Author's response to reviews

Title: Single and repeated moderate consumption of native or dealcoholized red wine show different effects on antioxidant parameters in blood and DNA strand breaks in peripheral leukocytes in healthy volunteers: a randomized controlled trial (ISRCTN68505294)

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

Please find the revised version of the above manuscript together with an itemized list of changes according to reviewers’ comments.

We are very grateful to the reviewers for their positive evaluation and constructive comments.

Reviewer # 1:

1. Only healthy subjects free of disease were included in the study. Presumably these people would not be under oxidative stress, given its association with various disease states, so would the authors have expected to see an improvement in antioxidant parameters?

We agree that an improvement in antioxidant parameters after consumption of polyphenol rich beverages could rather be expected in oxidative stress situations (Ref. 36) as shown in one of our previous studies with HIV infected patients and healthy subjects (Ref. 32). However, antioxidant effects after ingestion of one single dose of red wine in healthy, non-smoking subjects could be demonstrated in numerous studies (p 3, lines 8-12 / Ref. 7-12) and could therefore be expected. In contrast, the effects of repeated red wine (RW) and dealcoholized red wine (DRW) consumption on plasma antioxidant capacity and DNA strand breaks were difficult to predict, as the results of previous studies are contradictory (p 3, lines 12-15 / Ref. 15-25).

Despite these conflicting data, RW consumption is discussed to protect from cardiovascular disease (CVD) at least in part due to antioxidant effects of polyphenols (Ref. 1-3, 5). Since primary prevention would require a sustained antioxidant response, the aim of our study was to investigate, whether regular moderate intake of RW according to the consumption pattern typical for France, where possible protective effects of red wine consumption on CVD have been observed first, could improve antioxidant parameters in healthy subjects and if DRW could be an alternative polyphenol source without the problems associated with alcohol. These aspects are now included in the introduction (p 3, lines 17-21).

2. Although the authors state their reasoning for using a different volume of DRW versus RW, perhaps the analysis to confirm the polyphenol content would have been better being performed prior to the study being conducted. It does add a confounding factor to the results.
Indeed, the intake of equal amounts of polyphenols with RW and DRW would have improved the significance of our study as this would allow for evaluating the effect of alcohol on bioavailability and antioxidant activity of RW polyphenols in vivo more accurately. Unfortunately, the data on polyphenol concentration in DRW were not available before the start of the study. So we had to estimate the polyphenol content as described in the manuscript (p 6, section Study drinks). Even though the total phenolic content was similar in 200 mL RW and 175 mL DRW (293 vs. 272 mg catechin equivalents) intake of antioxidant equivalents and single polyphenols differed between the groups RW and DRW (values for total phenolics and TEAC were added to Table 2).

To account for this limitation our analysis focuses on the changes compared to baseline in each group, i.e. a separate analysis of the effects from ingestion of RW and DRW (p 9-10, section Statistical analysis). If the effects of RW and DRW were compared to each other, the possible influence of different polyphenol concentrations has been discussed (p 12, lines 17-19; p 15, lines 15-17).

3. The authors use a single serving of RW (200 mL), does this equate to a single serving that would be regularly consumed by the public, and if not, why was this dose chosen?

Since protective effects of RW are expected only after regular consumption of RW, we chose an amount that is not likely to cause negative effects on health due to the alcohol content, even after daily consumption for several years.

In the year 2000 when the study was planned, there was no international consent on the tolerable daily alcohol intake (and there still is none). The German Nutrition Society [L1] tolerates a maximum of 20 g/d for men and 10 g/d for women, whereas the United States Department of Agriculture (USDA) recommends a maximum daily ingestion of 1 standard drink (≈ 17.7 g alcohol) for women and 2 drinks (≈ 35.4 g) for men [L2]. The guideline of the Australian Nutrition Foundation is 1 – 2 standard drinks (10 g alcohol per drink) for women and 2 – 4 standard drinks for men, which corresponds to 10 – 20 g alcohol for women and 20 – 40 g alcohol for men [L3]. Hence, 250 ml of RW which is the standard volume of one glass of RW in Germany, would have provided 31.2 g alcohol, which clearly exceeds the German limits for both sexes. Hence, a lower dose of RW was chosen for the study. The ingestion of 24.6 g alcohol from 200 mL of the study wine was considered as compromise because the dose is within the range of most reference values, at least for men, and detrimental effects would not be expected for women for an intervention period of 6 weeks.
In our opinion it is not useful to investigate the effects of higher doses of RW (as seen in some other studies) to increase polyphenol intake as the detrimental effects of alcohol would outweigh any potentially protective antioxidant effect in the long term.

4. Were there any changes in dietary patterns throughout the study or between the groups?

Dietary records were kept, but are not presented. This is particularly important given the volunteers were allowed to consume 2 cups tea/coffee and 2 glasses of fruit juice.

The data on nutritional intake obtained from food records are now presented in the text (single dose: p 10, lines 5-7; dietary intervention trial p 11, lines 7-17) and also included in the discussion (p 14, lines 2-9).

For the single dose analysis 24 h food records were completed to check the compliance of the subjects to dietary restrictions (p 5, line 2), whereas in the dietary intervention trial possible changes of dietary patterns due to seasonal alterations should also be assessed (p 5, lines 20-23). The food records for the 6 weeks intervention trial could not cover changes compared to the subjects’ usual diet that occurred because of the restrictions in tea/coffee and fruit juice consumption, as the first food record was completed in the week prior to the intervention period when the dietary restrictions should already be followed (p 5, lines 15-16).

5. Phenolic acid increases were observed following the acute and chronic studies in the control groups. On page 14 of the discussion, the authors suggest this may have been due to the permitted foods which could have provided considerable but unknown amounts of phenolic acids. However, in the methods section, the authors point out that the volunteers were allowed to consume food from a buffet that was low in polyphenols. This seems to be a contradiction in their explanation?

We assume that the reviewer refers to the total phenolic content in plasma in this question, which indeed increased in the control group 360 min after consumption of one glass of water. Obviously our discussion was not precise enough in this point. We intended to clarify that 1) some “permitted foods” might have provided considerable amounts of polyphenols but they are considered to be low in polyphenols as appropriate analysis are missing so far, and 2) the ingestion of foods rich in polyphenols after the second blood sampling cannot be excluded since the subjects were allowed to eat outside the study center between the 90 and 360 min, but food intake was not recorded (p 5, lines 5-8). The discussion has been changed to clarify this point (p 15, lines 17ff). The first aspect is now also included on page 5, lines 2-3: "For this purpose they
received a list of acceptable foods which are considered to be low in polyphenols."

In contrast to the single-dose analysis, total phenolic content in plasma did not change in the control group in the dietary intervention trial (15.5 ± 1.4 vs. 15.3 ± 2.2 mg CE/L; p > 0.05; see Table 4).

6. Would the authors expect to see changes in vitamin C or E following RW or DRW intake?
What would be their explanation for this?

Yes, we expected changes in the concentrations of plasma vitamin C (in both studies) and serum vitamin E (only in the dietary intervention trial). **Rationale:** Vitamin C sparing effects of polyphenols *in vivo* have been described already in the 1930ies by Szent-Györgyi and co-workers. Flavonoids could stabilize ascorbic acid, reduce dehydroascorbic acid to ascorbic acid and lower the metabolic consumption of vitamin C [L4]. Vitamin C in turn is able to regenerate vitamin E by reducing the oxidized form. Thus, in addition to vitamin C sparing effects, an increase in serum vitamin E level could be expected in the long-term. In the single-dose study α-tocopherol in serum was not determined, as changes in the concentration of fat soluble vitamins will probably not occur within a few hours (Ref. 26).

However, data on vitamin C and vitamin E in serum/plasma after RW consumption are conflicting. Cao et al. (Ref. 7) observed an increase in serum vitamin C concentration after single consumption of 300 mL DRW in elderly women, which did not occur in other studies after a single dose of 113 mL DRW (Ref. 10) or 550 mL RW (Ref. 21). After two weeks of supplementation with red wine extract vitamin E concentration in plasma-serum increased in one study (Ref. 15) but not in others (Ref. 17, 20).

7. Would an increase in uric acid following RW simply reflect an increase in alcohol intake?

In the single-dose study we observed an increase of uric acid in plasma 90 min after consumption of RW, which is now included in the discussion (page 13, lines 7-12). This fact could be explained by the alcohol which 1) increases purine degradation and 2) reduces renal excretion of uric acid by increasing lactate concentrations ([L5]; Ref. 41). As RW is a source of purines, RW could also directly contribute to increased uric acid levels in plasma (Ref. 52). Furthermore, phenolic compounds could also interfere with uric acid secretion and re-absorption (Ref. 52) and lactic acid could be provided by DRW (Ref. 41).

However, results for plasma concentrations of uric acid (as well as albumin and bilirubin) were not discussed in detail in the present manuscript, as they were measured as secondary parameters.
to elucidate whether changes of TEAC after RW or DRW consumption are really induced by polyphenols or could be due to changes in other plasma antioxidants - an important aspect also demanded by others ([L6]; Ref. 14).

8. What about other measures of oxidative damage, for example, F2-isoprostanes, considered to be reliable and specific marker of in vivo oxidative damage.

In general single cell gel electrophoresis is a “tool that is highly efficacious in human bio-monitoring of plant food antioxidants” [L8]. For the present study DNA strand breaks in peripheral leukocytes were of special interest, as our aim was not only to investigate the antioxidant activity (this manuscript) but also immunomodulatory effects of RW and DRW in vivo (Ellinger et al., manuscript in preparation). Since DNA strand breaks in leukocytes are associated with apoptosis and the comet assay is also an effective tool for apoptosis investigation [L9, L10], it could support the interpretation of our immunological data. However, additional investigation on oxidized purin and pyrimidin bases and/or activity of DNA repair enzymes would have strengthened the significance of our results. We added this aspect to our discussion (p 14, lines 2-5).

We agree that determination of F2-isoprostanes is an excellent parameter for oxidative stress in vivo which is considered to be both biologically and methodologically valid [L11]. The direct measurement of oxidation products is probably more accurate and of higher physiologic relevance than indirect methods like TEAC. However, the technical equipment required for measurement of F2-isoprostanes was not available in our laboratory. Nevertheless, we thank the reviewer for their important hint and will consider this parameter for future studies as the investigation has become easier by ELISA-testkits which are available.

Reviewer 2:

1. Abstract. The method for DNA strand breaks should be written clearer. The reader cannot understand the word, “endogenous” DNA strand breaks, shown in Results of the abstract.

The expression “endogenous DNA strand breaks” is now avoided in the abstract (p 2). Modifications of the abstract are highlighted below:

[...] Methods
Total phenolic content and concentration of other antioxidants in plasma/serum, total antioxidant
capacity (TEAC) in plasma as well as DNA strand breaks in peripheral leukocytes were measured
A) before, 90 and 360 min after ingestion of one glass of RW, DRW or water; B) before and after
consumption of one glass of RW or DRW daily for 6 weeks in healthy non-smokers. DNA strand
breaks were determined by single cell gel electrophoresis (Comet Assay) in untreated cells or
after induction of oxidative stress ex vivo (300 μM, 20 min).

Results
A) Single consumption of RW or DRW transiently increased total phenolic content but did not
affect TEAC in plasma. DNA strand breaks in untreated cells increased after RW or DRW
ingestion, whereas H₂O₂-induced strand breaks were reduced by DRW. B) Regular consumption
of RW but not DRW increased total phenolics in plasma and decreased DNA strand breaks in
untreated cells without affecting plasma antioxidant capacity. [...]
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Values are means ± SD (minimum - maximum)

* Significantly different from baseline and from group RW and controls
4. *P*11, line 19. Which does the value of statistical significance (*p*=0.03) specify between TI and TM?

The value of statistical significance is now given for TI and TM separately (*p* 12, lines 1ff).

“Endogenous DNA strand breaks (Figure 2, Table 4) decreased only after regular consumption of RW (TI: *p*=0.03; TM: *p*=0.04), but not in group DRW and controls.”

References for this response letter:

L1 Deutsche Gesellschaft für Ernährung (DGE): *Referenzwerte für die Nährstoffzufuhr*. Frankfurt am Main: Umschau / Braus; 2000


L8 Aruoma OI: *Antioxidant actions of plant foods: Use of oxidative DNA damage as a tool for studying antioxidant efficacy*. *Free Rad Res* 1999, 30:419-27

