalf of all the authors,

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Reviewer 1

Major Compulsory Revisions,
1. The total population of the research were obtained from 2 separate populations, citrate vs. no anticoagulant and citrate vs. heparin, then the patients in the citrate groups were combined. Thus, comparison between no anticoagulant vs. heparin might be inappropriate. The separate comparisons of citrate vs. no anticoagulant and citrate vs. heparin without no coagulant vs. heparin might be more appropriate.

1. The reviewer addresses the issue that the citrate groups consist of patients from two different studies and indeed this is the case. However, before combining the groups, the two citrate groups were compared. There were no baseline differences between the groups concerning disease severity, laboratory values or other characteristics, we therefore feel that combining the groups was appropriate and that the comparison of the citrate group to the heparin and no anticoagulation group is valid.

Page 7 “Since there were no baseline differences between the two separate citrate groups (citrate vs. no-anticoagulation and citrate vs. heparin), the citrate data were pooled (Group 3)”.

2. In table 3, the baseline of the laboratory data, there were differences in platelet and creatinine among groups. This might affect the neutrophil function and platelet degranulation which might affect complement activation. The crossover of different methods in the same patients might be appropriate to study this topic.

2. The reviewer addresses the issue that there are baseline differences in platelet count and creatinine levels among groups. As indicated in the manuscript, the differences in platelet counts can be explained by the fact that patients with low platelet counts were considered at increased risk for bleeding and therefore did not receive anticoagulation, or were treated by citrate anticoagulation. The creatinine levels were lower in the no anticoagulation group and as stated in the manuscript, we therefore cannot exclude CVVH was started sooner in this groups. We do not think, however, that the baseline differences will have affected the neutrophil function. The study, however, was not designed to explore interactions of platelet degranulation and influence on complement activation, or influence of creatinine levels on this matter. This was a proof-of-principle study to address differences between the anticoagulation method and degranulation of neutrophils and levels of complement factor 5a.
We feel further research is needed to answer more detailed questions concerning the influence of platelet counts and creatinine levels on complement activation and degranulation of neutrophils, but we suspect on the basis of the literature that these effects are minimal.

3. The title is not appropriate, lower C5a level might not be caused by lower complement activation and lower MPO might not be the result of lower neutrophil degranulation (but lower endothelium excretion).

3. The reviewer feels the title is inappropriate. MPO can, apart from being released when neutrophils degranulate, also be excreted by endothelium. We address this issue on page 9 and 10: “Apart from being stored in granules in neutrophils, MPO is also widely distributed in the endothelium of blood vessels. The observed transient release of endothelium-bound MPO within minutes after bolus administration of heparin to patients agrees with the literature [16] and this phenomenon should be taken into account to consider plasma MPO as an indicator of bioincompatibility [21]. The concentration in inlet plasma of the heparin group, as an indicator of systemic MPO release, did not exceed that in the other groups, thus arguing against substantial endothelial release in our patients.”

If heparin would cause an increase in MPO excretion by the endothelium, it would be expected that plasma levels of MPO (inlet) would be higher in the heparin anticoagulation group, which is not the case. When looking at total mass production rate of MPO by extracting the MPO pre-filter of MPO levels post-filter, it is interesting that MPO production is higher in the heparin group. Furthermore, elastase production rates over the filter support this theory; it is highest when heparin is administered as anticoagulation and suggests that with heparin, there is more degranulation of neutrophils.

However, we have changed the title into the following:

“Citrate prevents heparin induced complement activation and neutrophil degranulation, when used for anticoagulation during continuous venovenous haemofiltration in critically ill patients.”

4. The higher Uf rate in citrate group might cause a lower contact time of blood and cellulose. The proper correction/presentation might be necessary.

4. The reviewer addresses the issue that the ultrafiltration rate in the citrate group is somewhat lower than in the other groups. We have stated the difference in ultrafiltration rate.
Correction of this difference in respect of contact time is difficult, since such a standard comparison is, to our knowledge, non-existing. However, when calculating the total mass production rate of C5a over the filter, we corrected for the ultrafiltration flow: \( M_{uf} = Q_{uf} \times C_{uf} \). \( M_{uf} \): mass ultrafiltration rate, \( Q_{uf} \): ultrafiltration flow rate, \( C_{uf} \): concentration in ultrafiltrate and \( M_{tp} = (M_0 + M_{uf}) - M_i \), \( M_i \): mass inlet rate, \( M_0 \): mass outlet rate, \( M_{uf} \): mass ultrafiltration rate, \( M_{tp} \): mass production rate.

Yet differences in ultrafiltration rate were low, we cannot exclude that this could have affected contact time of blood and cellulose and clearance of C5a. We have added the following to the discussion of the manuscript:

Page 9 “Due to higher ultrafiltration flows, concentrations of C5a in the ultrafiltrate were lowest in the citrate group, as was the sieving coefficient. We cannot exclude, however, that citrate anticoagulation was associated with diminished C5a clearance through the filter, but it seems more likely that lower ultrafiltration flow in this group was responsible for this finding.”

Page 11 “Ultrafiltration rate in the citrate group was somewhat higher than in the other groups, so that we cannot exclude an effect on filter and blood contact time and on clearance of C5a.”

Minor Essential Review,

1. The description in the result section should be rewritten. For an example, in result page 8 under “Elastase” topic, “The concentration at outlet increase over time”, but in the figure the concentration increase from 10 min to 60 min and then seems to be at the same level through 720 min.

1. The reviewer address the issue that the Elastase topic in the results is not clear. We have changed the following to the manuscript:

Page 8 “The concentration at outlet increased over time, mostly between 10-60 minutes, irrespective of the anticoagulation regimen.”

2. The correlation and the sepsis mortality data should be demonstrated.

2. The reviewer would like the sepsis mortality and correlation demonstrated. We have rewritten the Results section:
Page 9 “For patients with and without sepsis, there were no differences in C5a and elastase concentrations in inlet plasma over time. For MPO however, levels of MPO were higher in the sepsis group (166 (14-2670) ng/ml versus 84 (15-2611) ng/ml in non-sepsis, P=0.011). Concentrations of C5a did not differ between outcome groups. Plasma concentrations of elastase and MPO, however, were higher in ICU non-survivors than in survivors (elastase; 964 (155-7451) ng/ml versus 394 (28-4235) ng/ml, P<0.001 and MPO; 174 (29-2670) ng/ml versus 85 (14-1086) ng/ml, P<0.001).”

3. In the result section, the name of the group should replace the group number to make it easier to follow.

3. The reviewer address the issue that in the results section, the group number should be replaced by the group name (anticoagulation administered). We have applied the suggestion throughout the manuscript.

4. In figures, the symbol name should also be present in the figures.
5. Figure 1D. the gray color columns were not clearly seen.

4. and 5. We have made changes to the figures.

Level of interest: An article whose findings are important to those with closely related research interests

Quality of written English: Acceptable

Statistical review: No, the manuscript does not need to be seen by a statistician.
Reviewer 2.

Ad Abstract (Major)
1. The results are not clearly presented. With the present description they are difficult to understand for the reader. Please re-write the results.

1. The reviewer addresses the issue that the results in the abstract are not clearly presented. We have rewritten the abstract:

Page 2. “Net production of C5a across the filter was higher in the heparin group than in the other groups, but production decreased over time (P=0.007 vs other groups). The concentration of C5a in the ultrafiltrate decreased over time in all groups. Inlet (plasma) concentration of C5a was higher before starting CVVH in the citrate group, and increased most over time in the citrate group as well. Inlet C5a remained unchanged in the other groups. There was net production of elastase and MPO across the filter during heparin anticoagulation, while production was minimal and absent in the no anticoagulation and citrate group, respectively. During heparin anticoagulation, plasma concentrations of MPO at the inlet increased in the first 10 min of CVVH (P=0.024).”

Ad Introduction

2. P3 (Minor) Please ad ‘as’ before ‘observed’ to line 7 of paragraph 2. Please change ‘lacked a control group’ into as compared to heparin’ in line 12 of paragraph 2.

2. We have made the following changed to the manuscript:

Page 3 “As demonstrated in intermittent haemodialysis and in blood in vitro, calcium-chelating citrate anticoagulation may lower polymorphonuclear cell degranulation as observed with heparin; this may be partly independent of complement activation since cations, such as calcium, play a pivotal role in cell activation and degranulation [8-15].”

Page 3 “The single prior, albeit small, study suggesting prevention of neutrophil degranulation with citrate-CVVH as compared to heparin lacked a control group (no anticoagulation) and measurements of complement activation products [14].”
Ad Methods

3. P4 (Major) Please describe the criteria for using either no anticoagulation or citrate in patients with an increased risk of bleeding.

3. The reviewer addresses the issue that it is not clear when patients with an increased bleeding risk were treated with citrate of no anticoagulation. We have added the following to the manuscript to clarify:

Page 4 “The patient population and methods for this study are reported elsewhere [3,5]. In brief, the first study started one year before the availability of a custom-made citrate-based replacement fluid. From March 2004 to September 2005, patients admitted to the ICU who developed AKI necessitating CVVH but did not receive heparin due to a high bleeding tendency, either received anticoagulant-free CVVH (n = 13) or, after becoming available in 2005, regional citrate anticoagulation (n = 10) and were prospectively followed.”

4. P5 (Minor)

Please change ‘available’ in line 2 into ‘used’.

4. We have adapted the manuscript as follows:

Page 5 “For lactate- or bicarbonate-based CVVH, commercially prepared buffer solutions were used (BH504 [lactate-based] or HF32bic [bicarbonate-based] in n=6 Group 1 and n=5 in Group 2, Dirinco, Rosmalen, the Netherlands).”

5. Please describe the criteria for the use of lactate or bicarbonate replacement.

5. The reviewer addresses the issue that the criteria for the use of lactate or bicarbonate-containing replacement fluids is not clear. We have added the following to the manuscript:

Page 5 “Patients with high serum lactate levels (>5 mmol/L) were routinely treated with bicarbonate-buffered rather than lactate buffered CVVH.”

5. P7 (Major)
Ad C5a “The concentration in the inlet plasma was higher in Group 3”. Please mention explicitly that this was already the case at the start of CVVH (as suggested elsewhere), because from the figure it seems that this difference developed during CVVH, especially in the last 12-h of treatment. I wonder by which test the authors determined a significantly higher baseline concentration, because neither in the methods section nor in the results section a comparison between individual time points is mentioned. This could be of importance, also for other pattern differed between groups. Please explain.

5. The reviewer addresses the issue that it is not clear which test were used to compare individual time points. Individual time points were compared using Kruskal-Wallis tests. We have added the following to the methods:

Page 7 “Groups were compared for data at individual time points using the Kruskal-Wallis test.”

Also, as suggested, we have added the following to the C5a section:

Page 8 C5a “The concentration in inlet plasma was higher in the citrate group before initiation of CVVH than the other Groups (P=0.009) [Fig. 1].”

6. The reviewer addresses the issue that the manuscript contains only the p-values when concerning sieving coefficient, and not the calculated sieving coefficient. We have added the following to the manuscript:

Page 8 “The sieving coefficient was low and lower in group 3 than the other groups (no anticoagulation 0.49 (0.05-9.45), heparin 0.35 (0.12-11.2), citrate 0.10 (0.02-0.62), P<0.001) and decreased in time in all groups (P<0.001).”

7. C5a directly correlated with elastase and MPO in the inlet plasma”. This is remarkable given the different patterns between the mediators over time (increase of MPO in the first 10-min in
the H-group; increase in C5a in the last 12h in the C-group and an increase in MPO and elastase in the last 12-h in the NA group. Could the authors please comment on this?

7. The reviewer finds it remarkable that C5a correlated with elastase and MPO in the inlet plasma. Indeed, levels of C5a at inlet correlated with inlet levels of elastase and MPO irrespectively of time points (elastase $r_s = 0.37, P<0.001$; MPO $r_s = 0.30, P<0.001$). To clarify, we have changed that manuscript as follows:

Page 9 “Correlations. C5a directly correlated with elastase and MPO concentrations in inlet plasma for all groups and time points together (elastase $r_s = 0.37$, $P<0.001$; MPO $r_s = 0.30$, $P<0.001$). No such correlation was found across the filter.”

8. P9 (Major)
“We also suggest that citrate prevents endothelial release of MPO by heparin”. Please change this sentence into “We also suggest that heparin triggers endothelial release of MPO, while CVVH with citrate or no anticoagulation do not.” Or something alike. However, to my opinion the authors cannot conclude this from the present data, because they took no sample after the heparin bolus but before connection of the circuit. They can discuss this a finding from other studies, that this may be the case in the present study, but that unfortunately they cannot conclude this from the present data. At least this is my interpretation.

Page 10 “Apart from being stored in granules in neutrophils, MPO is also widely distributed in the endothelium of blood vessels. The observed transient release of endothelium-bound MPO within min after bolus administration of heparin to patients agrees with the literature [16] and this phenomenon should be taken into account to consider plasma MPO as an indicator of bioincompatibility [21].”

The reviewer states we did not take a sample after administration of heparin. However, the first sample was taken before administration of the first heparin bolus, and the following sample was taken 10 minutes after CVVH. There is a remarkable increase of MPO in the first 10 minutes of therapy in the heparin group only. Although we cannot exclude neutrophil degranulation in the filter being partially responsible for this finding, it seem in concordance
with other literature that the increase in MPO was, at least partly, caused by release of MPO by the endothelium. Nonetheless, we have adapted the manuscript as suggested:

Page 9. “We also suggest that heparin triggers endothelial release of MPO, while CVVH with citrate does not.”

9. (Minor) Please reformulate the sentence starting with “These findings..” (line 2).

9. We have reformulated the sentence:
Page 9: “These findings argue in favour of citrate being superior to heparin as anticoagulation in CVVH in terms of biocompatibility.”

10. “At initiation of CVVH and throughout the study, inlet levels of C5a were highest in the citrate group”. See above.

10. We have commented to this remark at point 5.

11. Major: The authors first suggest that elastase and MPO release was possibly associated with complement activation in the filter. However, this suggestion is not supported by the data of the present study nor by data from hemodialysis patients. Therefore, this is a theoretical association not supported by clinical data. Please reformulate this argumentation.

11. The reviewer comments on the issue that the argumentation it is not clear at present concerning complement activation in the filter being responsible for elastase and MPO release from neutrophils. We comment on this matter in the Discussion:

Page 10 “Total mass production rate of C5a in the filter was highest and thus most decreased over time in the heparin group. Therefore, increased neutrophilic degranulation evoked by heparin, illustrated by relatively high production of elastase and MPO shortly after connecting the filter, could be associated, at least in part, with complement activation in the filter, in contrast to observations during haemodialysis [17,18]. However, there was no correlation between complement activation and neutrophil degranulation across the filter whereas such correlation was present in patient’s plasma. Moreover, there was no evidence for neutrophil degranulation in the filter in the citrate and no anticoagulation group. The latter agrees with the idea that degranulation of neutrophils is, at least partly, calcium dependent and independent of complement activation [8,12,13,17,19].”
As stated, we cannot exclude complement was, at least in part, responsible for neutrophils degranulation.

12. Minor: Same paragraph: “Conversely, there was no evidence for neutrophil degranulation in the filter in the other groups.” I would suggest saying that there was not evidence for neutrophil degranulation in the citrate group and minimal degranulation in the NA group. This makes the later argumentation more consistent.

12. We have adapted the manuscript as follows:

Page 10: “Moreover, there was no evidence for neutrophil degranulation in the filter in the citrate group and there was minimal degranulation in the no anticoagulation group.”


13. We have adapted the manuscript as suggested.

14. Major: Please delete from the conclusion that citrate prevents MPO release from the endothelium. Although this may be the case, this cannot concluded from the present study. Inlet concentrations were not different between groups.

14. The reviewer addresses the issue that it should be deleted from the conclusion that citrate prevents endothelial release of MPO caused by heparin. We have commented on this matter before. However, we have made the following changes to the manuscript:

Page 10 “In conclusion, regional citrate anticoagulation during CVVH in critically ill patients with AKI resulted in less complement activation and neutrophilic release of potentially harmful degranulation products elastase and MPO in the filter when compared to heparin-CVVH.”

Level of interest: An article of importance in its field

Quality of written English: Needs some language corrections before being published

Statistical review: Yes, but I do not feel adequately qualified to assess the
statistics.

**Declaration of competing interests:**
I have no other competing interests than being interested in the field.

In addition to the Referees' comments, could you please also address the following editorial points:

1. Ethical Approval
   In the methods section, can you please include the full name(s) of the ethical committees that approved these studies.

   1. Page 4 “Study protocols were approved by the local medical ethical committee and performed in accordance with the Declaration of Helsinki.”

2. Abstract
   Please can you include the study objectives in the Background section of the Abstract.

   2. We have changed the abstract as follows:

   Page 2 “During continuous venovenous haemofiltration (CVVH), regional anticoagulation with citrate may be superior to heparin in terms of biocompatibility. Study objectives were complement activation (reflected by circulating C5a), neutrophil degranulation and endothelial myeloperoxidase (MPO) release.”

3. Figure Legends
   Please can you remove the figure legends from the uploaded images. Figure legends should be separate from the images. They should be included in the manuscript document and appear after the References.

   3. We have removed the legends from the figures and included them in the manuscript after the References.

4. Formatting
   Please also ensure that your revised manuscript conforms to the journal style ([http://www.biomedcentral.com/info/ifora/medicine_journals](http://www.biomedcentral.com/info/ifora/medicine_journals)). It is important that your files are correctly formatted.