**Author’s response to reviews**

**Title:** Clinical and Genomic Safety of Treatment with Ginkgo biloba L. leaf extract (IDN 5933, Ginkgoselect®Plus) in Elderly: A Randomised placebo-controlled clinical trial [GiBiEx].

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**Author’s response to reviews:**
Dear Dr. Liu,

Thank you for considering our manuscript for publication. My colleagues and I found the remarks raised by the reviewers to be helpful and, we made the changes accordingly. Our response to all of the reviewers’ comments is attached.

We respectfully submit a revised version of the manuscript for publication in BMC Complementary and Alternative Medicine.

Yours sincerely,

On the behalf of all authors

Stefano Bonassi, PhD
Point-by-point responses to the reviewers’ comments

Reviewer #1 (Shungte Kao):

1. Please explain why the evaluation of hepatotoxicity did not include bilirubin, ALKP, prothrombin time and AFP.

The USA National Toxicology Program (NTP) technical report on the toxicology and carcinogenesis studies of ginkgo biloba extract (cas no. 90045-36-6) in F344/n rats and B6C3F1/n mice (gavage studies) observed both in treated rats and mice lesions in the liver such as hyperplasia and increased incidences of liver cancers in male and female mice. Indeed they concluded that Ginkgo biloba extract caused cancers of the liver in male and female mice. Therefore early indication of an increased risk of liver cancer was our first safety concern. In the 2016 Stepien et al [Cancer Epidemiology 40 (2016) 179–187] reported positive associations between circulating liver biomarkers in sera such as gamma-glutamyl transferase, GGT; alanine
aminotransferase, ALT; aspartate aminotransferase, AST; alkaline phosphatase, ALP; total bilirubin and the risks of developing hepatocellular carcinoma and intrahepatic bile duct carcinoma. Not all markers were associated to intrahepatic bile duct carcinoma, and bilirubin was among them. The authors concluded that elevated liver enzymes are good pre-diagnostic markers of livers cancers and therefore for this reason we evaluated only GGT; ALT; AST.

2. Please explain why there was no evaluation of hepatotoxicity.

Serum activities of ALT (EC 2.6.1.2) and AST (EC2.6.1.1) are key indicators of drug-induced liver toxicity in regulated safety studies involving laboratory animals and patients, and for general patient monitoring [reviewed in Vet Clin Pathol. 2013 Dec;42(4):535-8]. Based on the level of elevation of transaminases or alkaline phosphatase and the ratio (R) of elevation of baseline ALT to baseline alkaline phosphatase (ALT/ULN)/(ALP/ULN), drug-induced liver injury is classified as either hepatocellular, cholestatic or mixed types. The degree of elevation in liver enzymes has poor correlation with severity of liver disease.[J Clin Exp Hepatol. 2012 Sep;2(3):247-59]

However, as described in the revised manuscript (Pag. 11, lines 1-2) our objective was not the hepatotoxicity but the risk of liver cancer. As explained above, we paid special attention to safety concerning the risk of developing livers cancers in individuals treated with Ginkgo Biloba. However liver transaminases (AST and ALT) are typically related to hepatic injury from either hepatitis infection, NAFLD (non-alcoholic fatty liver disease), liver cirrhosis or other causes, and therefore we changed the term hepatotoxicity with the more general definition of liver injury [Cleve Clin J Med 77 (3) (2010) 195-204].

3. Please describe only the assessment of expression patterns of genes putatively associated to early events of hepatic carcinogenesis and ignore the common associated cancer.

There is extensive evidence on gene expression in liver carcinogenesis. Recent papers such as Jen et al [J Biol Chem. 2013, 288:14451-62] showed that p53 was down-regulated in diethylnitrosamine treated mice after 6-25 weeks, or Yang et al. [FASEB J. 2001; 15 : 1507-16.] showed that the mRNA levels of c-myb and Sp1 is increased in human hepatocellular carcinoma.

In addition, the mechanism of hepatocarcinogenesis in GBE exposed animals is much more complex, involving alterations in H-Ras and Ctnnb1 mutation spectra, and WNT pathway disregulation, when compared to spontaneous liver cancer, as shown by Hoeneroff et al [Toxicol Pathol. 2013 Aug;41(6):826-41.]. These events are not always early but are highly specific in hepatic carcinogenesis, including GBE induced cancer, and therefore we decided to evaluate down/up-regulation of p53, Ctnnb1 and c-myb associated to GBE treatment. Moreover, when we
started the analysis, we could not predict anything about the possible involvement of gene changes occurring in blood cells obtained from patients potentially undergoing liver cancer development. Most of the reported literature data on gene expression in hepatocarcinoma actually refers to liver tissue, whereas a very limited set of experimental data are available on gene expression on lymphocytes of patients undergoing liver cancer development at early stages. Based on these evidences, we believe we cannot exclude the involvement of c-myb, p53 and ctnnb1 in our analysis.

4. The study was the elderly and suffering from a variety of diseases, the DNA damage and genomic instability may be exist, how to makes differential diagnosis. We agree with the reviewer on this limitation of the study. Nevertheless, the study was not designed to make differential diagnosis among diseases, but rather to measure possible variations in DNA damage and genomic instability associated to the treatment with GBE (under the assumption of independency from existing diseases).

JOHNSON STANSLAS (Reviewer 2): General comments

1. Too many acronyms in the text without providing their full names (definitions) at first mention. The list of abbreviations is only found almost close to end of the document.

All acronyms used in the text have been spelled out at first mention.

2. It is good to get the language edited.

The text has been carefully reviewed

Specific comments

Abstract

1. NTP - in full.

The acronym has been spelled out as requested, i.e., US National Toxicology Program (Page xx, line xx)
2. Provide more details of Ginkgoselect®Plus such as

a. how it was prepared.

b. standardisation of the extract.

Details about GBE preparation and standardization are now reported (Page 2 lines 9-12)

3. Elaborate further how clinical (adverse clinical effects, hepatotoxicity) end points were determined.

The critical safety endpoints of the study were dealing with liver carcinogenesis, however the presence of adverse health effect (specific for liver functionality or systemic) was a complementary endpoint of evident clinical interest. Specific questions were selected from the literature on human studies in individuals treated with GBE. The choice of liver enzymes was mostly based on the evidence cited above (Stepien et al., 2016) that that elevated liver enzymes are good pre-diagnostic markers of livers cancers.

4. From which biological sample the genomic studies were conducted?

Gene expression profiles were evaluated in lymphocytes (now more explicitly reported in methods (Page 8, line 12)

Introduction

1. "the compound has been.." - not a compound but rather best referred as extract!

The term compound has been replaced by the term extract (Page 3, line 16)

2. Technical Report published by the US National Toxicology Program - provide the supporting literature.

The National Toxicology Program (NTP) is an interagency program within the Public Health Service (PHS) of the Department of Health and Human Services (HHS) and is headquartered at the National Institute of Environmental Health Sciences of the National Institutes of Health (NIEHS/NIH). The studies described in the Technical Report series are designed and conducted
to characterize and evaluate the toxicological potential, including carcinogenic activity, of selected substances in laboratory animals (usually two species, rats and mice). Substances selected for NTP toxicity and carcinogenicity studies are chosen primarily on the basis of human exposure, level of production, and chemical structure. NTP Technical Reports are indexed in the NIH/NLM PubMed. The NTP technical report on the toxicology and carcinogenesis studies of ginkgo biloba extract (cas no. 90045-36-6) in F344/n rats and B6C3F1/n mice (gavage studies) is a comprehensive study of 191 pages reporting a large literature of biological effects of ginkgo biloba extract cited in our manuscript at the item n. 28.

Materials and Methods

1. Was the blood thinning effect of Ginkgoselect®Plus determined?

We did not monitor this parameter since we excluded those individual at higher risk of bleeding, i.e., those subjects with previous report of increased bleeding tendency under treatment with anticoagulant and anti-aggregant drugs.

2. Is 6 months trial sufficient to provide the safety profile of this product when in reality it is consumed for a longer time? Do note that the toxicological study by US National Toxicology Program was done for 2 years!

This one is not a study on the possible carcinogenicity of GBE, but a safety study in which early events of genomics risks are evaluated. As regards the micronucleus and the comet assays, there is extensive literature that even an exposure of few days may alter the background frequency of DNA damage.

3. State how randomization was performed.

In the revised Materials and Methods the randomization procedure was described in more detail (Page 5, Lines 25-27).


The certificate of analysis of Ginkgoselect®Plus was attached as supplementary file n.1.

5. NMT? 5 ppm
Not More Than 5 Parts Per Million. The acronym been spelled out in the revised version of the manuscript.

6. Provide the questionnaire as a supplementary file.

The questionnaire (in the original language) has been attached as supplementary file n. 2.

7. The numbers do not add up "A total of 19 subjects (28.8%), withdrew from the study for the following reasons: death (19), discharge (10), admission to another hospital for worsening state of health (4), and discontinued treatment (4)". I believe the correct number of death is 1.

Yes, the correct number is 1 and the typo has been corrected.

8. State how many males and females in each group.

In the revised Materials and methods at page 6, lines 18-29, we reported the number of males and females for each group.

9. MR, GR, and RR?

The initials referred to the doctors among the authors that were in charge of clinical assessment. However, since this information may be misinterpreted it was removed.

10. SPSS version?

In the revised version of Materials and Methods (Page 10, line 9) we reported the version of the software (version 16.0).

11. What is the cause of death of a patient in the Ginkgoselect®Plus group?

The patients died of acute pancreatitis in chronic renal failure patient. The competent organisms (ISS-Istituto Superiore di Sanità and the local ethics committee) were timely informed. The doctors in charge of the patient ascertained that the death had nothing to do with the treatment
with GBE. A note about this case has been added to the revised Materials and Methods session (Page 6, line 16-17; Page 10, lines 22-24).

12. CBMN? assay.

CBMN assay is the standard acronym for the assay. However, for the sake of non specialized readers we used the term micronucleus (MN) assay instead of CBMN.

Results

1. "Up-regulated in 3 out of 8 IDN 5933-treated patients, down-regulated in 3 other GBLE-treated patients - what is the difference between IDN5933 and GBLE"?

Ginkgo biloba L. leaf extract, IDN 5933, and Ginkgoselect®Plus, are synonyms, as explained in the introduction and in the Materials and Methods. Occasionally we used variants to avoid repetitions. This sentence is actually quite confusing, and therefore we changed the text. We reviewed the whole manuscript reducing the use of variants to the GBLE main definition.

2. Why no statistical analysis is shown in "Table 5.

Table 5 was removed but the row data are attached in Supplementary material section. The statistical value (p > 0.05 Fisher's exact test of independence) was reported in the revised Results session (Page 12, lines 4-6; 9-11).

3. Expression profiling of c-myb and p53 genes"? However, in the text it is mentioned that no significant difference was observed - what statistical test used for this purpose?

Given the supervised approach to data analysis, and the emphasis on the up or down regulation of gene expression, we evaluated the distribution of subjects with significantly altered profiles for the candidate genes with the Fisher's exact test of independence.

Discussion

1. A specific heading on Limitations of Study should be provided immediately after the "Discussion" section.
In the revised version of the manuscript a specific paragraph on the limitations of the study has been added to the Discussion (Page 14, lines 23-28; Page 15, lines 1-6).

2. Upload raw data of MN, comet assay and gene expression studies as supplementary files. Row data are now uploaded as supplementary files (n.3, 4, 5).