Author's response to reviews

Title: Association of plasma F2-isoprostanes and isofurans concentrations with erythropoiesis-stimulating agent resistance in maintenance hemodialysis patients

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Dear Dr. Cupisti,

We are grateful to Drs. Del Vecchio and Bellizzi for their thoughtful comments and reviews of our manuscript entitled “Plasma isofurans concentrations are associated with erythropoiesis-stimulating agent resistance in maintenance hemodialysis patients,” and thank them for the opportunity to improve the manuscript. Below, we have provided responses to the reviewers’ comments and have also outlined the substantive changes made in response to these comments:

Reviewer #1 (Dr. Del Vecchio)

Major Compulsory Revisions:
1. The major point is that by definition it is possible to talk of response to ESA only in treated patients. Indeed, observational studies have clearly demonstrated that the patients who do not receive ESA treatment have a far better outcome and different characteristics than ESA treated patients. Looking at table 1, I noticed that the majority of the patients with ERI <3.78 were untreated. For this reason the analysis should be performed after taking out untreated patients (you may consider them in a supplementary analysis).

- We agree with the reviewer that patients who do not receive ESA treatment may be different in both measured and measured ways from patients who receive ESA treatment. Accordingly, and in response to this revision request, we have removed the 88 patients who had at least one study visit at which they were not receiving ESA therapy. The total size of the cohort is thus now 165 patients, and this change is now reflected in all tables and figures as well as the text. Supplemental Table S1 has been updated as well to incorporate the additional excluded patients. The original data from Tables 2 through 5 that report the results of the analyses on the full 253 patients have now been moved to the Supplementary Materials, and have been retitled Tables S2 through S5. A brief description of the results of these new sensitivity analyses has been added to the results section of the manuscript (page 13, line 13 to 22).

- We note that the overall conclusions and findings in both the baseline as well as the time-averaged analyses have not changed substantially as a result of this change in the study cohort makeup. We thank the reviewer for this suggested change, and in the opportunity to increase the external validity of our findings.

2. Another point is antioxidant treatment. This is a randomised trial and thus the authors need to analyse the direct association among antioxidant treatment and both markers of oxidative stress and ERI.

- We apologize for not being clearer regarding the reporting of the effect of the antioxidant treatment on outcomes in the PATH trial. The main results from the PATH trial have previously been published by our group (Himmelfarb et al., JASN, 2014). This publication is cited as reference #27 in the manuscript. This original paper analyzed the effect of an antioxidant treatment (mixed tocopherols plus α-lipoic acid) versus placebo on markers of inflammation and oxidative stress. Additionally, as a secondary analysis,
the effect of treatment assignment on ESA resistance was analyzed. To summarize the main findings of the paper, there were no significant differences between the antioxidant treated group and the placebo group with respect to concentrations of inflammatory markers (hsCRP and IL-6) or oxidative stress markers (F2-isoprostanes and isofurans). This finding was robust even after adjustment for baseline values, diabetes, history of cardiovascular disease, race, age and medication use. Additionally, there were no significant differences between groups at baseline or at each time point in the study with respect to ESA dose adjusted by blood hemoglobin concentration. An area under the curve (AUC) analysis confirmed this null finding. We have included the data on ESA resistance in this document as Table 1, and are found in the original Himmelfarb et al. paper as Table 4).

• Given that these data analyzing the effect of the antioxidant treatment on outcomes have been published previously, we have not included them in our manuscript. However, in our analyses, we have accounted for the experimental treatment exposure by adjusting for treatment assignment in our regression models.

3. More data should be added to Table 1. In particular, considering the possible factors influencing ESA response, information is needed on:
   • Serum albumin
   • Dialysis adequacy
   • PTH levels
   • Iron treatment
   • TSAT (this variable was used in the statistical analysis
   • Randomisation to study treatment

   We agree with the reviewer that our Table 1 was missing important data on baseline characteristics that would aid the reader in understanding differences among subgroups. We thank the reviewer for pointing this out, and we have added the requested data to Table 1, including % randomized to study treatment, serum albumin, dialysis adequacy as measured by Kt/V, PTH, weekly IV iron dose, and transferrin saturation.

Minor Essential Revisions
1. The abbreviation HD is more widely used than MHD
   • We have replaced MHD with HD or maintenance HD as appropriate throughout the abstract and the body of the paper.

2. The possible role of study treatment should be considered in the discussion section
   • We agree with the reviewer that it is important to address the possible impact of study treatment. We have added two sentences to the last paragraph of the discussion that read as follows: “Though the results from the PATH trial showed no significant impact of antioxidant treatment status on ESA resistance over study follow-up, it is possible that treatment status may impact the association between inflammatory or oxidative stress markers and ERI. We have attempted to account for this by controlling for trial group assignment in our multivariable regression models, by the possibility of residual confounding remains.”
3. The patients enrolled in this study were younger and with a mean BMI higher than that commonly observed in everyday clinical practice. Please discuss the role of age and BMI on ESA response

- We agree that it is important to consider the role of age and BMI on ESA response, and appreciate the reviewer pointing this out. We have added four sentences to the limitations paragraph in the discussion section that acknowledges that our cohort has somewhat lower age and higher BMI than the general ESRD population, and discusses how we have addressed the influence of these factors on ERI in our study. We have also added three new citations relevant to this issue. The added sentences read: “Fourth, patients enrolled in the PATH trial were slightly younger and had a higher mean BMI than the general United States ESRD population, which may limit generalizability. Recent work, however, has shown that age does not predict ESA response in either pediatric or adult patients undergoing dialysis. Additionally, though BMI is associated with ESA dose in HD patients, the correlation is weak, and calculation of ERI incorporates body weight. Additionally, we have adjusted for both age and BMI in our final adjustment models.”

Discretionary Revisions:
1. Discussion: there is an interesting study about carbamylate albumin and ESA hyporesponse. Please considering quoting and commenting it

- We thank the reviewer for bringing the paper by Kalim et al. to our attention, and we agree that it is very much relevant to our discussion. We have added a citation and brief reference to the study on page 16, lines 16-20.

Reviewer #2 (Dr. Bellizzi)

Major Comments
1. The study enrolled 253 out of 353 patients from the PATH study, either treated or untreated control subjects. Authors performed an analysis based on the time-averaged exposure, that is measuring the levels of markers of Oxidative Stress at the baseline and at the end of the study; thus, in some subjects enrolled in the present study, the OS is influenced by the intervention treatment of PATH. It seems more adequate to include in such time-averaged analysis only control subjects of PATH (no effect of treatment). On the contrary, changes of the markers of OS with treatment should be better correlated with ERI changes (see below).

- We thank the reviewer for suggesting this additional analysis. We agree that in formulating time-averaged exposure variables in a randomized trial such as PATH, the treatment assignment has the potential to influence the exposure. In our analyses, however, we included both treated and untreated subjects for 3 reasons. First, the results of the main PATH trial analysis that have been previously published (Himmelfarb et al., JASN, 2014) showed that there was no significant difference in markers of oxidative stress or inflammation over follow-up between the antioxidant treatment group and the placebo group. Second, in our adjusted analyses, we included treatment group assignment as a covariate for adjustment, thus accounting for any remaining confounding by treatment assignment. And third, eliminating half the cohort from the analyses
substantially reduces the power of the study to detect associations between biomarker exposures and ESA resistance.

- With these caveats, we have repeated the time-averaged analyses among control subjects only, and included the results of these analyses as Supplemental Tables S6 and S7. Of note, likely given the limited sample size with the restricted cohort, there was no detected significant association oxidative stress marker quartile and ESA resistance. Patients with time-averaged hsCRP in the highest quartile did have higher mean ERI compared to those in the lowest quartile. An explanation of this sensitivity analysis and its results has been added to the results sections (page 14, lines 1-10).

2. To better understand the differences among subgroups at baseline (Table 1), either data on Kt/V, PTH, transferrin saturation, serum albumin, mean corpuscular volume of RBC and % of patients on ESA for each ERI quartile, or normal values for markers of inflammation and oxidative stress, or statistical differences (p for trend; p vs. reference) should be added.

- We agree with the reviewer that our Table 1 was missing important data on baseline characteristics that would aid the reader in understanding differences among subgroups. We thank the reviewer for pointing this out, and we have added the requested data to Table 1, including % randomized to study treatment, serum albumin, dialysis adequacy as measured by Kt/V, PTH, and transferrin saturation, as well as weekly IV iron dose, which was requested by reviewer #1. Unfortunately, data on mean corpuscular volume of RBCs was not collected in the study, and thus was not available to present. Additionally, to improve clarity and so as to not include results from the primary analysis in the table of baseline characteristics, we removed the primary exposures (hsCRP, IL-6, F2-isoprostanes, and isofurans) from Table 1.
- Per Major Revision#1 requested by Reviewer #1, we removed from the study cohort patients who were not treated with ESA during the duration of the follow-up period. Thus, all patients included in the study were treated with ESA. Correspondingly, we did not report % of patients on ESA for each ERI quartile given that this would be 100% for all quartiles of ERI.
- We have added p-values to Table 1 as requested that report on tests for heterogeneity among the different ERI quartiles. These data are now visible in the far right column of Table 1.

3. For each analysis it is reported a model adjusted for possible factors of ESA resistance. Some major factor of ESA resistance in MHD, however, have not been considered; at least Kt/V, PTH and Ferritin should be included in the adjusted analysis. Maybe a multivariate analysis including all the factors associated with ESA resistance could be useful.

- We thank the reviewer for pointing out additional covariates to include for adjustment in our final adjustment models. We have added Kt/V and parathyroid hormone (PTH) as covariates to Models 2 and 3 in our baseline and time-varying analyses. Updated estimates, confidence intervals and P-values can now be found in Tables 2 through 5.
- Importantly, inclusion of covariates for adjustment should either reflect consideration of confounding due to association with both the exposure and the outcome, or an attempt to increase precision. In either case, inclusion of covariates in the causal pathway between
the exposure (in this case inflammation or oxidative stress) and outcome (ERI) should not be done, as this can obscure a true relationship by introducing a Type II error. Serum Ferritin is highly likely to be in the causal pathway certainly between hsCRP, IL-6 and ESA resistance given its role as an acute phase reactant, and may also be in the pathway between oxidative stress and ESA resistance. Because of this, we have excluded serum ferritin as a covariate in our final adjustment models. For similar reasons, we have also not included serum albumin as a covariate in the final models.

4. The cubic splines correlation was used instead of linear correlation because the relationships between the markers of oxidative stress and ERI is not linear. Authors should give some more details on the modalities they evaluated the non-linearity. What does it means “Unadjusted restricted cubic splines … were superimposed on scatter plots”? Also, statistical differences should be added for Figures 2 and 3.

- We thank the reviewer for pointing out that we have not provided sufficient explanation regarding our model selection. We have now inserted a paragraph in the methods section of the manuscript which fully describes our model diagnostics and rationale for not modeling the exposures as continuous variables in our linear regression models. The paragraph has been inserted on page 8, lines 4 through 14
- We apologize for the confusion regarding our description of the cubic spline construction that the reviewer has referenced in the methods section of the manuscript. To clarify, we constructed cubic splines with four degrees of freedom along with 95% confidence intervals to as an illustrative representation of the functional form of the association between each inflammatory and oxidative stress biomarker and ESA resistance, given that our model diagnostics described above had revealed a non-linear relationship between biomarkers and ERI. Splines were superimposed in figures 2 and 3 on scatter plots of baseline biomarker values (x-axis) and ERI (y-axis), where each dot represents an individual patient. In our revised manuscript, we have replaced the unadjusted splines with splines adjusted for our Model 3 covariates to achieve consistency with our quantile regression methods. These changes and clarifications have been inserted into the text of the the methods section (Page 9, lines 1 through 5).
- We have added P-values for the comparison of mean ERI for quartile 4 versus quartile 1 (reference) to Figures 2 and 3, and have also subdivided the figures with dashed lines to emphasize where the data in each quartile lie. We hope that these modifications have improved the impact of the figures.

5. It would be very interesting to know the relationship between baseline exposition factors and ERI changes along the study; this might suggest a possible impact on clinical outcome.

- We thank the reviewer for suggesting this additional interesting analysis. Accordingly, we analyzed the association of quartiles of baseline markers of inflammation and oxidative stress with change in ERI was performed using a generalized estimating equation model including interaction terms for exposure quartile and study visit. These data are now reported in Table 4 in the revised manuscript. Data are presented as predicted change in ERI per month (i.e. study visit) by baseline biomarker quartile. Trend test P-values are presented which test the significance of a trend in changes of ERI
per study visit over quartiles of each baseline marker. Additionally, statistical comparisons were made between quartiles 4 and 1 for each biomarker exposure. This analysis is now described in the methods section on page 9, lines 7 through 15. As shown, there is a trend toward increasing change in ERI over study follow-up with increasing quartile of baseline inflammatory or oxidative stress quartile. For hSCRP, IL-6 and isofurans (but not F2-isoprostanes), there was a greater change in ERI per month for patients with baseline markers in the highest compared to the lowest quartiles. However, the trend test P-values for all exposures remained above the pre-determined level of significance of 0.05. We have described these findings in the relevant sections of the results which describe the time-averaged analyses. In addition, we have briefly discussed this in the discussion section.

6. Besides the higher quartile, also the middle-low quartile of isofuran is significantly different from reference (Table 3); this means that the relationship between ERI and isofuran is not-linear. Authors should comment on this point for the possible interpretation of mechanisms leading from OS to ESA resistance.

   - We agree with the reviewer that our results suggest a non-linear relationship between isofurans concentrations and ERI, and indeed this result persisted even after making the changes to the cohort construction and adjustment models suggested by both reviewers. We have added a new paragraph with new citations relevant to this finding, and have suggested a possible explanation. The new paragraph has been inserted into the discussion section on Page 16 line 21.

7. Authors associate the relatively high isofurans levels with a greater tissue oxygen tension due to higher ESA resistance. This mechanism needs more discussion; indeed, haemoglobin levels were not different among ERI quartiles and it’s not clear why tissue oxygen tension should be greater with higher ESA resistance. Authors should deeply address this central issue, taking into account also the non-linear relationship between isoprostane and ERI.

   - We apologize for not adequately discussing this potential mechanism that might explain the differential findings for isofurans and F2-isoprostanes. We have added a number of sentences to the relevant paragraph in an effort to clarify our thought process, and have also added new references, though we note that the proposed mechanism is theoretical only. We hope that the improved paragraph has increased readability and interpretability. Changes have been made on page 18, lines 4 through 20.

   - In addition, please see the preceding paragraph and our response to your comment #6 above regarding the non-linear relationship between isofurans and ERI.

8. Authors state that they cannot conclude on the causality of the association and further studies should test if intervention to decrease oxidative stress leads to reduction of ERI. I would suggest two analyses. First, the relationship between markers of OS at baseline and changes of ERI along the study might add information on causality. Second, this study comes from a RCT where antioxidant therapy was assigned to MHD patients vs. controls; the comparison of ERI changes between intervention group (treatment for OS) and controls might assess such effect of intervention to decrease oxidative stress on the reduction
of ERI.

- We thank the reviewer for suggesting these additional analyses. As suggested, we have conducted a secondary analysis using a generalized estimating equation model approach to assess the relationship between baseline markers of oxidative stress and inflammation with changes in ERI along the course of study-follow-up. We have described this in more detail in answer to your comment #5 above. As mentioned, we have added descriptions of this analysis to the relevant areas of the methods and results sections.

- With respect to the impact of assigned antioxidant treatment status on ESA resistance, we note that Reviewer #1 has asked a very similar question in Major Comment #2. Again, we apologize for making it clearer that this had been reported previously by Himmelfarb et al. in a prior investigation (Himmelfarb et al, JASN, 2014). Please see answer to Reviewer #1 Major Comment #2 for complete explanation.

**Minor Comments:**

1. **The Title of the paper should not include the conclusion of the study.**
   - We have changed the title to a non-declarative form. It now reads: “Association of plasma F2-isoprostanes and isofurans concentrations with erythropoiesis-stimulating agent resistance in maintenance hemodialysis patients.

2. **In Table 1 it seems there is a mistake in the limits of ERI quartiles (first or second) and, as well, the median ESA dose for the first quartile should be checked.**
   - We apologize for the error in the labels for the limits of the ERI quartiles in Table 1 and very much thank the reviewer for pointing this out. Given that the sample size and study cohort changed somewhat following the change requested by Reviewer #1 in Major Comment #1, we have updated all the ERI quartile labels, and have verified them for accuracy. We have also verified that the median ESA dose (in units/kilogram/week) reported in Table 1 is correct for all ERI quartiles.

3. **Isofuran correlation with CRP is very weak or even absent.**
   - We agree that the correlation between hsCRP and isoruans is very weak, and have added a qualifier to the sentence in the results section to emphasize this. Additionally, we have added the correlation coefficient of 0.01 between isofurans and IL-6 to emphasize the absence of this correlation as well.

4. **The long introduction on the mechanism how inflammation influences the ESA resistance can be reduced improving the readability of the discussion.**
   - We have reduced the word count of the first paragraph in the discussion from 192 to 126. We believe this change has improved the flow of the discussion.

5. **The last conclusion of the discussion, “Additionally, lipid ……” seems too speculative and not fully supported by data and can be deleted.**
   - We have deleted this sentence in the concluding paragraph.
**Table 1.** ESA use, unadjusted and adjusted for hemoglobin concentration, at each time point between two treatments

<table>
<thead>
<tr>
<th>Time Point</th>
<th>Placebo Group</th>
<th>α-Tocopherol+ALA Group</th>
<th>P Value&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Patients (&lt;i&gt;n&lt;/i&gt;)</td>
<td>ESA Use</td>
<td>Patients (&lt;i&gt;n&lt;/i&gt;)</td>
</tr>
<tr>
<td>Unadjusted</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enrollment</td>
<td>165</td>
<td>36,000</td>
<td>160</td>
</tr>
<tr>
<td>Baseline</td>
<td>164</td>
<td>36,000</td>
<td>159</td>
</tr>
<tr>
<td>1 mo</td>
<td>154</td>
<td>37,200</td>
<td>137</td>
</tr>
<tr>
<td>2 mo</td>
<td>147</td>
<td>36,000</td>
<td>126</td>
</tr>
<tr>
<td>3 mo</td>
<td>137</td>
<td>36,000</td>
<td>118</td>
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<tr>
<td>4 mo</td>
<td>130</td>
<td>36,000</td>
<td>116</td>
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<tr>
<td>5 mo</td>
<td>125</td>
<td>36,000</td>
<td>114</td>
</tr>
<tr>
<td>6 mo</td>
<td>124</td>
<td>40,800</td>
<td>114</td>
</tr>
<tr>
<td>Adjusted for hemoglobin</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Enrollment</td>
<td>165</td>
<td>3214</td>
<td>159</td>
</tr>
<tr>
<td>Baseline</td>
<td>163</td>
<td>2070</td>
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</tr>
<tr>
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<tr>
<td>6 mo</td>
<td>123</td>
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</table>

<sup>a</sup>P value was obtained from nonparametric Mann–Whitney U tests. ESA dose in units.