Author's response to reviews

Title: NOX2-generated oxidative stress is associated with severity of ultrasound liver steatosis in patients with non-alcoholic fatty liver disease

Authors:

Maria Del Ben (maria.delben@uniroma1.it)
Licia Polimeni (licia.polimeni@gmail.com)
Roberto Carnevale (roberto.carnevale@uniroma1.it)
Simona Bartimoccia (simona.bartimoccia@gmail.com)
Cristina Nocella (cristina.nocella@gmail.com)
Francesco Baratta (francesco.baratta@gmail.com)
Lorenzo Loffredo (lorenzo.loffredo@uniroma1.it)
Pasquale Pignatelli (pasquale.pignatelli@uniroma1.it)
Francesco Violi (francesco.violi@uniroma1.it)
Francesco Angelico (francesco.angelico@uniroma1.it)

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Author's response to reviews: see over
To the Editor

BMC Gastroenterology

Please, find enclosed a carefully revised version of our manuscript “**NOX2-generated oxidative stress is associated with severity of ultrasound liver steatosis in patients with non-alcoholic fatty liver disease.**”

The manuscript has been extensively revised taking into consideration all the comments, questions and suggestions of the reviewers. A point to point reply to the criticisms of each Reviewer has been prepared.

The revised text clearly indicates where changes have been made by using a red font. In particular, following the Editorial Board Comments, the manuscript has been revised particularly in relation to comments and criticisms of Reviewer 4.

The following major changes have been performed:

- The concept of the multiple parallel hit theory has been introduced in the Introduction and in the Discussion
- Two new multivariate analyses have been performed: the first assessing independent predictors of the severity of NAFLD assessed by serum cytokeratin-18 levels; the second assessing the independent predictors of systemic oxidative stress, assessed by urinary 8-isoprostane levels. The results of the two analyses have been reported in the Results and commented in the Discussion.
- We evaluated differences between subjects with probable NASH and those with low probability of having HASH according to the noninvasive scoring system NAFLD fibrosis score.

Other minor changes have been performed in the Abstract, Core tips, main text (see our point to point reply to Reviewers), references and tables
A detailed reply has also been given to all other issues raised by the 4 Reviewers and appropriate changes in the text and in the tables have been done.

Finally, the manuscript has been revised for typos and grammatical errors by a native speaker of English.

**POINT-TO-POINT REPLY**

**Reviewer 1:**

Begoña Ochoa

Given that few studies have reported increased circulating levels of oxidative stress markers in patients with non-alcoholic fatty liver and no published study has been performed with newer markers of systemic oxidative stress, the authors aim to assess the relationship between urinary 8-iso-prostaglandin F2α, a well-admitted marker of oxidative stress, and serum soluble NOX2-derived peptide (sNOX2-dp) and the severity of liver steatosis in subjects with non-alcoholic fatty liver. Overall, the study is designed and performed...
appropriately, including an ample cohort of patients, although it elicits the drawbacks that the authors themselves point out in the Discussion section. In summary, the study demonstrates that the levels of urinary 8-iso-PGF2α were independent predictors of non-alcoholic fatty liver and a strong association of urinary 8-iso-PGF2α and of serum sNOX2-dp with the severity of steatosis at ultrasound was also observed. These findings are interesting and valid in clinical practice.

Minor essential revisions:

The authors conclude "We demonstrated an increased NOX2-generated oxidative stress in subjects with non-alcoholic fatty liver. Oxidative stress was independent from obesity, diabetes and metabolic syndrome and increased with the severity of liver steatosis at ultrasound." None of the sentences is right. Serum oxidative stress markers are general markers of the oxidant-antioxidant balance of the whole body and no measurement of free radicals derived from NOX2 has been performed. Neither "such oxidative stress" can be said that is independent of the conditions alluded. Therefore, conclusions should be rewritten properly.

Reply: Following the suggestion of the Reviewer, the conclusions have been rewritten both at the end of the Summary and of the Discussion.

Although the authors report that subjects underwent routine biochemical evaluation, they should clearly state how urine and blood collection and treatment was performed, otherwise experimental conditions may be not reproducible.

Reply: We have described in the Laboratory measurement section how blood samples and urine spot samples were collected and treated.

Tables. Parameters should be clearly defined in footnotes in all tables. Revise commas and dots.

Reply: Parameters have been clarified in footnotes in all tables. Commas and dots have been carefully revised.

Level of interest: An article whose findings are important to those with closely related research interests

Quality of written English: Acceptable

Statistical review: No, the manuscript does not need to be seen by a statistician.

Reviewer 2

Amedeo Lonardo

1. The “two-hit theory”, although often quoted, is not the only nor the most updated one. Other pathogenic and histogenic views have been proposed and may be discussed ( Wanless IR, Semin Liver Dis.2004;24:99-106; Lonardo A, et al. Clinical physiology of NAFLD: a critical overview of pathogenesis and treatment Expert Review of Endocrinology and metabolism 2010 ; Tilg H, Hepatology. 2010;52:1836-46.). In particular, the Authors may be willing to discuss that simple steatosis and NASH are probably two unrelated disorders

Reply: The multiple parallel hit theory has been introduced in the Introduction and in the Discussion. We have also introduced a sentence stating that simple steatosis and NASH may be two different disorders. Two references (10 an 11) have been added.

2. The Authors may be willing to discuss that, at variance with other more recent and validated indices (Ballestri S, et al Liver Int. 2012;32:1242-52.), the ultrasonographic Hamaguchi score does not specifically predict NASH.

Reply: We acknowledge that Hamaguchi score does not specifically predict NASH. A sentence has been added in the limitations of the study (see Discussion)

3. The Authors need to be more prudent: Statistical association between US evidence compatible with steatosis and markers of increased oxidative stress does not imply casualty. Moreover, the two quoted studies (Pacana T, Curr Opin Clin Nutr Metab Care 2012,15:641–648. and Sanyal AJ, New Engl J Med 2010,362:1675–1685.) are not guidelines. Finally, based on comparative analysis, the statement that guidelines support antioxidant treatment should be deleted (Nascimbeni F, J Hepatol. 2013;59:859-71.).

Reply: The Reviewer is right. The two quoted references are not guidelines. They have been replaced with reference 13 (Chalasani N et al. – AASLD guidelines) which suggests vitamin E supplementation for the treatment of NASH in non diabetic subjects.

4. This submission states that “Liver steatosis was defined according to Hamaguchi criteria based on the presence of abnormally intense, high level echoes arising from the hepatic parenchyma, liver-kidney difference in echo amplitude, echo penetration into deep portion of the liver and clarity of liver blood vessel structure [32-33].” Accordingly, reference 32 needs to be deleted.

Reply: Reference 32 has been deleted

5. The statement “.....ultrasound, which is a qualitative method inadequate to quantify less than 30% liver fat content [36].” conflicts with more recent studies (Dasarathy S, J Hepatol. 2009;51:1061-7).

Reply: The proportion has been modified in 20%, according to Desarathy et al. A new reference (38) has been added.

Level of interest: An article whose findings are important to those with closely related research interests

Quality of written English: Acceptable

Statistical review: Yes, but I do not feel adequately qualified to assess the statistics.

Reviewer 3:

Carmine Finelli
Major Compulsory Revisions

• What is the concentration of 8-iso-prostaglandin F2# (8-iso-PGF2#) in patients with NAFLD without METS?

Reply: This data is already reported in table 3

• Are patients smoking?

Reply: Only 20.8% of subjects were current smokers. There were no significant differences in mean urinary 8-isoprostanes and serum NOX2 levels between smokers and non smokers.

• What is the renal function of patients?!

Reply: All subjects had normal renal function. No significant differences in serum creatinine were found between subjects with NAFLD and those without.

In fact, the prostaglandin, 8-iso-PGF2#, is an isoprostane that is produced by the non-enzymatic peroxidation of arachidonic acid in membrane phospholipids. Measurement of 8-iso-PGF2# is a reliable tool for the identification of subjects with enhanced rates of lipid peroxidation. Enhanced formation of 8-iso-PGF2# has been reported in association with several cardiovascular risk factors, as well as atherosclerosis. Cigarette smoking is a risk factor for renal function alteration in healthy smokers and is characterized by a high eGFR and a high urinary protein associated with an increase in the 8-iso-PGF2# and hs-CRP. (Sauriasari R, Sakano N, Wang DH, Takaki J, Takemoto K, Wang B, Sugiyama H, Sato Y, Takigawa T, Takahashi N, Kanbara S, Hitomi Y, Nakamura H, Ogino K. C-reactive protein is associated with cigarette smoking-induced hyperfiltration and proteinuria in an apparently healthy population. Hypertens Res. 2010 Nov; 33 (11): 1129-36.)

Patients with NAFLD have almost all damage endothelial (dysfunction). This damage is known to be correlated with elevated concentrations of 8-iso-PGF2# (Nassar H, Furu kado S, Tanaka M, Miwa K, Okazaki S, Sakaguchi M, Mochizuki H, Kitagawa K. The relation between carotid plaque echogenicity and oxidative stress marker 8-iso-prostaglandin F2#. Ultrasound Med Biol. 2012 Mar;38(3):487-91.). Should it be evaluated perhaps?!

Reply: Although important, we believe that he study of surrogate markers of arteriosclerosis (FMD, IMT, ABI) is out of the aims of this study.

In addition, lacking a healthy control group !!!

Reply: In our study we compared patients with NAFLD with patients without NAFLD consecutively recruited in a outpatient clinic for metabolic and cardiovascular diseases. In our opinion, to compare patients with NAFLD with patients without NAFLD and the common risk factors (real healthy controls) would have been misleading.

Minor Essential Revisions: The interpretation (discussion and conclusion) should be reevaluated in the light of these results, as well as any statistical analysis.

Tables are not easily accessible to the reader and should be semplicate and divided.

Reply: We have revised the tables and improved the variable description.

Discretionary Revisions
• Why the authors not describe patients without METS?

Reply: Clinical and biochemical characteristics of NAFLD patients with and without METS are reported in table 3

Level of interest: An article of outstanding merit and interest in its field

Quality of written English: Acceptable

Statistical review: Yes, and I have assessed the statistics in my report.

Reviewer 4:

Sven Francque

Major Compulsory Revisions

The authors report on the parameters of oxidative stress in patients with NAFLD. The question is relevant. The paper is well written and nice to read. Methodology is overall well-elaborated and adequate. Some issues need to be addressed:

According to the correlation table, the urinary PGF2alpha strongly correlates with BMI and inversely with adiponectin, but to a lesser extent with the US Hamaguchi score. In the regression analysis looking at the dichotomous question NAFLD vs. no NAFLD, BMI appears not to be an independent variable.

This asks for some comments: First NAFLD is not always NASH, and in terms of inflammation and disease, NASH is considered more dangerous than simple steatosis. The question is whether we expect oxidative stress to be increased in a patient with mild steatosis and without steatohepatitis. So the question remains whether we want to oppose NAFLD vs. no NAFLD (which means that mild degrees of steatosis are also included) or whether the relationship with the severity of the steatosis is more relevant. So I would like to see a regression analysis assessing factors independently related to the severity of the NAFLD, and a regression analysis assessing factors independently related to the degree of oxidative stress as based on the parameters used in this paper.

Reply: Following the suggestion of the Reviewer, we performed a first multiple linear regression analysis assessing factors independently related to the severity of NAFLD assessed by serum cytokeratin-18 serum levels, a marker of apoptosis reflecting liver disease severity. The multivariate analysis showed an independent association of urinary 8-isoprostanes and the severity of NAFLD assessed by cytokeratin-18 serum levels (independent predictors were also BMI and serum adiponectin).

In addition, following the Reviewers suggestion, we also performed a further multiple linear regression analysis to evaluate factors predicting the degree of systemic oxidative stress assessed as urinary levels of 8-isoprostanes. In this analysis, BMI and cytokeratin-18 (i.e NAFLD severity) were independent predictors of increased oxidative stress after controlling for confounding factors.
The results of both regression analyses have been reported at the end of the Results and commented in the Discussion.

Furthermore non-invasive scores for the diagnosis and severity of NASH and fibrosis. Most of these scores can be calculated with the parameters that were recorded in these patients. I would therefore like to see an analysis in relation to these scores, and a comparison nash vs. no nash (it can be argued that, as there is no biopsy as golden standard, these scores can be used (with all their inherent limitations)).

Reply: Among non invasive scoring systems for the diagnosis and severity of NASH and fibrosis, NAFLD fibrosis score is the most validated one and identifies liver fibrosis in patients with NAFLD. It is able to accurately separate patients with NAFLD with and without advanced fibrosis (NAFLD fibrosis score greater than 0.676 and lower than -1.455, respectively) (Angulo P et al. The NAFLD fibrosis score: a noninvasive system that identifies liver fibrosis in patients with NAFLD. Hepatology 2007). We performed an analysis in relation to this score. In our population, patient with probable NASH had higher levels of markers of oxidative stress, but the difference didn’t reach statistical significance. This is likely due to the very small number of patients in this group (only 15 patients). Therefore, we decided not to include the analysis in the paper.

It would also be nice to include waist in the analyses, as visceral obesity seems to be more relevant than just the BMI.

Reply: We performed new multivariate analyses including waist circumference instead of BMI as independent variable. The results were similar.

The question remains unanswered, as this is a cross-sectional analysis (this should clearly be discussed by the authors) whether oxidative stress, caused by obesity, contributes to the pathophysiology of NAFLD (or perhaps more NASH) or whether the inflamed liver contributes to the oxidative stress (and hence long term complications).

Reply: The question raised by the Reviewer has been discussed in the Discussion as a possible limitation of the study.

Discretionary Revisions: A minor remark: overall the paper is very well written, but some of the tables need some correction (cytocheratin 18 e.g.).

Reply: All tables have been carefully reviewed.

Level of interest:

An article whose findings are important to those with closely related research interests

Quality of written English: Acceptable

Statistical review: No, the manuscript does not need to be seen by a statistician.