Author’s response to reviews

Title: Impact of high drinking water nitrate levels on the endogenous formation of apparent N-nitroso compounds in combination with meat intake in healthy volunteers

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Author’s response to reviews:

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To:
Editors-in-Chief
Philippe Grandjean, University of Southern Denmark, Denmark
David Ozonoff, Boston University School of Public Health, USA
Editors-in-Chief – Environmental Health

Maastricht, 18th of May 2019

Dear Prof. Grandjean, dear Prof. Ozonoff, dear editors-in-chief,

Dear Reviewers,

Thank you very much for providing the acceptance of our manuscript entitled “Impact of high drinking water nitrate levels on the endogenous formation of apparent N-nitroso compounds in combination with meat intake in healthy volunteers’ by Simone G. van Breda, Karen Mathijs, Virág Sági-Kiss, Gunter G. Kuhnle, Ben van der Veer, Rena R. Jones, Rashmi Sinha, Mary H. Ward and Theo M. de
Kok, in your journal Environmental Health.
We have taken all comments of the reviewers into consideration and provide here our response including a detailed description of all the changes we have made accordingly. All changes have been marked in red in the revised manuscript using track changes. A reference list of used literature to substantiate our response is provided at that end of this document. Here, the reviewers’ comments are placed first, after which we provide our response.

Reviewer #1:

This is a very well written manuscript with clear objectives, appropriate methods and conclusions matching the objectives. It is a relevant contribution to the potential link between exposure to nitrate in drinking water and meat consumption, and exposure to carcinogenic chemicals.

Some minor comments that could be considered by the authors for improvement include:

1. The authors downplay the results on the comet assay in the discussion. This is a relevant finding suggesting an effect modification by type of meat intake, which is not developed in the discussion. This finding is relevant since the outcome (genotoxicity) is closer to cancer risk than ATCN. The way results are discussed are particularly unbalanced since on the other hand, the authors highlight in the abstract and develop in the discussion the findings on the potential role of vitamin C, which are based on weak associations. The readers may wonder the reason of this unbalanced discussion of findings. Could it be because limited validity or reliability of the comet assay results? If this is the case, limitations should be mentioned in the discussion to guide the interpretation of findings. If results are reliable, they warrant to be developed in the discussion in the framework of previous evaluations, interpretation etc.

Response to comment 1:
Caco-2 cells were exposed to faecal water of the participants, and genotoxicity was measured by means of the comet assay. Tail intensity levels increase after exposure, however, due to the large variation in the measurements, no statistically significant differences could be established between the participants who consumed either processed red meat or non-processed white meat. Consumption of red processed meat in combination with drinking water containing low nitrate levels resulted in an increased tail intensity level as compared to baseline measurements, but not as compared to consumption of processed red meat in combination with drinking water containing high nitrate levels or compared to consumption of unprocessed white meat in combination with either low or high levels of nitrate in drinking water. Statistically, indeed, effect modification of the effect of nitrate levels in drinking water by meat type is demonstrated, but the biological significance of this cannot be explained. We have added a sentence describing this at the discussion section.

2. Page 10, line 273. "Only after the low drinking water nitrate period…” the authors probably mean "only after the HIGH drinking water nitrate period".

Response to comment 2:
The sentence is correct. The level of DNA damage was higher after the low drinking water nitrate period and not after the high drinking water nitrate period, in the processed red meat group. The biological significance is not clear.
Reviewer #2:

Review of: Impact of high drinking water nitrate levels on the endogenous formation of apparent N-nitroso compounds in combination with meat intake in healthy volunteers. General impressions: Well-written and an important contribution to understanding the impact nitrates have on human health. Looking forward to a follow-up study with a larger and perhaps a more refined/controlled trial. Some of my questions are "devil's advocate" in nature, as a bit of the background is not spelled out for the reader.

Comments abstract:

1. Why are you focusing on meat (other than it hasn't been studied before)? You might mention that cured meat can contain high levels of nitrates or what the USDA and/or EU limit is.

Response comment 1 abstract:

The rationale behind the study and the choice for the focus on consumption of different meat types in combination with drinking water nitrate levels is explained in more detail in the background of the manuscript. In order to make this clearer in the abstract, cured meat is added as potential source of nitrate.

2. Any a priori reason why nitrate from meat and water would have an interactive effect and instead of being additive? Does the source of nitrate impact its composition? Did you conduct a test of interaction?

Response to comment 2 abstract:

Red meat in particular contains relatively large amounts of haem iron as compared to white meat. Haem iron is a component of haemoglobin and myoglobin. It is the iron-containing chemical compound in red meat, also providing its pigment or colour. While dietary iron is crucial to good health, haem is also toxic in the digestive system. It has its own toxicity, but also acts to promote the formation of N-nitroso compounds (NOCs), by catalysing the chemical reaction in which NOCs are formed out of NOC precursors (1, 2). This is the reason why nitrate from meat and water could have an interactive effect instead of only being additive: the haem present in the red meat can also act as a catalyst for the formation of NOCs derived from NOC precursors generated from nitrate in drinking water, leading to a higher level of endogenously formed NOCs from this dietary source as compared to consumption of an equal amount of drinking water without consumption of red meat containing haem iron.

Did you conduct a test of interaction?
No, we did not conduct a test of interaction.

3. Please mention the results of the analyses of genotoxicity in faecal water (Comet-assay for DNA breakage) and compliance markers. What are compliance markers?

Response to comment 3 abstract:
Compliance markers are markers which provide information on the adherence of the participants to the intervention. For intake of meat, 3-methylhistidine and 1-methylhistidine were measured as biomarker for unprocessed white meat (3) and processed red meat intake (4), respectively. We have added the data to the manuscript (abstract, methods, results, and Table 1 and 3). Results demonstrate the compliance to the intervention as a statistically significant increase in 3-Methylhistidine excretion in subjects consuming unprocessed white meat as compared to baseline levels, and a significant decrease in 3-Methylhistidine levels in subjects consuming processed red meat and drinking water containing high levels of nitrate as compared to baseline, was observed. The latter could be explained by the absence of consumption of white meat for two weeks, which could lead to this lower level of 3-Methylhistidine levels in the urine of the subjects. Furthermore, 1-Methylhistidine levels were significantly increased in subjects consuming processed red meat.

Comments background

1. Would reiterate the point about haem iron that is currently in the beginning of the discussion section in the background as well.

Response to comment 1 background:

This has been added to the background section.

2. There are regions of the world where nitrate levels in drinking water are high. You might mention this, in addition to your discussion of low levels, as it pertains to your exposure.

Response to comment 2 background:

This has been added to the background section.

3. Is excretion a good thing since your body isn't retaining these NOCs? Or is this a proxy for measuring formation of these compounds? Please clarify.

Response to comment 3 background:

Excretion of NOCs in the urine or faeces has been reported to be a suitable procedure for estimating human daily exposure to both endogenous and exogenous NOC (5-8), and is a biomarker of cancer risk (8-11).

Comments methods:

1. Briefly describe "healthy."

Response to comment 1 methods:

Healthy is regarded as absence of any aberrations or insufficiency of the gut, liver, kidney, heart or
lungs. In addition, symptoms of any type of inflammation in the gut or liver, anaemia or any metabolic of endocrine aberrations, should not be present. This has been added to the method section.

2. You mention multiple types of meat. Was everyone on the same day-to-day meat diet or were they allowed to vary? Should the participants have been given their entire diet to eat instead of just the meat items and 2L of water?

Response to comment 2 methods:

Everybody was on the same day-to-day meat diet which consisted of multiple types of meat. In addition to this, they received 2L of bottled water with a standard level of nitrate. We have further clarified this in the method section. Participants were allowed to eat other foods of their choice and this was captured using the daily food diary.

3. Were the participants coached on how to use the food diary software?

Response to comment 3 methods:

Subjects received training before the study started on how to use the food diary software. Furthermore, they visited the department several times throughout the study period during which the food diaries were checked by the responsible investigator for correctness. In case of incorrect use, subjects were made aware of this and instructed again.

4. What do we think the compliance with the protocol was? Did they consume more or less water than prescribed? Additional water is not listed as a prohibited drink.

Response to comment 4 methods:

Subjects were not allowed to drink any additional water than the provided water. Furthermore, they were requested to drink the whole amount of 2L, spread throughout the day. If any water or meat were left at the end of the day, they could report that on a feedback form. We determined that compliance high, as there was frequent feedback and contact with participants throughout the study when food diaries were checked, and when new meat products and water bottles were provided. Subjects had to bring back the empty water bottles and packing material for the meat products at their next visit for recycling which also provides some indication of their actual use of the products. Biomarkers of meat intake show indeed an increase in consumption for both meat types. Furthermore, nitrate excretion during the high drinking water nitrate period was significantly higher as compared to the level of excretion during the low drinking water nitrate periods, which indicates increased consumption.

5. Please indicate if you analysed data as intent-to-treat or not.

Response to comment 5 methods:

We did not analyse data as intent-to-treat. There was one drop out after week 1 in the processed red
meat group. As a number of analyses require paired analyses, it was decided to leave out the data which was gathered at baseline and after week 1.

6. Inconsistent use of abbreviations (litres versus L) on line 132, 169 (hours versus h), and 177 (hours versus h).

Response to comment 6 methods:
This has been corrected and the whole document was checked for inconsistencies.

7. Line 145: 24h should be 24 h

Response to comment 7 methods:
This has been corrected and the whole document was checked for inconsistencies.

8. Line 168: define CV.

Response to comment 8 methods:
CV = coefficient of variation. This has been changed in the manuscript.

9. Line 186: define CLD.

Response to comment 9 methods:
CLD = chemiluminescence detector. This has been added to the manuscript.

10. Inconsistent use of quotation types between lines 213 and 214.

Response to comment 10 methods:
This has been revised and the whole document was checked for inconsistencies.

11. Not a fan of p values, although I understand some still rely heavily on these. Would prefer if you reported point estimates and confidence intervals. Can you present both?

Response to comment 11 methods (and response to comment 2 results; and comments 1, 2 and 5 Figures and Tables):
In order to be consistent, we have added the SEM to the mean values where these were missing. Adding the confidence intervals would therefore be redundant; also, as these do not provide information on statistical significance, and the p-values do, we provide the exact p-value instead of “n.s.”.
12. What is the purpose of the baseline levels? Have you completely removed nitrates from the diet or had participants restrict their meat consumption during the baseline time?

Response to comment 12 methods, comment 6 results, and comment 3 discussion:

We have included the baseline measurement, as a number of comparisons are pairwise, meaning that each subject acted as its own control. This enables the evaluation of the meat intake. Each subject has its own dietary pattern and the intervention adds meat and changes the level of nitrate in drinking water. In case the baseline measurements are taken out, the effect of the meat cannot be evaluated as such. The major conclusion of the study, that nitrate in drinking water adds significantly to level of endogenous nitrosation, is based on the comparison between the outcomes of week 1 and week 2, and is not based on the comparison with baseline levels.

13. Is the white meat unprocessed? You refer to "processed red meat" often but rarely use "unprocessed white meat." Please restate the "unprocessed" nature of the white meat throughout the main text.

Response to comment 13 methods:

The white meat is unprocessed. We refer at some parts to non-processed white meat. We have adjusted this to unprocessed white meat and have applied this to the whole manuscript.

14. Does timing of ingestion of NOC inhibitors matter? I'm wondering if someone ate a big salad or a bag full of oranges recorded in your food diary on day 7, and then you collected their feces and urine how that would impact your results.

Response to comment 14 methods:

Timing of ingestion of NOC inhibitors probably does matter, as does the ingestion of NOC precursors, on the formation of NOCs. It has been shown by Mirvish et al. that ascorbic acid is one of the most effective inhibitors of intragastric nitrosation. When it is administered together with nitrosating agents and a variety of secondary amines, formation of NOC is inhibited (12-14). In the present study, ingestion of NOC precursors in the form of meat and drinking water was spread throughout the day, thereby providing a continuous resource of NOC precursors into the body. Ingestion of NOC inhibitors was not regulated. However, there are numerous N-nitroso compounds that can be formed after ingestion of NOC precursors, which all have different properties. Their chemical formation and nitrosation rate will be dependent on different factors such as pH, temperature, presence of nitrosation derivates, etc. Furthermore, the kinetics (half-life) is also very different for each NOC, and varies between hours and days (6, 13). This means that after a diet which provides a stable level of NOC precursors for several days, NOCs will be present in the body as parent compounds, and different kinds of metabolites, in varying amounts. Ingestion of NOC inhibitors will only have an effect of newly formed NOC, which will only be a relatively small part of the NOCs that are present in the body at that moment.

15. Have you considered doing fixed-effects regression?

Response to comment 15 methods:
We evaluated a random effects models in addition to our fixed linear models. However, likelihood ratio tests comparing models with and without random terms were not significant, so we opted to present the simpler models.

16. Is your linear regression simple or have you adjusted for any relevant confounders?

Response to comment 16 methods:

The models did not include covariates, as gender, age, and smoking since these factors were not related to nitrate or ATNC excretion.

Comments results:

1. Inconsistent spacing of p values between lines 233 and the rest of the text.

Response to comment 1 results:

This has been revised and the whole document was checked for inconsistencies.

2. Again, please present point estimates and 95% CIs.

Response to comment 2 results:

I refer here to my response to comment 11 methods.

3. Glad you included Figure 1. It wasn't clear from the introduction that there are two randomized treatment groups and the only variable changing between them is the nitrate exposure. Initially I thought that the participants were also changing their meat consumption in a cross-over style study. Please clarify early on. May consider using different verbiage such as "arms" instead of groups to differentiate or reword using more references to week 1 and week 2.

Response to comment 3 results:

We have adjusted the phrasing in an early paragraph to clarify this.

4. Did you control for any of the other foods consumed in your models? What is the purpose of the food diary if you don't?

Response to comment 4 results:

The models were not controlled for any other foods consumed, as the number of subjects per group is not high enough (low power). Smoking, coffee, tea and alcohol intake was not allowed during the study and also the use of antibiotics in the prior month and during the study was not allowed. The purpose of the food diaries was to estimate intake of vitamin C and nitrate.
5. "...however, ATNC levels and excretion of urinary nitrate increased significantly following the high drinking water nitrate period (Figure 2A and 2B, respectively; p < 0.01 and p < 0.05 for ATNC levels for red and white meat, respectively; p < 0.05 and p < 0.001 for urinary nitrate excretion for red and white meat, respectively)." Increased compared to what reference group? Week 1 or baseline?

Response to comment 5 results:

The levels of ATNC and excretion of urinary nitrate were significantly higher during the high drinking water nitrate period as compared to the low drinking water nitrate period. We have clarified this in the particular paragraph.

6. "Only after the low drinking water nitrate period, DNA damage was significantly higher in the processed red meat group compared to baseline levels (p<0.05) (Table 3)." What is the purpose of comparisons to the baseline levels? Do we know what people were consuming at baseline? You could be giving them additional nitrates or reducing their nitrate exposure in your study, but we have no idea which.

Response to comment 6 results:

I refer here to my response to comment 12 methods.

7. Your results and tone towards their importance do not align between what is written in the abstract and the main text. "Furthermore, a near significant positive correlation was found between the ratio of nitrate/vitamin C intake (including drinking water) and the level of ATNC in faecal water of subjects in the processed red meat group." - from the abstract "In the processed red meat group, a positive correlation was observed between ATNC levels in faecal water and the ratio of nitrate and vitamin C, but this was mainly driven by one subject and not statistically significant (R=0.27, p= 0.15) (Figure 3A)." - from the results section

Response to comment 7 results:

We have adjusted our tone in the abstract to what is described in the main text.

Comments discussion:

1. You mention Vitamin E here but did not mention it in the results. Any reason why?

Response to comment 1 discussion:

In the present study, only the effect of vitamin C was taken into account as possible nitrosation inhibitor, although vitamin E and a number of polyphenols could also exert this effect. However, estimating vitamin C and different polyphenols from food diaries is challenging.

2. Do you think the results would change if you had unprocessed red meat included as a treatment arm?
Response to comment 2 discussion:

Unprocessed red meat does not contain additional nitrite and nitrate as compared to processed red meat. However, it does contain haem iron, which in combination with high drinking water nitrate levels, could lead to formation of NOCs. The theoretical formation of NOCs after consumption of unprocessed red meat in combination with high drinking water nitrate levels would therefore be expected to be higher as compared to unprocessed white meat in combination with high drinking water nitrate levels, but lower as compared to processed red meat in combination with high drinking water nitrate levels.

3. "Furthermore, our results emphasize the importance of taking both nitrosation precursors as well as nitrosation inhibitors into account in the assessment of the nitrate intake on cancer risk." I am unsure of where you did this in your own work. Are you referring to the ratio of nitrate and Vitamin C to NOCs? Please clarify.

Response to comment 3 discussion:

Nitrosation precursors which are taken into account are the nitrate in drinking water, the nitrate and nitrite, and haem iron in processed red meat; vitamin C is taken into account as nitrosation inhibitor.

4. I would refrain from the comparisons to baseline unless you had a washout period within the baseline week.

Response to comment 4 discussion:

I refer here to my response to comment 12 methods.

5. "Although it is not directly related to the drinking water standard as the concentration varies by the person's weight, this level of nitrate which is used in the drinking water exceeds the regulatory limit of 50 mg/L nitrate by the WHO." Very confused by this sentence. Please clarify.

Response to comment 5 discussion:

We have adjusted the sentence in order to make it clearer:
“The ADI level it is not directly related to the drinking water standard as the allowable intake varies by the person’s weight. However, the level of nitrate which was used in the drinking water exceeded the regulatory limit of 50 mg/L nitrate by the WHO.”

6. "Taking into account dietary vitamin C intake in our study, we found a positive, although not statistically significant, association between endogenous ATNC-formation among subjects consuming relatively high levels of nitrate and low levels of vitamin C." Wasn't this driven by one person? Can you really make this claim?

Response to comment 6 discussion:

This correlation was indeed mainly driven by one person. We have toned down our conclusion in the
manuscript and provided explanations in the discussion why we could not establish a strong association between vitamin C intake, nitrate intake and ATNC formation. This is probably because vitamin C was not administered in a controlled manner (dose and timing) to the subjects by means of supplementation, but was assessed by means of food diaries. Mirvish et al have showed that vitamin C should be ingested at the same time or within a few hours with the nitrosation precursors in order to prevent inhibition of nitrosation (12-14).

7. Any explanations as to why you failed to observe changes to the cells? This must have been time-consuming work and it barely is mentioned. I would either remove this portion of the text, or preferably, include more about the null implications.

Response to comment 7 discussion:

This has been explained in our response to the comments of reviewer 1 and is repeated here for reviewer 2: Caco-2 cells were exposed to faecal water of the participants, and genotoxicity was measured by means of the comet assay. Tail intensity levels increase after exposure, however, due to the large variation in the measurements, no statistically significant differences could be established between the participants who consumed either processed red meat or non-processed white meat. Consumption of red processed meat in combination with drinking water containing low nitrate levels resulted in an increased tail intensity level as compared to baseline measurements, but not as compared to consumption of processed red meat in combination with drinking water containing high nitrate levels or compared to consumption of unprocessed white meat in combination with either low or high levels of nitrate in drinking water. Statistically, indeed, effect modification of the effect of nitrate levels in drinking water by meat type is demonstrated, but the biological significance of this cannot be explained. We have added a sentence describing this at the discussion section.

8. Please clarify whether the nitrate concentrations presented are N-NO3 or NO3-.

Response to comment 8 discussion:

Nitrate concentrations presented are NO3-.

Comments conclusions:

1. Your list of abbreviations is missing quite a few items.

Response to comment 1 conclusions:

We have added all missing abbreviations.

Comments Figures and Tables:

1. Provide point estimates in addition to the p values.

Response to comment 1 Figures and Tables:
I refer here to my response to comment 11 methods.

2. Table 1: Define SEM.

Response to comment 2 Figures and Tables:

We have defined SEM in the Figures and Tables.

3. Table 1: Line 528 has an erroneous ellipse and I don't see what this footnote is in reference to.

Response to comment 3 Figures and Tables:

We have deleted this from the table.

4. Table 1: Are any of these characteristics different between the two groups?

Response to comment 4 Figures and Tables:

There are no statistically significant changes between the study groups at baseline. This is also stated at the results section under “Study population”.

5. Table 2: Please provide the standard error or deviation.

Response to comment 5 Figures and Tables:

We have added the SEM in Table 2.

6. Table 2: Please provide the nitrate concentrations instead of, or in addition to, "low" and "high."

Response to comment 6 Figures and Tables:

Nitrate concentrations are added to Table 2.

7. Table 2: Is "Overall" including the baseline? Where are those data?

Response to comment 7 Figures and Tables:

“Overall” indicates the high and low drinking water nitrate periods combined. This is the mean level across the whole intervention period. We have added this explanation to the table.

8. Table 2: It looks like Vitamin C doesn't differ between arms or weeks on study. This might be another way to discuss the findings that with elevated nitrate in the drinking water and stable Vitamin C intake, NOCs changed.
Response to comment 8 Figures and Tables:

We have added this finding to the discussion section.

9. Table 3: Please reverse the order of the columns so they are the same as Table 2.

Response to comment 9 Figures and Tables:

We have reversed the columns.

10. Table 3: Please state the p value instead of n.s.

Response to comment 10 Figures and Tables:

We have added all p-values for the three different comparisons to Table 3 (low versus high, low versus baseline, and high versus baseline).

References


7. Ohshima H, O'Neill IK, Friesen M, Bereziat JC, Bartsch H. Occurrence in human urine of new sulphur-containing N-nitrosamino acids N-nitrosothiazolidine 4-carboxylic acid and its 2-


