Author's response to reviews

Title: Cardiovascular disease relates to intestinal uptake of p-cresol in patients with chronic kidney disease

Authors:

Ruben Poesen (ruben.poesen@uzleuven.be)
Liesbeth Viaene (liesbeth.viaene@uzleuven.be)
Kristin Verbeke (Kristin.Verbeke@med.kuleuven.be)
Patrick Augustijns (Patrick.Augustijns@pharm.kuleuven.be)
Bert Bammens (bert.bammens@uzleuven.be)
Kathleen Claes (kathleen.claes@uzleuven.be)
Dirk Kuypers (dirk.kuypers@uzleuven.be)
Pieter Evenepoel (pieter.evenepoel@uzleuven.be)
Björn Meijers (bjorn.meijers@uzleuven.be)

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

We hereby submit the revised version of our manuscript entitled ‘Cardiovascular disease relates to intestinal uptake of p-cresol in patients with chronic kidney disease’ to *BMC Nephrology*.

We thank the editorial board and reviewers for their constructive and valuable comments. Please find below our point-by-point responses to these comments. We hope that in its present form the manuscript will be suitable for publication in *BMC Nephrology*.

The authors declare that there are no conflicts of interest. Björn Meijers is Associate Editor of *BMC nephrology*.

Kind regards,

B. Meijers
Reviewer 1:

Major Compulsory Revisions

1) Multivariate Cox proportional hazards regression models:
1a) Please identify the minimum set of variable that you will consider to be confounders a priori. In my opinion, these include age, diabetes, protein intake and eGFR. Other variables to consider: proteinuria, BMI, prevalent CVD. 1b) Consider sequential models with addition of these variables in the above order. Check to see if the addition of the model improves model fit of nested models.

We definitely agree with the reviewer that this study is limited by the relatively low number of events, which was also stated in the limitations section of the manuscript. We acknowledge that it is therefore difficult to conclude a truly independent association between 24h urinary excretion of PCS and outcome, and weakened our conclusion in the manuscript (see also question 3). As the number of events is below 30 and assuming the arbitrary number of approximately 10 events per predictor included in the model, we were not allowed to include more than 3 variables in each model.

Therefore, we first considered all variables potentially associated with outcome (20 variables, see methods section), only remaining those that were associated with outcome in univariate analysis (P < 0.20), and subsequently using these variables to build different models. We believe that this is the least unbiased approach as reasonably possible. As suggested by the reviewer, we also built additional sequential models with a set of variables that could be considered confounders *a priori*: age, diabetes, protein intake and eGFR.

With respect to cardiovascular disease:
Starting with 24h urinary excretion of PCS, age, diabetes, and protein intake improved model fit, while eGFR did not. Again, 24h urinary excretion of PCS remained associated with cardiovascular events during follow-up with HR 1.103 (1.006 – 1.209) (P 0.04).

These findings were added in the manuscript:
“We also built sequential models with addition of variables that were considered confounders a priori, i.e., age, presence of diabetes mellitus, protein intake and eGFR. Again, 24h urinary excretion of PCS remained associated with cardiovascular events during follow-up (HR 1.103 (1.006 – 1.209), P 0.04).”
With respect to overall mortality:
Starting with 24h urinary excretion of PCS, age, and protein intake improved model fit, while diabetes and eGFR did not. In this model, there was a clear, albeit non-significant, trend of association between 24h urinary excretion of PCS and survival (HR 1.098 (0.987 – 1.222), \( P \) 0.09), protein intake: HR 0.973 (0.942 – 1.004), \( P \) 0.09, age: HR 1.058 (1.017 – 1.101), \( P \) 0.005).

These findings were added in the manuscript:
“As for cardiovascular disease, we also built sequential models with addition of variables that were considered confounders a priori, i.e., age, presence of diabetes mellitus, protein intake and eGFR. Again, there was a clear, albeit non-significant, relationship between 24h urinary excretion of PCS and overall mortality (HR 1.098 (0.987 – 1.222), \( P \) 0.09).”

1c) Is there a time-interaction that is being modeled? It is mentioned in methods and there seems to be a time interaction between the highest tertile and other tertiles based on the KM plot (at ~21 months). Please consider stratifying by time <18 months and >18 months.

As stated in the methods, we formally tested the proportionality assumption in each model. This assumption was never violated for 24h urinary excretion of PCS.

1d) Table 3 and 4 with trivariate models are confusing – if you choose this approach then consider reporting all possible trivariate combinations – however, I would much rather see the sequential models as described in 1a above.

We agree with the reviewer that the trivariate models might be a bit confusing, but, as already explained (see question 1a), due to the relatively low number of events and the general rule of approximately 10 events per predictor to avoid overfitting, we were restricted to 3 variables for each model. We tried grouping variables, significantly associated with outcome in univariate analysis, into arbitrary and more or less logical categories (e.g., “renal function”: eGFR and proteinuria, “cardiovascular risk factors”: prior cardiovascular disease and diabetes mellitus, “nutrition”: albumin and BMI, “mineral metabolism”: PTH and Ca). Again, we acknowledge that this is certainly a limitation of our study.

I think the reviewer will agree with us that it would be an enormous effort to check all possible trivariate combination with 24h urinary excretion of PCS (i.e., 45 models for survival – 66 models
for cardiovascular disease). However, we performed the additional analyses as asked by the reviewer in question 1a and 1b.

1e) Consider sensitivity analyses with tertiles of p-cresol sulfate with the lowest tertile as reference.

We performed additional analyses with tertiles of 24h urinary excretion of PCS. With respect to cardiovascular disease, there was a significant association with tertiles of 24h urinary excretion of PCS in the unadjusted analysis (highest vs. lowest tertile: HR 3.011 (1.084 – 8.365), \( P = 0.03 \)), and a trend, albeit non-significant, in the sequential model (see question 1a) (highest vs. lowest tertile: HR 2.512 (0.854 – 7.473), \( P = 0.09 \)). In contrast, the association was less clear in the survival analysis, both in the unadjusted (highest vs. lowest tertile: HR 1.567 (0.607 – 4.042), \( P = 0.35 \)) and sequential model (highest vs. lowest tertile: HR 1.432 (0.506 – 4.056), \( P = 0.49 \)). This might suggest a more graded and rather continuous risk associated with higher urinary excretion of PCS.

2) Is there information on prevalent gastrointestinal disease and antibiotic use available at baseline?

We agree with the reviewer that presence of both gastrointestinal disease and antibiotic use may be of relevance as this could potentially influence intestinal generation and absorption of p-cresol.

Therefore, we reviewed patients’ medical charts and we only observed a total of 6 patients with a history of colorectal surgery, colorectal malignancy or inflammatory bowel disease. In addition, 11 patients were treated with antibiotics at baseline, of which prophylactic treatment for urinary infection was most commonly used. We believe the number of these patients is too small to investigate for potential differences in outcome or 24h urinary excretion of PCS.

3) Discussion: It is difficult to conclude an “independent” association particularly due to the small number of events. Just association will suffice. Also, are the first CVD events truly “new-onset” as 26.5% of the cohort had prevalent CVD.

We agree with reviewer 1 that “association” is the more appropriate statement. We left out “independent” in both the discussion and conclusion section.

In addition, we agree with the reviewer that we investigated the association between 24h urinary excretion of PCS and a new cardiovascular event during follow-up, rather than “new-onset” cardiovascular disease. As prior cardiovascular disease is a risk factor for a subsequent
cardiovascular event, we adjusted for this in the model (see table 5), with 24h urinary excretion of PCS still remaining a significant predictor of cardiovascular events during follow-up. Notably, there was also a clear trend of correlation between 24h urinary excretion of PCS and prior cardiovascular disease.

To avoid confusion, we changed the manuscript where necessary.

4) Discussion 4th paragraph: Another explanation for difference between the study by Wu et al and this study may be cultural and dietary differences and should be discussed.

We agree with the reviewer that, besides racial differences, there might be other explanations for the observed differences between the study by Wu et al and our study, including cultural and dietary factors. The true impact of these genetic and environmental factors, however, remains speculative and need further investigation.

A comment was added to the manuscript:
“We can, however, not exclude the possible impact of genetic or environmental differences as our cohort mainly consists of Caucasian patients in contrast to the Asian patient group studied by Wu et al.”

5) Discussion: I would like to see a paragraph describing the synthesis, absorption, metabolism and excretion of p cresol. It does not have to be detailed but should succinctly describe what is known and what is unknown. This paragraph could then be followed by a description of the factors describing inter-individual variability.

As pointed out in the manuscript, pharmacokinetics of p-cresol are still largely unknown. At current, we know that p-cresol is generated in the large intestine following bacterial fermentation of tyrosine, a process that probably depends on the amount of fermentable carbohydrate vs. protein. Then, p-cresol is absorbed in the large intestine, although mechanisms governing this process (i.e., active transport vs. passive diffusion) are unexplored. After absorption, p-cresol is almost fully metabolized to PCS by sulfate conjugation, or to p-cresyl glucuronide by glucuronide conjugation. Which kinds of enzymes are responsible for this process and whether the large intestine and/or liver are involved, is also unknown. In addition, serum concentrations of p-cresyl glucuronide are substantially lower than those of PCS, theoretically pointing to diminished generation/conjugation or higher total clearance. With respect to renal clearance, we also know
that PCS is secreted by renal tubular cells; the renal fate of p-cresyl glucuronide is less clear, although at least a higher glomerular filtration (due to a diminished albumin binding) can be assumed. In addition, whether there is non-renal clearance of both molecules (i.e., intestinal efflux and bile secretion) is again unknown.

As the manuscript mainly focused on the intestinal contribution to PCS serum levels, we would like to avoid extensively describing theoretical factors underlying metabolism and clearance of p-cresol, especially because this remains highly speculative. A short paragraph is already present in the introduction section, and a fuller theoretical approach of factors specifically determining total intestinal uptake of p-cresol, including its interindividual variability, is presented in the discussion section.

6) Discussion: Please discuss the negative studies of the association between serum p-cresol sulfate and outcomes and if your study helps explain these prior studies.

In the past, serum PCS has been repeatedly associated with overall mortality and cardiovascular disease, this in patients with varying degrees of renal dysfunction, and by several unrelated research groups (see, among others, ref 3-6). Recently, a study by Melamed et al (BMC nephrology 2013) was published, demonstrating a lack of association between serum PCS and outcome in hemodialysis patients in a subcohort of the CHOICE study. We believe that this discrepancy might be partly explained by their use of total serum PCS as predictor of cardiovascular disease instead of free and active serum PCS. In agreement, an association with cardiovascular disease in the same cohort could be observed by Shafi et al when including free PCS (Abstract ASN 2013). This reference has been added to the manuscript.

Nevertheless, serum PCS accumulates when renal function falls and eventually fails, making it rather difficult to fully adjust for the effect of renal failure and to investigate the sole impact of this solute. Therefore, we examined the direct relationship between 24h urinary excretion of PCS, as a surrogate of its total intestinal uptake and the body’s exposure to p-cresol/PCS, again observing and confirming the association between this solute and outcome.

On the other hand, to the best of our knowledge, the association between PCS and CKD progression in a clinical cohort was only reported by the group of Wu. As discussed in the manuscript, we didn’t observe this association between PCS and progression of renal disease in our cohort. We believe this should encourage other research group to examine this potential
association in their cohorts and to explore determinants underlying this apparent discrepancy, which, as also noted by the reviewer, could be cultural, dietary, racial, ...

Reviewer 2:

Major Compulsory Revisions

1) If the authors have the data, which they should, it would be nice to see if there is a correlation in table 2 between urinary excretion of PCS and serum bicarb levels. This can be added to table 1 as well and tested in the models. Since protein intake is so closely correlated to PCS levels (and should be correlated with serum bicarb levels as protein provides an acid load).

The authors thank the reviewer for this valuable suggestion. Serum bicarbonate levels were added to table 1 and tested for correlation in table 2. However, there was no correlation between serum bicarbonate levels and 24h urinary excretion of PCS (P 0.82), although a trend, albeit not significant, could be observed when correlating serum bicarbonate levels with 24h protein intake (P 0.12).

2) I wonder why phosphate was not included in the cox proportional models of 1st cardiovascular event - there is more evidence to the association of phosphate and outcomes than some of the other things included (PTH)

As the number of variables included in the cox proportional hazard models was limited due to the relatively low number of events, we only included variables that were associated with outcome in univariate analysis (P < 0.20), which was not the case for serum phosphate.

We performed additional cox proportional hazard analyses including 24h urinary excretion of PCS, serum phosphate, and eGFR. In contrast to 24h urinary excretion of PCS, serum phosphate was not significantly associated with overall mortality and cardiovascular disease in these models. However, we agree with the reviewer that there is substantial evidence showing an association between serum phosphate levels and outcome, but this is mainly demonstrated after multivariate adjustment. As we were unable to perform a full multivariate adjustment, this might explain the apparent lack of association between serum phosphate and outcome in this cohort.
Minor Essential Revisions

1) page 10 - the section in the middle of the page is probably better titled Correlations of 24 hr urinary excretion rather than Determinants as this is better reflects what is found in the paragraph.

As suggested by the reviewer, we changed the title of the paragraph in “Correlations of 24h urinary excretion of PCS”.

2) Please provide the number of events for tables 5 and 6 somewhere in the table so people don’t have to refer back to the text.

As suggested by the reviewer, the number of events was added to table 5 and 6.

3) How many participants experienced a progression of kidney disease? It may be nice to put this on page 12 so that the reader can compare the power available for the different outcomes.

Progression of renal disease, defined as progression to renal replacement therapy and/or doubling of serum creatinine during follow-up, was observed in 55 patients.

A comment was added to the manuscript:

“Progression of renal disease was observed in 55 patients during follow-up. However, there was no association between 24h urinary excretion of PCS and renal disease progression (P 0.19).”