Author's response to reviews

Title: Strong association between non alcoholic fatty liver disease (NAFLD) and low 25(OH) vitamin D levels in an adult population with normal serum liver enzymes.

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Author's response to reviews:

Dr Lin Lee,

Please find enclosed the revised version of our manuscript entitled: “Strong association between non alcoholic fatty liver disease (NAFLD) and low 25(OH) vitamin D levels in an adult population with normal serum liver enzymes” (MS: 1972360686520661) together with a point by point list of the changes made in response to all the reviewers’ comments. In the manuscript all the changes made have been written in red.

We hope that you will now find our work suitable for publication in your journal.

Best regards

Maria Gisella Cavallo

I) Reviewer: Kathleen M Botham

Reviewer's report:

The aim of this study was to test the hypothesis that non alcoholic fatty liver disease (NAFLD) is associated with hypovitaminosis D in subjects with insulin resistance and related metabolic disorders, but without previously diagnosed liver disease. 162 out of 262 patients referred to the Diabetes and Metabolic Diseases clinic in Rome were found to have NAFLD, and these subjects were found to have significantly lower levels of serum 25 hydroxyvitamin D compared to subjects without NAFLD independently of age, sex, serum triglycerides, HDL and glycaemia. The study appears to have been well carried out with appropriate study design and analyses. This topic is of interest, but is not entirely new, as an association between low serum vitamin D and NAFLD has been reported previously in patients with existing liver disease.
Minor essential revisions

1. The English grammar and paragraph structure needs attention throughout the manuscript.
We have now revised the grammar and the paragraph structure throughout the manuscript.

2. The authors refer to vitamin D and calcitriol, are these terms interchangeable?
It should be made clear if they intend a difference between them, if there is no difference, they should stick to one or the other.

In our manuscript calcitriol was considered as synonymous of vitamin D. We have now substituted the term “calcitriol” with “vitamin D” throughout the text, as suggested by this reviewer.

3. Results. Data given in the tables should not be repeated in the text.
4. We have removed from the text the clinical and biochemical parameters of study sample described in table 1; in addition table 4 was considered repetitive and therefore removed.

II) Reviewer: Jane Lynch

1. Is the question posed by the authors new and well defined? This question is of interest because it addresses adults at risk for fatty liver who do not present with elevated liver enzymes as the risk factor for NAFLD. It is estimated that at least one third of patients with fatty liver do not have elevated ALT. Although the hypothesis is well defined, the introduction includes a description of the current theories of both NASH and NAFLD which could be summarized to allude to the role of NAFLD to possible early mechanisms of insulin resistance. This rationale would then explain why a better understanding low vitamin D’s role in fatty liver would be relevant to metabolic syndrome research and for potential prevention of cirrhotic liver disease. Because low vitamin D is known to be more prevalent in obesity, one would expect a positive correlation with metabolic syndrome and this is not the focus of this paper.

We have now summarized in a sentence and reference the theories about the role of NAFLD in early mechanisms of insulin resistance omitting their full description from the Introduction: “However, recent investigations hypothesized a primary role of fatty liver in determining insulin resistance [20]”, as suggested by this reviewer.

2. Are the methods appropriate and well described, and are sufficient details provided to replicate the work? The study hypothesis was that there was a direct association between hypovitaminosis D and the presence of NAFLD in subjects with various degrees of insulin-resistance and related metabolic disorders. The Surrogate measure to estimate the degree of fatty liver was abdominal ultrasonography to quantify fat in the liver as well as a Fatty-Liver-Index correlate of NAFLD (reference 25). The use of the ultrasound surrogates is not ideal, especially in obese patients, however it is a practical approach commonly used in
practice. The remaining assessment for other causes of liver disease was certainly extensive however this would more typically be done for patients with findings of an elevated AST, ALT or other evidence of liver disease in the initial evaluation of a patient with elevated liver function tests. The studies for HOMA IR to provide measures of insulin resistance are appropriate and interesting in this paper. No measure of insulin secretion or sensitivity testing is available because these require OGTT derived data for the equations.

We thank the referee for her comments, and we understand that further clarification/modification are not required.

3. A) Are the data sound and well controlled? Of the 262 consecutive subjects, 52 had T2DM. Of the patients with T2DM, there was a 3:1 ratio of positive NAFLD. The data regarding the subgroup with T2DM was addressed in table 5 where the study sample was stratified according to serum 25(OH) vitamin D quartiles. The prevalence of NAFLD, MS and its components was increased in the lowest 25(OH) vitamin D quartile whereas the prevalence of T2D was similar in the bottom and top quartiles with a p value of .001. The clinical significance of this data on only a total of 52 T2DM individuals who all had relatively low vitamin D levels was based on the mean overall group differences of 14.8 and 20.5 which had a SD of 9 in each. I think this study would be stronger with this group treated as a different cohort or analyzed separately from the non DM patients.

We fully agree with this reviewer that our results could be stronger after analysing T2D subgroup separately from non-DM patients. The results of the comparison of T2D vs non-diabetic subjects and the logistic regression analysis performed in the whole population have been added in a specific paragraph of Results section: “We also analyzed our study population according with the presence of T2D. Patients affected by T2D had serum 25(OH) vitamin D levels similar to non diabetic patients (17 ± 10.2 ng/ml vs 17.5 ± 8.8, p= n.s.); the logistic regression analysis performed in the whole population confirmed that vitamin D was not a determinant of diabetes (p=n.s.).”

B) If there is an independent association between MS and low vitamin D, it is not surprising that the cohort with NAFLD (BMI 31.3 and waist circumference 105.9) had lower vitamin D than those without NAFLD (BMI 25.8 and waist circumference 90.2) The data probably needs to be analyzed with a multivariate analysis to control for the role of the BMI and/or waist circumference to be more compelling that this relationship described is not one related to body size and MS. However, it is interesting that the association between NAFLD and low 25(OH) vitamin D levels was independent from age, sex, triglycerides, HDL and FBG in a multiple regression analysis model. The subgroup of normal weight subjects (n=70) with a BMI< 25 are interesting and would reinforce the findings of less correlation with lipid profiles since these were normal weight individuals referred to the clinics for suspected MS. It is surprising to see the very high correlation between ultrasound and FLI index in this study knowing the issues with surrogate ultrasound measures for fatty liver in obese patients.

We are aware that the adiposity, as estimated by BMI and/or waist circumference, is tightly associated with MS and may represent a confounding
factor in determining the association between low vitamin D and NAFLD. We have now added a new multivariate logistic model including age, sex, 25(OH) vitamin D and BMI, as suggested by this reviewer, confirming the independent inverse correlation between 25(OH) vitamin D and the presence of NAFLD. These sentences have been added in the Results section: “The association between NAFLD and low 25(OH) vitamin D levels (...) is not affected by BMI in a multivariate logistic model comprising sex, age, 25(OH) vitamin D and BMI (25(OH) vitamin D: OR 0.93, C.I. 0.88-0.99, p=0.02; BMI OR 4.4, C.I. 2.2-8.9, p<0.001)” and in the Discussion: “We performed a logistic multivariate analysis also adjusting for BMI demonstrating that the association between NAFLD and 25(OH) vitamin D persists after BMI adjustment”.

4. Does the manuscript adhere to the relevant standards for reporting and data deposition? Yes.

5. Are the discussion and conclusions well balanced and adequately supported by the data? Similar to my comments regarding unfocused introductory information, the final discussion should likewise focus on the topic of this specific paper. For example, the following discussion content is not one addressed in this paper and could be summarized to a sentence with reference alluding to the concerns that vitamin D may play a role in both NAFLD and cirrhosis outcomes. “Since insulin-resistance is known to promote fibrosis progression [37,38], it can be speculated that vitamin D may ameliorate hepatic outcomes not only though an anti-inflammatory action but also by an insulin-sensitizing effect. Thus, it was largely demonstrated that calcitriol exerts an insulin-sensitizing action by increasing the expression of insulin receptors in peripheral tissues and facilitating the insulin-mediated glucose transport.

We have now focused the discussion on the specific topic of this paper, as suggested by this reviewer. For this reason we summarized the hypothetical role of vitamin D in liver diseases in a sentence and references omitting its specific molecular activities, as follows: “Vitamin D levels inversely associated with the presence of dysmetabolic conditions in our study as well as in other published reports [5-7] and may play a role in both NAFLD and cirrhosis outcomes though its anti-inflammatory and insulin-sensitizing activities [37,38]”. 

6. Do the title and abstract accurately convey what has been found? Yes.

7. Is the writing acceptable? There are many vocabulary errors and typos which could easily be corrected with minor editing by authors familiar with scientific English language.

We have corrected the grammar and typographical errors that we were able to find throughout the manuscript.

III) Reviewer: William Barlow

Major compulsory issues:

1. The paper was scientifically interesting, but I found the presentation of results
to be somewhat confusing. I believe the goal is to suggest a possible path between vitamin D levels and subsequent NAFLD. Of course, in observational data one can only show an association, rather than cause. Nonetheless, the most appropriate analysis is logistic regression on the primary outcome NAFLD using predictors such as vitamin-D levels, age, etc. This is what is actually done in Table 3, showing a strong association of vitamin-D with NAFLD after adjustment for other variables. However, those results are not described in the abstract nor correctly referenced in the methods section. Instead, the abstract presents the mean vitamin D levels by NAFLD status (OK but not what is being tested in the analysis), rather than the odds ratio for a single unit change in vitamin-D. The methods section described this analysis incorrectly as multiple linear regression, rather than logistic regression on a dichotomous outcome.

We have now reported also the results of the logistic regression analysis on the primary outcome NAFLD (as dichotomous variable) and vitamin D in the abstract: “Patients with NAFLD (n=162,61.8%) had reduced serum 25(OH) vitamin D levels compared to subjects without NAFLD (14.8 ± 9.2 vs 20.5 ± 9.7 ng/ml, p<0.001, OR 0.95, IC 95% 0.92-0.98)”. The statistical procedures have now been correctly referenced in the methods section: “Logistic regression was used to estimate the predictive value of 25(OH) vitamin D and metabolic parameters on the presence of NAFLD, considered as dichotomous variable”, as suggested by this reviewer.

2. Similarly, there was an analysis of NAFLD scoring using linear regression (Table 2) assuming it was continuous but it seems to only have three ordered values. Ordinal regression would be better here.

The analysis of NAFLD scoring has now been performed by means of an ordinal regression, as correctly suggested by the reviewer; the results, in line with those previously obtained, are shown in Table 4 and in the Results section: “(…) we performed an ordinal regression analysis that showed a significant association between NAFLD score, the components of MS and the insulin resistance degree, measured by means of HOMA-IR”. We have also amended the Method section accordingly: “Ordinal regression was used to detect the association between predictor variables and presence and degree of NAFLD (0: absence, 1: mild, 2: moderate, 3: severe)”.

3. Finally, there are some analyses relating clinical predictors to each other. Table 4 has FLI as the outcome, but it is just estimated from a logistic regression model based on triglycerides, ggt, and waist circumference so seems removed from the main question unless FLI is commonly used in this area as a measure.

FLI is a simple and validated predictor of hepatic steatosis that in this study tightly correlated with US-detected fatty liver. This finding may be of interest considering the recent issues in identifying clinical surrogates of US-detected fatty liver, especially in obese patients and in those with normal liver enzymes. However, we are aware that FLI is just an estimated value derived from clinical and biochemical parameters and seems removed from the context of this specific paragraph. For this reason we described the findings about FLI in a separate paragraph of the Results section, after the primary outcomes of the study, and
removed the related table from the manuscript.

4. Similarly, Table 5 showed the lower and upper quartiles of vitamin D by other factors. I actually prefer to see all 4 quartile values with a trend test, but recognize that the approach used here is commonly done.

We have now shown the clinical and biochemical characteristics of all the 25(OH) vitamin D quartiles in table 5 as well as the trend test for each parameter, according with this reviewer’s comment.

5. Finally, Table 6 is back to a logistic regression on NAFLD. It would be better to put all the primary analyses (NAFLD as dichotomous outcome) together and then make it clear the other analyses are secondary. Table 1 is needed first as a description of the population.

We re-organised the Results section in paragraphs reporting: i-study population description, ii-primary outcomes results (logistic analyses with NAFLD considered as dichotomous variable), iii-secondary outcomes.

6. It was somewhat unclear when log-values of continuous variables were used, versus untransformed, values.

We have now explained in the Methods section and in the table legends when log-values of continuous variables were used: “Because HOMA-IR, FBG, triglycerides, AST, ALT, GGT and alkaline phosphatase were skewed, we used natural logarithmic transformations of these variables before performing both means comparison (Student's T-test), ordinal and multivariate regression analyses”.

7. I doubt that any scientific conclusions would change after redoing the statistical section. The methods need to be in agreement with what was actually done. There needs to be a primary question which I believe is the association of vitamin-D and other variables with the dichotomous outcome NAFLD. The other analyses can be added afterward if they add something that is not known.

As the referee already pointed out, no scientific conclusion has substantially changed after modifying statistical procedures according to his suggestions. Thus, the methods section has been modified according with the final analyses performed in our study. Also, in the Results section, we first described the results of primary outcomes (NAFLD as dichotomous variable) and then added the secondary analyses, as specified in point 5 of the answers to referee.

Minor compulsory issues:
8. Please spell out NAFLD in the abstract the first time.
NAFLD was spelt out in the abstract the first time.

9. There are a few typographical errors that need to be fixed.
We have corrected the typographical errors that we were able to find throughout the manuscript.