***Reviewer #1***

We would like to thank the reviewer for reviewing our manuscript entitled “Dietary nitrate intake is associated with decreased incidence of open-angle glaucoma: The Rotterdam Study" (MS# nutrients-1760125). We appreciate the critical review and suggestions to improve our manuscript. Enclosed please find our revised manuscript, which incorporates the comments and suggestions of both reviewers. In this letter we will address the comments of reviewer #1 point by point.

**What was the definition of visual field loss that defined glaucoma?**

The definition of glaucomatous visual field loss was elaborated in the corresponding reference. However, we agree that we were very brief in explaining glaucomatous visual field loss in the manuscript. Therefore we made the following changes to elaborate:

In lines 89-98, we changed:

“iOAG was defined as glaucomatous visual field loss in at least one eye with reproducibility of the defect, independent of IOP”

To:

“All participants underwent visual field testing using the Humphrey Field Analyzer (HFA; Carl Zeiss Meditec, Jena, Germany). A second supra-threshold test was performed when a visual field defect appeared to be present. Details have been described elsewhere. [1] If the second supra-threshold test showed at least one overlapping abnormality in the same hemifield, Goldmann kinetic perimetry (RS-I-1 and RS-I-3; Haag-Streit) or full-threshold HFA (all other cohort visits) was performed on both eyes. If abnormalities were consecutive and reproducible, thus present on the Goldmann or full-threshold test and on both supra-threshold tests, visual field loss was considered to be present. Defects had to be in a consistent hemifield and a least one depressed test point had to have exactly the same location on all fields.”

In lines 98-100, we changed:

“All other possible causes of visual field loss were excluded.”

To:

“Glaucoma specialists examined fundus photographs, ophthalmic examination reports, medical histories, and MRI scans of the brain to exclude all other possible causes of visual field loss. Discrepancies were resolved by consensus.”

In lines 103-104, we added:

“For IOP, three measurements were taken from each eye, of which the median value was recorded. [2] For iOAG cases, we used IOP measurements of the affected eye. If both eyes were affected or unaffected, a random eye was selected. ***IOP was not included in the definition of iOAG.***”

**Was glaucoma also defined by an objective finding, such as the optic disc finding or imaging of the optic disc?**

OCT images were taken with SD-OCT (Topcon Corp., Tokyo, Japan) since 2007. This means that OCT images are not available for the first four visits of RS-I, and the first two visits of RS-II. Additionally, only part of the images are focused on the disc and even less are reliably segmented. Fundus images were available and were used rather to exclude other cases of visual field loss in participants that showed visual field defects than to observe optic disc abnormalities.

**Were the incident cases of glaucoma or controls being treated in any case prior to their diagnoses?**

Thank you for this comment. We understand that it might be useful to present the number of treated glaucoma cases in our baseline characteristics table. Unfortunately, detailed information on prior treatment/surgery for glaucoma is limited (only present for RS-I), and therefore we did not include this variable as covariate in our models. It is thus possible that incident glaucoma cases have received treatment for glaucoma in between two research visits. However, since all participants were (confirmed by examination) free of OAG at baseline, treatment for glaucoma could not have been longer than maximum of one follow-up visit. Although not having this data is a limitation of this study, we do not think it is an important confounding factor, since we do not expect that glaucoma treatment would influence one’s diet. More importantly, dietary information was collected at baseline, with all participants free of OAG. Therefore, if glaucoma presence or glaucoma treatment would have an effect on dietary intake, this would not be applicable in our study. We have added a short sentence explaining this in lines 312-316 of the manuscript.

**"As we did not observe an association between dietary nitrate intake and IOP, nitrate may protect against glaucoma via other, IOP-independent, mechanisms." The authors should avoid making statements such as "nitrate is protective" since what they have found is a statistical association.**

We agree that, although our findings confirm earlier reported associations between dietary nitrate intake and OAG, intervention studies are necessary to investigate and confirm the association between dietary nitrate intake and iOAG. Therefore, we will change all references to “protective (effect)” to “association/associated with”.

**There is very little association between ischemia--reperfusion and glaucoma pathogenesis hence the statements on page nine are irrelevant.**

Thank you for this suggestion. Although the evidence for the association between ischemia--reperfusion and glaucoma pathogenesis might be scarce, this is only one possible pathway that is addressed in explaining the inverse association between dietary nitrate intake and glaucoma incidence. Nevertheless, we will adjust the manuscript (lines: 271-288) so that this statement is less strong and the focus is more on (a combination with) other pathways, rather than this pathway alone.

**If I am calculating correctly it has been over 20 years since the incident cases average followup occurred, that is 10-20 years after the start of a study in 1991.  How much might the diet of persons in this population have changed since then?  Thus, is this study still relevant to today? 30 years ago eating more vegetables was not the vogue it is today.**

We agree that the baseline dates of all three cohorts (RS-I, 1991-1993; RS-II, 2000-2001; RS-III, 2006-2008) are already quite some years ago and that dietary patterns in general have changed over time. However, since dietary recommendations are not standard of care for glaucoma patients, we believe that this would likely lead to non-differential misclassification (that causes a bias towards the null hypothesis), since we would expect that glaucoma cases would change their diet similarly to our control population and vice versa.

We thus feel that this study and its findings are still highly relevant today since nutrition has become ever more important in medical sciences. Ophthalmology is one of the disciplines which has gained enormous insights from these recent developments. However, to date, no nutritional recommendations have been made for glaucoma patients and research into the association between nutrition (on each level, e.g. diets, food groups, nutrients) and glaucoma is scarce. If associated, this may support diet recommendations in patients that show first signs of developing glaucoma and/or people at-risk for glaucoma as well, impacting millions of people worldwide.

Especially since diet changes into a vegetarian of vegan diet are becoming more popular, we think that understanding the potential beneficial effects of these changes, thus the effects of vegetables and their nutrients, is more important than ever. We do agree that these findings should be replicated or validated in another (preferably, an intervention study) study before dietary nitrate intake should be considered as an important public health implication.

To address your question about the change of dietary patterns over time more thoroughly:

Dinnisen et al. [6] recently published an article in *Nutrients* where they described changes in dietary intakes of Dutch adults between 2007-2010 and 2012-2016, and evaluated these changes by age, gender, and education. They demonstrated that there was no significant increase in vegetable intake for Dutch adults aged 19–69 years between 2007–2010 (n = 2106; DNFCS 2007–2010) and 2012–2016 (n = 1540; DNFCS 2007–2010) for the total population. There was also no difference when they compared the change in vegetable intake over time per sex or per age category (51-69 years being closest to the baseline age used in our study). Only in the group with the highest educational level, the mean consumption of vegetables increased over time. However, since there was no significant difference in education level in our study between cases and controls (Table 1), we assume that changes in vegetable intake would be similar between both groups, leading to non-differential misclassification. Moreover, we adjusted for education level in our additional model.

Regarding dietary nitrate intake from non-vegetable sources, this article states that the intake of red or processed meat decreased over time. This was applicable to both males and females and seen for all age groups. Again, we would expect that this decrease is similar for glaucoma cases and controls.

Another Dutch study [7], starting in 1986 (which is more close to the first visit of RS-I) stated that “Another potential limitation that should be considered is whether the food frequency questionnaire at baseline was a reliable estimate of past and future diet. The stability of dietary habits over time was evaluated from five annually repeated questionnaire administrations in independent random samples of the cohort. The mean intakes barely changed and the correlation between two measurements decreased only slightly over an increasing time interval. It was concluded that the single food frequency questionnaire measurement characterizes dietary habits for a period of at least 5 to 10 years.” [8]

Nevertheless, since both studies are not directly translatable to the Rotterdam Study, we have assessed the association between the dietary nitrate intake and iOAG over cumulative follow-up periods to provide insight into the stability of the found estimates over time. Please see our comment regarding your next suggestion for more details.

**This study would have been that much stronger if a follow-up food questionnaire had been administered.  The long space between the questionnaire and the incidence of glaucoma (only mentioned at the last part of the limitations) is quite a major limitation.**

We agree that the lack of follow-up food questionnaires is a major limitation of this study. Please find an additional supplementary figure (Figure S1; original Figure S1 has now become Figure S2) below and in the supplements of the manuscript, to show that we assessed the association between the dietary nitrate intake and iOAG over cumulative follow-up periods (x-axis) to provide insight into possible reversed causality. The persistence of the association over time implies that reverse causality is unlikely. The effect of (baseline) dietary nitrate intake on glaucoma appears to be relatively stable over time. We therefore consider our results reliable, although we agree limitations are present and these findings should be interpreted with caution. In addition to the new supplementary figure, we have added information about this analysis to the methods (lines 152-153), results (209-211), and discussion (296-298).



**Figure S1.** Multivariable-adjusted odds ratios with corresponding 95% confidence intervals (CIs) for incident open-angle glaucoma per 10 gram/day increase in total dietary nitrate intake (A), nitrate intake from vegetables (B), and nitrate intake from non-vegetable food sources (C), shown per cumulative follow-up interval. Model 1: adjusted for body mass index, total energy intake, diet quality, physical activity, and follow-up time. Model 2: model 1 additionally adjusted for intraocular pressure. Model 3: model 1 additionally adjusted for education level and smoking status.

**Past studies of the Rotterdam population mention several other risk factors which were not taken into account in this study including myopia, cup-to-disc ratio, and family history of glaucoma, as well as other features such as exfoliation. How big is the nitrate association compared to these?**

Thank you very much for assessing the covariates included in these analyses so thoroughly. Unfortunately, family history of glaucoma was only available for a subset of the first cohort, and not for the second and third cohort. Therefore, we were unable to include this variable as a covariate, as this would severely limit our power (528 participants of 1038 participants; 133 glaucoma cases and 389 controls).

As cup-to-disc ratio is highly correlated with glaucoma diagnosis, we feel that cup-to-disc ratio should not be included in the model. Moreover, cup-to-disc ratio is not associated with dietary nitrate intake, and therefore cannot be considered as confounding factor. By including cup-to-disc ratio into the model, we would adjust the association between dietary nitrate intake and glaucoma by “glaucoma presence” and thus diminish any existing association.

To meet your comment/suggestion, we have added baseline spherical equivalent (proxy for myopia, excluding aphakic or pseudophakic participants) to model 3. As you can see in the results below (original results visualized in Figure 2 in the manuscript), adjusting additionally for SE did not change the results of the continuous nitrate intake analyses and did only marginally change the results of the quintile analyses, leading to a more significant finding for Q5 as compared to Q1. Because both analyses are similar, and since myopia is associated with education level, we chose to keep the original model 3 in the manuscript, adjusting for education level, but without adjustment for SE.

To address your question, in lines 318-323, we will change:

“Finally, although the analyses were adjusted for several confounders, residual confounding cannot completely be excluded.”

To:

“Although the analyses were adjusted for multiple confounders, we were unable to adjust for other possible confounders such as family history of glaucoma due to a lack of data. We did consider the risk factor myopia, for which we adjusted by including education level into model 3. We also included spherical equivalent into the model (data not shown), but this did not change the results. Lastly, residual confounding cannot completely be excluded.”

Original model 3: model 1 additionally adjusted for education level and smoking status.

|  |  |  |  |
| --- | --- | --- | --- |
|  | OR | Lower | Upper |
| **Total dietary nitrate** |  |  |  |
| Continuous (per 10mg/day) | 0.95 | 0.91 | 0.98 |
| Quintile 1 | 1 | 1 | 1 |
| Quintile 2 | 0.86 | 0.5 | 1.48 |
| Quintile 3 | 0.77 | 0.44 | 1.33 |
| Quintile 4 | 0.78 | 0.45 | 1.35 |
| Quintile 5 | 0.39 | 0.2 | 0.74 |
| **Dietary nitrate intake from vegetables** |  |  |  |
| Continuous (per 10mg/day) | 0.95 | 0.91 | 0.98 |
| Quintile 1 | 1 | 1 | 1 |
| Quintile 2 | 0.79 | 0.46 | 1.36 |
| Quintile 3 | 0.83 | 0.49 | 1.42 |
| Quintile 4 | 0.79 | 0.45 | 1.37 |
| Quintile 5 | 0.39 | 0.2 | 0.73 |
| **Dietary nitrate intake from non-vegetable food sources** |  |  |  |
| Continuous (per 10mg/day) | 0.65 | 0.42 | 0.99 |
| Quintile 1 | 1 | 1 | 1 |
| Quintile 2 | 0.97 | 0.57 | 1.67 |
| Quintile 3 | 0.95 | 0.55 | 1.62 |
| Quintile 4 | 0.66 | 0.37 | 1.18 |
| Quintile 5 | 0.69 | 0.39 | 1.24 |

Alternative model 3: model 1 additionally adjusted for *spherical equivalent*, education level and smoking status.

|  |  |  |  |
| --- | --- | --- | --- |
|  | OR | Lower | Upper |
| **Total dietary nitrate** |  |  |  |
| Continuous (per 10mg/day) | 0.94 | 0.90 | 0.98 |
| Quintile 1 | 1 | 1 | 1 |
| Quintile 2 | 0.92 | 0.53 | 1.62 |
| Quintile 3 | 0.73 | 0.41 | 1.31 |
| Quintile 4 | 0.76 | 0.43 | 1.35 |
| Quintile 5 | 0.34 | 0.17 | 0.67 |
| **Dietary nitrate intake from vegetables** |  |  |  |
| Continuous (per 10mg/day) | 0.94 | 0.91 | 0.98 |
| Quintile 1 | 1 | 1 | 1 |
| Quintile 2 | 0.86 | 0.49 | 1.51 |
| Quintile 3 | 0.79 | 0.45 | 1.39 |
| Quintile 4 | 0.80 | 0.45 | 1.42 |
| Quintile 5 | 0.34 | 0.17 | 0.66 |
| **Dietary nitrate intake from non-vegetable food sources** |  |  |  |
| Continuous (per 10mg/day) | 0.62 | 0.40 | 0.97 |
| Quintile 1 | 1 | 1 | 1 |
| Quintile 2 | 0.96 | 0.55 | 1.68 |
| Quintile 3 | 1.00 | 0.57 | 1.73 |
| Quintile 4 | 0.64 | 0.35 | 1.16 |
| Quintile 5 | 0.65 | 0.35 | 1.19 |

**Overall the finding seems to be an association without a rationale since in effect of nitric oxide presumably related to past nitrate intake would, by their own discussion, change eye pressure or blood pressure yet neither variable was associated with the incident glaucoma.**

In the introduction section we have suggested that dietary nitrate might have both IOP-dependent, or blood pressure dependent, and IOP-independent effects that could affect glaucoma risk. Unfortunately, we were only able to assess the effect of dietary nitrate intake on IOP and blood pressure. In the most ideal situation, we would have assessed the effect of dietary nitrate intake on retinal nerve fiber layer (RNFL) thickness. Unfortunately, due to limitations as described above, we were not able to do so. Since we found no significant effects of dietary nitrate intake on IOP and blood pressure, we suggest that IOP-independent effects of dietary nitrate intake might influence glaucoma risk. This should be further investigated in future studies.

1. Springelkamp, H.; Wolfs, R.C.; Ramdas, W.D.; Hofman, A.; Vingerling, J.R.; Klaver, C.C.; Jansonius, N.M. Incidence of glaucomatous visual field loss after two decades of follow-up: the Rotterdam Study. *Eur J Epidemiol* **2017**, *32*, 691-699, doi:<https://dx.doi.org/10.1007%2Fs10654-017-0270-y>.

2. Dielemans, I.; Vingerling, J.R.; Hofman, A.; Grobbee, D.E.; de Jong, P.T. Reliability of intraocular pressure measurement with the Goldmann applanation tonometer in epidemiological studies. *Graefes Arch Clin Exp Ophthalmol* **1994**, *232*, 141-144.

3. Wolfs, R.C.; Borger, P.H.; Ramrattan, R.S.; Klaver, C.C.; Hulsman, C.A.; Hofman, A.; Vingerling, J.R.; Hitchings, R.A.; de Jong, P.T. Changing views on open-angle glaucoma: definitions and prevalences--The Rotterdam Study. *Invest Ophthalmol Vis Sci* **2000**, *41*, 3309-3321.

4. Ramdas, W.D.; Rizopoulos, D.; Wolfs, R.C.; Hofman, A.; de Jong, P.T.; Vingerling, J.R.; Jansonius, N.M. Defining glaucomatous optic neuropathy from a continuous measure of optic nerve damage - the optimal cut-off point for risk-factor analysis in population-based epidemiology. *Ophthalmic Epidemiol* **2011**, *18*, 211-216.

5. Ramdas, W.D.; Wolfs, R.C.; Hofman, A.; de Jong, P.T.; Vingerling, J.R.; Jansonius, N.M. Heidelberg Retina Tomograph (HRT3) in population-based epidemiology: normative values and criteria for glaucomatous optic neuropathy. *Ophthalmic Epidemiol* **2011**, *18*, 198-210.

6. Dinnissen, C.S.; Ocké, M.C.; Buurma-Rethans, E.J.M.; van Rossum, C.T.M. Dietary Changes among Adults in The Netherlands in the Period 2007-2010 and 2012-2016. Results from Two Cross-Sectional National Food Consumption Surveys. *Nutrients* **2021**, *13*.

7. Balder, H.F.; Goldbohm, R.A.; van den Brandt, P.A. Dietary patterns associated with male lung cancer risk in the Netherlands Cohort Study. *Cancer Epidemiol Biomarkers Prev* **2005**, *14*, 483-490.

8. Goldbohm, R.A.; van 't Veer, P.; van den Brandt, P.A.; van 't Hof, M.A.; Brants, H.A.; Sturmans, F.; Hermus, R.J. Reproducibility of a food frequency questionnaire and stability of dietary habits determined from five annually repeated measurements. *Eur J Clin Nutr* **1995**, *49*, 420-429.