Author’s response to reviews

Title: Elevated plasma copeptin levels identify the presence and severity of non-alcoholic fatty liver disease in obesity.

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Version: 1 Date: 18 Feb 2019

Author’s response to reviews:

Dear Professor Recchioni

Please find attached the revised version of our manuscript entitled “Elevated plasma copeptin levels identify the presence and severity of non-alcoholic fatty liver disease in obesity” along with a point-by-point response to all the comments raised by the reviewers. All the changes made have been highlighted in the manuscript.

We hope that you will now consider our paper suitable for publication in BMC Medicine.

Best Regards,

Maria Gisella Cavallo

Olle Melander
Reviewer 1

The study by Barchetta entitled "Elevated plasma copeptin levels identify the presence and severity of non-alcoholic fatty liver disease in obesity" has been reviewed. The study investigates the correlation of copeptin with non-alcoholic fatty liver disease in obesity. Overall, the study design is well described and structured. The manuscript is well written and easy to understand.

1. My first concern is that the authors support their conclusions based on statistical analysis that may not be the appropriate to approach such complex disease. T-tests and correlation tests could not be enough robust to assess a clinically relevant association between copeptin and NAFLD.

We thank the reviewer for all the suggestions on the statistics which did help us to improve the data presentation and strengthen the study findings. We have performed new tests and analyses, according to his comments, listed point by point below and described in both the manuscript’s Statistics and Results section.

1.1 As it is showed in table 1b there is a significant difference of copeptin concentrations between NAFLD and NAFLD, but it seems that there is also a big difference in terms of percentage of metabolic syndrome. Given the importance of this component I would recommend the authors to perform separated analyses in both groups with and without metabolic syndrome.

We agree with the reviewer that the presence of metabolic syndrome, which is typically associated with NAFLD and correlated with higher copeptin levels in several investigations, may represent a major confounder when exploring the association between NAFLD and copeptin. For this reason, following the recommendations of the reviewer, we have now performed separated analyses in both groups with and without metabolic syndrome, analyzing the presence of NAFLD and NASH in relation to copeptin levels. These new analyses, although affected by the sample size reduction for the division in four subgroups, overall confirmed our study results: “When comparing plasma copeptin levels between obese+/NAFLD+ and obese+/NAFLD-patients in relation to the presence of MS, the finding of higher copeptin in presence of NAFL – both NAFLD and NASH – was confirmed in the MS group (n= 42): NAFLD-: 7.1 ± 2.7 vs NAFLD+: 10 ± 5.2, p= 0.024; NASH-: 7.7 ± 3.7 vs NASH+: 12.2 ± 5.7, p= 0.007 and slightly confirmed in the significantly smaller subgroup of obese patients without MS (n= 18): NAFLD-: 5.9 ± 2.4 vs NAFLD+: 8 ± 2.4, p= 0.06; no MS group: NASH-: 6.5 ± 2.6 vs NASH+: 8.5 ± 0.2, p= 0.01” (page 9, lines 3-8).
Furthermore, we have carried out a new partial correlation analysis between plasma copeptin and diagnosis of NAFLD, adjusted for the presence of MS, which showed correlation’s coefficient = 0.32, p= 0.017: “Furthermore, the association between higher copeptin levels and NAFLD persisted significant when assessed in the partial correlation analysis adjusted for presence of MS (correlation’s coefficient = 0.32, p= 0.017)”, (page 99, lines 9-11). The relationship between higher copeptin and NAFLD has been also tested in new multivariate models including each individual metabolic parameter which may be associated with NASH, confirming the existence of an independent association between higher copeptin and NASH after adjusting for multiple metabolic confounders: “In order to identify determinants of NASH (yes/no, dependent variable) in our study population, we built multivariate logistic regression models including age, sex, T2DM and components of MS, as expressed as either the numbers of MS’ components –ranging from 0 to 5- or entering each metabolic parameter as a continuous variable in a conditional forward logistic regression”, page 8, lines 5-9; “Finally, in a logistic regression model adjusted for age, sex, renal function, presence of T2DM and MS’ components, copeptin levels predicted the presence of NASH at the liver biopsy (Table 4). The association between copeptin and NASH persisted statistically significant also after further adjustment for each individual metabolic parameter (BMI, FBG, triglycerides and HDL-c), entered as continuous variables in progressive conditional forward regression models (Copeptin-Standardized β coefficient= 0.64, p= 0.035, Odd Ratio= 1.97, 95%C.I. 1.05-3.69; Cox & Snell R2= 0.56; Supplementary Table 3S)” (page 10, lines 8-14; see also response #1.3 and Table 3S).

1.2 Table 3 shows a comparison of clinical characteristics of participants belonging to quartile 1 versus quartile 4 of plasma copeptin concentration. The 4 quartile exhibited a greater percentage of participants with NAFLD (42% vs 15%); nonetheless the authors fail to show if this difference is not due to a sex difference. Given the importance of this component I would urge the authors to perform an analysis in sex-stratified quartiles.

We thank the reviewer for this valuable comment. In our study population, the prevalence of NAFLD was significantly higher in individuals belonging to the IV vs I quartile of plasma copeptin, and the prevalence of male sex was higher in the IV copeptin quartile than in the I quartile. This latter finding reflects results from several previous observations, which found greater mean copeptin concentration in male than female individuals. Thus, we agree with the reviewer that sex may represent a confounder when investigating the association between higher copeptin levels and NAFLD. Although we also considered an analysis in sex-stratified quartiles as one of the best options for exploring this possibility, however our study sample was not powered for performing this additional stratification, as some subgroups would have included very few subjects. Therefore, we have now carried out new analyses for exploring correlates of NAFLD in our study population and we found no association between sex and NAFLD (standardized β coefficient: -0.01, p= 0.86, OR 0.92 (95% C.I. 0.37-2.27). Furthermore, we have
entered the variable sex in all the new multivariate models confirming that sex did not represent a confounder in the association between NASH and higher copeptin levels. These analyses are described in the Results section and in new Tables: “The presence of NAFLD correlated with higher copeptin levels and, as expected, with all the clinical parameters associated with MS, such as greater BMI, waist circumference, FBG and the presence of T2DM and atherogenic dyslipidaemia, whereas no association was found between NAFLD, sex and age (Supplementary Table 1S)” (page 9, lines 13-17; Supplementary Tables S1 and S3).

Moreover, we have added a new paragraph in the Discussion commenting on the important association between sex and circulating copeptin levels: “In line with previous investigations, we observed an association between male sex and greater copeptin concentration. In order to exclude the possible interference of sex distribution behind the association between copeptin and NAFLD, we first explored the association between NAFLD and sex, finding no relationship between these two variables. Thereafter, we built several sex- and age-forced multivariate models confirming that sex did not represent a confounder in the association between NAFLD and higher copeptin levels.” Discussion (page 11, lines 5-13).

1.3 Moreover, it also calls the attention the results showed in table 4, where the association of Metabolic syndrome (B 3.46 and 95% C.I. O.R. 1.11 - 909.1) seems more significant than copeptin (B 0.5 and 95% C.I. O.R. 1.02-2.93). Once again, given the importance of this comportment I would recommend the authors to perform the same association test using several models with the related variables (perhaps in an additive fashion considering the sample limitations). I would also suggest the authors to present the results as standardized betas coefficients.

As the reviewer punctually observed, in our population the association between metabolic syndrome and NAFLD was statistically significant in the multivariate logistic regression analysis, showing B: 3.46 and O.R. 31.5 with 95% C.I. 1.11 - 909.1. However, because of this very wide 95% confidence interval, the p value of this correlation is not extremely small (p= 0.043), being comparable to the one reported for copeptin levels, as shown in the old Table 3. Moreover, following the reviewer’s suggestion, we have now calculated the β standardized coefficients for all the covariates, which was not done before since not provided in the output for logistic regression analyses in traditional statistical packages (i.e. SPSS, SAS), unlike for linear regression analyses. The β standardized coefficient calculation has been now performed using the formula by King, J.E. (2007): “Standardized coefficients in logistic regression. Paper presented at the annual meeting of the Southwest Educational Research Association, San Antonio, TX” (paper available online at http://www.ccitonline.org/jking/homepage/). The new analysis showed a β standardized coefficient for predicting NASH of 0.36 for MS and 0.54 for copeptin (new Table 4, page 22).
We do agree with the reviewer that MS may represent an important confounder when exploring possible determinants of NAFLD in our study population. For this reason, in the previous analysis we entered as a covariate the number of MS’ components, ranging from 0 to 5, to give a measure of the metabolic impairment rather than considering just the presence/absence of MS as such. Moreover, in that model we also considered the presence of type 2 diabetes and dyslipidemia separately, as they are known risk factors for NAFLD. Furthermore, following the reviewer’s recommendation, we have now carried out several new analyses entering the parameters of MS as continuous variables in an additive fashion through a stepwise procedure, confirming the presence of an independent association between higher copeptin levels and the diagnosis of NASH. All the new statistical procedures are described in the Statistics and the Results in the appropriate section: “In order to identify determinants of NASH (yes/no, dependent variable) in our study population, we built multivariate logistic regression models including age, sex, T2DM and components of MS, as expressed as either the numbers of MS’ components –ranging from 0 to 5- or entering each metabolic parameter as a continuous variable in a conditional forward logistic regression”, page 8, lines 5-9; “Finally, in a logistic regression model adjusted for age, sex, renal function, presence of T2DM and MS’ components, copeptin levels predicted the presence of NASH at the liver biopsy (Table 4). The association between copeptin and NASH persisted statistically significant also after further adjustment for each individual metabolic parameter (BMI, FBG, triglycerides and HDL-c), entered as continuous variables in progressive conditional forward regression models (Copeptin- Standardized β coefficient= 0.64, p= 0.035, Odd Ratio= 1.97, 95%C.I.= 1.05-3.69; Cox & Snell R2= 0.56; Supplementary Table 3S)”; page 10, lines 8-14; see also response #1.3 and Table 3S.

2. Even though the correlation described in this manuscript is novel, I would recommend the authors to give mention to previous studies that have explored the association of copeptin with advanced liver disease and contextualize such findings with their own results. i.e. Plasma copeptin as biomarker of disease progression and prognosis in cirrhosis. J Hepatol. 2016;65: 914-20.; Copeptin as an indicator of hemodynamic derangement and prognosis in liver cirrhosis. PLoS One. 2015;10:e0138264.; Plasma copeptin, a possible prognostic marker in cirrhosis. Liver Int. 2013;33:843-51.

These important studies suggested by the reviewer were actually already mentioned in the Discussion (References 24, 36 and old reference 37), along with the one by Tawfik and collaborators on the potential role of copeptin as biomarker of liver cirrhosis and its major complications (Reference 25). Indeed, we have now discussed them more in detail contextualizing their findings with our results and added a new reference on this topic (new Reference 37: Kerbert AJC, Verspaget HW, Navarro ÀA, Jalan R, Solà E, Benten D, et al. Copeptin in acute decompensation of liver cirrhosis: relationship with acute-on-chronic liver failure and short-term survival. Crit Care. 2017;21(1):321. doi: 10.1186/s13054-017-1894-8):
VP/V1aR system has been widely studied in relation to the progression to cirrhosis and hepatic decompensation of several acute and chronic liver diseases [24-25, 36-38]. “Indeed, the secretion of VP is involved in advanced stages of chronic liver diseases; VP counteracts the reduction of the arterial pressure induced by splanchnic vasodilatation and subsequent increased peripheral vascular resistance. In this scenario, although the non-osmotic secretion of VP may temporary preserve the arterial blood volume, it is associated with the development of ascites, hepato-renal syndrome and detrimental clinical outcomes. Thus, Solà and collaborators [24] first demonstrated that plasma copeptin represents a prognostic factor for disease progression, clinical decompensation, and prognosis in patients with cirrhosis [24], and these findings were confirmed in other investigations [36-38]” (page 12, lines 8-16).

3. Previous works have studied the association of copeptin with certain scores of liver disease such as the Child-Pugh score. Therefore, I highly recommend the authors to conduct and extra analysis and evaluate the association of copeptin with a NAFLD score such as the one described by Angulo 2007 (Hepatology. 2007 Apr;45(4):846-54.)

As the reviewer pointed out, the NAFLD fibrosis score is a very useful score able to predict the presence of hepatic fibrosis in individuals affected by NAFLD on the basis of indirect signs such as BMI, blood transaminases, platelet counts, albumin and presence of diabetes mellitus. This score demonstrated high positive predictive value for advanced biopsy-proven liver fibrosis, so representing a useful tool for risk stratification in large populations and for the identification/follow-up of subjects candidate to liver biopsy. Since all our study participants underwent intraoperative liver biopsy as for inclusion criteria, the additional analyses suggested by the reviewer have been performed by considering scores derived from liver histology, which is the gold standard for grading and staging NAFLD and NASH. Thus, we found that higher copeptin levels are positively associated not only with the percentage of macro- and microvesicular steatosis and lobular inflammation, as already reported, but also with more severe scores of NAFLD Activity Score (NAS) and the Steatosis, Activity, Fibrosis score (SAF). These new results are now reported in both the Results section and Table 2: “In obese individuals, circulating copeptin levels positively correlated with the percentage of macro- and microvesicular steatosis, lobular inflammation, the NAS score for diagnosis of NASH, and the SAF score for fibrosis (Table 2)”, page 9, lines 18-20.

4. My last recommendation to the authors is to properly replace the term gender for sex, as I suspect it is the case.
The term gender has been replaced with sex throughout in the manuscript and tables. We apologize for the mistake.

So, overall I am positive about this study, but I would urge the authors to accommodate the recommended changes.

Reviewer 2

In this study cross-sectional study, Barchetta I and coll., analyzed the correlation between plasma copeptin levels and the NAFLD severity (biopsy-proven) in obese patients. The study demonstrates that copeptin levels predict the presence of biopsy-proven NAFLD in obese individuals independently of other variables analyzed. The study is well conducted and the results shown well justify the conclusions reported.

Only a few minor observations:

On page 6 line 21 it is repeated "using a"

This repetitions has been now deleted.

Hypertension is one of the parameters evaluated for diagnosis of MS. The authors reported the blood pressure levels but we have no information on how many obese patients were hypertensive and how many were taking any antihypertensive drug.

In our study population, 67% of obese subjects were hypertensive and they were all treated with antihypertensive agents. We thank the reviewer for this comment and we have now added this new information in Table 1a and Table 1b, along with new information on antidiabetic and cholesterol-lowering agents (Table 1a and Table 1b, pages 14-16).
Reviewer 3

In the current work, Barchetta et al assessed the role of plasma copeptin to identify the presence of NAFLD and NASH in a cohort of obese individuals. They concluded that plasma copeptin levels were higher in patients with NAFLD and NASH compared to patients without NAFLD and NAFL.

However, there are several issues that should be analyzed more carefully:

1. Data discrepancies: just as an example, note that mean HOMA-IR was 4.5 for obese individuals (n=60), but only 3.6 and 4.1 when divided in no NAFLD (n=28) and NAFLD (n=32). How is it possible that when combining the no NAFLD and NAFLD patients the mean actually goes up by that much? Please provide an explanation. This is observed in several variables.

We thank the reviewer for his comment, which allow us to better explain and present our data. As stated in the statistics section, several variables, such as plasma copeptin, triglycerides, FBG, AST, ALT, GGT, FBI, HOMA-IR and HOMA-%B, had skewed distribution and were log-transformed before the analyses. We understand that our data presentation in the tables could be misleading for the readers, as for non-normally distributed variables the mean value is not a good measure of central tendency and the median should be preferable. Indeed, as shown in the table, this discrepancy disappears when considering the median (25th-75th percentile) value instead of mean SD (HOMA-IR: entire group: (2.8 (2.2-4); subgroup no NAFLD: 2 (1.5-3.6) and subgroup NAFLD: 2.9 (2.4-3.8)). We are sorry that our data presentation was not clear and have now provided median (25°-75°) values for skewed and mean SD values for non-skewed variables in both the manuscript and tables.

2. If, as stated in the manuscript, the aim was to explore the relationship between plasma copeptin and the presence/severity of NAFLD and NASH, I would suggest combining Tables 1 and 2 to show patients' characteristics in 3 groups: 1) non-obese (n=60), 2) obese-no NAFLD (n=28) and 3) obese NAFLD patients (n=32). Use ANOVA to compare the 3 groups and Bonferroni's adjustment (or other) for pairwise comparisons.
As correctly reported by the reviewer, the primary aim of this study was to investigate the relationship between plasma copeptin and the presence/severity of NAFLD and NASH. For this reason, we recruited patients candidate to bariatric surgery undergoing intra-operative liver biopsy as this procedure represents the gold standard for assessing NAFLD/NASH. In our study, we also provided data on an additional cohort of sixty non-obese controls without US-assessed NAFLD, for giving a standard of copeptin levels in absence of obesity and metabolic impairment, in this way making our study design more complete. Therefore, we presented patients characteristics in two different tables, the first one for providing the clinical characteristics of all the study participants, the second one for showing in detail differences between obese patients with/without NAFL at the biopsy. Thus, comparing copeptin levels between the three subgroups represented by: obese patients with or without NAFLD at the biopsy and non-obese without NAFLD at the US scan, was not the main purpose of this study. The results of an additional comparison between copeptin levels in NAFLD patients vs controls have been shown for completeness and for corroborating the main study finding.

However, we understand and apologize if this has been somehow misleading. Therefore, we have now re-written the first paragraph of the Results section providing first the main study finding and then additional results. In this context, we have now performed a multiple comparison by ANOVA test, as suggested by the reviewer, and this analysis confirmed that copeptin levels were significantly different between the subgroups (F= 4.09, p= 0.019). The post-hoc adjustment showed that obese NAFLD patients have significantly higher copeptin levels than both obese non NAFLD patients (p= 0.022) and non-obese non NAFLD individuals (p= 0.034). No difference was shown between copeptin levels in obese non NAFLD vs non obese non NAFLD (p= 0.98). This new analysis is reported in the second paragraph of the results section: “Obese patients with biopsy-proven NAFLD (obese+/NAFLD+, 53%) had significantly higher copeptin levels than obese individuals without NAFLD (obese+/NAFLD-) (obese+/NAFLD+: 9.5 ± 4.9 pmol/L vs obese+/NAFLD-: 6.4 ± 2.6 pmol/L, p= 0.004). Moreover, copeptin levels were also greater in obese+/NAFLD+ individuals when compared with non-obese subjects without MS and NAFLD (obese-/NAFLD-, mean ± SD copeptin: 7.4 ± 5.1 pmol/L; p=0.01). The presence of significantly greater copeptin levels in obese+/NAFLD+ than both obese+/NAFLD- and obese-/NAFLD- was confirmed by post-hoc adjusted ANOVA test (model F= 4.09, p= 0.019; p= 0.022 and p= 0.034)” (page 8, lines 18-24; page 9 lines 1-2).

3. Obese individuals undergoing bariatric surgery and healthy non-obese individuals are 2 different extremes of the obesity spectrum. Regardless of this, we see no difference in many metabolic parameters such as fasting insulin and HOMA-IR. How do authors explain this observation?
As shown in Table 1a, the two study cohorts were represented by obese and normal weight individuals comparable for sex and age. As expected, a large number of clinical and biochemical parameters were significantly different between these two groups, including BMI, waist circumference, systolic and diastolic blood pressure, HDL-cholesterol, triglycerides, AST, ALT, fasting blood glucose, prevalence of type 2 diabetes and metabolic syndrome. Conversely, as pointed out by the reviewer, fasting insulin – and the derived calculated parameters HOMA-IR and HOMA β% – were comparable between the study subgroups. Although the cross-sectional design of this study and the metabolic evaluations performed do not allow to provide conclusive explanation for this finding, several considerations should be done.

First, individuals in the control group have been recruited among subjects referring to our outpatient clinics for metabolic evaluation, as stated in the methods, and resulted negative for presence of metabolic syndrome (NCEP ATPIII criteria), NAFLD and type 2 diabetes. Although this metabolic characterization provides accurate information on patients’ phenotype in the context of clinical practice and for our study purposes, however, it does not allow ruling out definitively all possible early-stage and/or preclinical conditions, detectable for example by dynamic tests, which were beyond the object of our study. Indeed, the main clinical parameters differed significantly between the two subgroups, as shown in the manuscript and listed above, making our metabolic profiling reasonably reliable.

Moreover, obese participants were around 44-year-old and, besides being affected by severe obesity, they had a prevalence of type 2 diabetes of 14%, metabolic syndrome of 70% and biopsy-proven NAFLD just over 50%. Thus, we can speculate that at least one third of these patients had not (yet) any clinically relevant metabolic impairment but obesity at the time of the study enrolment, making plausible the observation of few parameters overlapping between obese and non-obese individuals. We thank the reviewer for his comments and have now added a new paragraph discussing this point in the Discussion section: “In our population of obese individuals, the prevalence of MS reached 70% whereas T2DM was diagnosed in 14% and biopsy-proven NAFLD in just over 50% participants. Therefore, we may speculate that, according to the metabolic profiling, at least one third of these patients had not (yet) clinically relevant metabolic impairment but obesity, at the time of study recruitment, thus explaining the observation of some overlap in few parameters, such as fasting insulin and its derived indexes, between obese and non-obese subjects” (page 11, lines 2-7).

4. HOMA-IR of 3.3 (and insulin of 17 uU/mL) is relatively high for non-obese, "metabolically healthy" patients (as stated in the manuscript). Indeed, it is not significantly different from the 4.5 in obese individuals. This is extremely surprising. Please justify and expand on this in the discussion.
We thank the reviewer for this comment. In our study population, FBI and HOMA-IR did not differ significantly between obese and non-obese individuals and possible explanations for this observation have been provided in the Response #3. Non-obese subgroup had median (range) HOMA-IR= 2.9 (2.1-4.8) and FBI= 13 (9.1-25.3) uU/mL which, although do not described conditions of overt metabolic disease, make plausible to hypothesize the presence of some preclinical condition associated with reduced insulin sensitivity in a few cases. However, the interpretation of calculated indexes such as the HOMA-IR, well applicable and useful for the screening of large populations, should be considered with caution when applied to smaller cohorts, as the one recruited for our study. Considerations on these aspects have been now expanded in the Discussion, as appropriately suggested by the reviewer (page 11, lines 2-7, see response 3). Moreover, we understand that the expression “metabolically healthy” may not be fully representative of our non-obese population and, therefore, we have now substituted the expression with “not affected by metabolic syndrome” throughout the manuscript.

5. There were no differences in copeptin in obese vs. non-obese individuals. However, several studies have suggested that copeptin is associated with obesity and insulin resistance. How do authors explain these discrepancies in their results? Were controls not as "healthy" as originally presumed?

This study aimed at investigating the relationship between plasma copeptin and the presence/severity of biopsy-proven NAFLD/NASH in obese patients; thus, this study was powered for testing this primary hypothesis, as appropriate. Although the investigation of copeptin in relation to obesity and insulin resistance is an interesting issue and object of research, as pointed out by the reviewer, this evaluation did not represent the purpose of our study. Therefore, considerations on aspects beyond the study endpoint, are speculative and not specifically powered for being considered conclusive. However, beside this aspect, it is worthy to note that in our study population, obese patients with NAFLD/NASH had higher copeptin levels than non-obese individuals, whereas this difference was not significant when comparing obese non-NAFLD vs non-obese non-NAFLD individuals, corroborating our hypothesis of a major role of NAFLD and NASH in leading the association between dysmetabolism and greater copeptin concentration. For aspects regarding the metabolic phenotype of the non-obese study group, please see also responses to points #3 and #4.
6. Patients with NAFLD had lower insulin levels than patients without NAFLD (this by itself is perplexing). Now, FPG was similar in the 2 groups, so it strange than HOMA-IR was slightly higher in the NAFLD group (even when not significantly). Also, we would expect a higher IR in patients with NAFLD. Please explain your interpretation of these results. Why do authors believe that TG levels were not that different between No NAFLD and NAFLD patients? Or SBP and BMI slightly lower in NAFLD? (While due to small sample size these differences are not statistically significant, the direction of their trend is strange).

In our study population, patients with NAFLD did not have lower insulin levels than patients without NAFLD, as indicated by the respective median (25th-75th percentile) FBI (µU/l) values reported in Table 1b: no NAFLD= 9 (6.3-15.5) µU/l vs NAFLD 11.5 (10.5-14) µU/l, p= 0.66. Similarly, also FBG, HOMA-IR and TG levels were slightly higher in the NAFLD group [FBG = 99(87-125) mg/dl; HOMA-IR = 2.9 (2.4-3.8); TG = 136 (117.2-164)] in comparison with the no NAFLD one [FBG = 95(92-101.5) mg/dl; HOMA-IR = 2(1.5-3.6); TG = 101 (73.7-121.5)], although these differences were not statistically significant (p= 0.60, p= 0.77, p= 0.47, respectively), as already reported in the Table and correctly observed by the reviewer. As affirmed in the response #1, we do apologize if our data presentation of mean SD values –along with the median (25th-75th percentile) for skewed variable – was somehow misleading and we have now entered median (25th-75th percentile ) values for skewed and mean SD values for non-skewed variables in both the manuscript and tables.

As for BMI and SBP, although considerations are merely speculative because the mean differences observed are not statistically significant, some remarks may be done. One the one hand, in a population of morbidly obese individuals, the presence of NAFLD may not necessarily be associated with greater and greater BMI within the tight range of severe obesity; in contrast, NAFLD is supposed to correlate with presence of metabolic impairment, as the case of our study cohorts where the prevalence of MS was almost double in NAFLD vs no NAFLD subgroup (81 vs 46%, p= 0.004). On the other hand, the slightly lower mean SBP values in the NAFLD group may be due to significantly higher prevalence of use of anti-hypertensive agents in this subgroup in comparison with the no NAFLD group (81 vs 36%, p= 0.003), in line with the higher prevalence of MS reported. We apologize if data on treatments were not specified in the first version of this manuscript and have now added this important information in the revised version.

7. It is unclear to this reviewer why you would present a correlation between a continuous and a dichotomous variable like in Table 2 (e.g. copeptin and NASH yes/no).
The correlation between continuous and dichotomous variable is a traditional, well-accepted procedure able to provide a measure of the association between a continuous parameter, such as copeptin levels, and the presence of a condition, as NASH in the example of the reviewer. Likewise, this is also the case of analyses carried out for identifying clinical parameters associated with sex, as a traditional example of dichotomous variable. Moreover, in the specific case of NASH, this analysis has represented the first step for subsequent multivariate models specifically designed for identifying independent predictors of NASH (yes/no) at the liver biopsy, in our study population. Besides, when investigating correlates of plasma copeptin levels, we also took in consideration, along with several “continuous” clinical variables, continuous histological scores, such as the NAS and SAF scores.

8. Provide the information of how many patients of the 32 with NAFLD had NASH and how many had borderline NASH.

This information has now been added in the Results and Figure 1: (page 9, lines 21-22; page 23, line 19).

9. Provide information regarding diabetes and lipid-lowering medication use.

We thank the reviewer for the comment and have now provided information on diabetes, lipid-lowering and antihypertensive medication use in the manuscript (Table 1a and 1b, pages 14-17).

10. Expand Table 3 to include all quartiles to assess gradual changes.

We thank the reviewer for this suggestion; table 3 has now been expanded accordingly (Pages 20-21).

11. This manuscript only shows an association or higher levels of copeptin in specific subgroups. Authors should try to assess whether these differences are important enough to use copeptin as a biomarker of NASH. What was the sensitivity, specificity, PPV and NPV to diagnose NASH?

We thank the reviewer for his comment and have now built a ROC curve showing that higher copeptin (entered as a continuous variable) predicted NASH at the liver biopsy with an AUC= 0.822, SE= 0.057, 95% CI= 0.71-0.93 and p= 0.0007 and sensitivity= 92% - specificity= 70% for plasma copeptin greater than 8.06 pmol/L. This new analysis is described in the Statistics section (“Moreover, the predictive value of plasma copeptin for NASH identification was estimated by the area under receiver-operating characteristic curve (AUROC), with a 95% confidence interval (C.I.)”, page 8, lines 9-11), in the Results (“Furthermore, higher copeptin concentration predicted the presence of NASH at the liver biopsy in the ROC curve with
AUROC= 0.822 (95% C.I.: 0.71-0.93, p<0.001, Supplementary Figure 1); page 10, lines 4-6) and shown in the Supplementary Figure 1.

12. Power calculation was done between NAFLD and No-NAFLD groups. It should be mentioned in the power calculation section that because copeptin levels were surprisingly higher in the non-obese group compared to the obese No NAFLD group, the same calculation between NAFLD and non-obese would result in the need of 90 patients per group to have a power of 80%.

The primary endpoint of this study was to investigate the relationship between plasma copeptin and the presence/severity of NAFLD and NASH, which for our study has been assessed by liver histology, the gold standard method for this diagnosis. Indeed, the post-hoc sample size calculation has been performed on the basis of mean ± SD copeptin levels found in subjects with and without biopsy-proven NAFLD/NASH. Conversely, the comparison of copeptin levels between non-NAFLD obese patients and non-NAFLD non-obese subjects belonging to the control group did not represent the purpose of this study. We apologize if this point was not clearly stated in the manuscript and have now specified it better in the revised version of this manuscript: “As far as we know, this is the first study investigating plasma copeptin levels in relation to the presence of NAFLD. Thus, for confirming the statistical power of our findings, we performed a post-hoc sample size calculation on the basis of mean ± standard deviation (SD) copeptin levels in obese subjects with and without biopsy-proven NAFLD which showed that twenty-seven subjects in each group would have been sufficient to reach the statistical significance with power= 80% and α error= 0.05”; page 7, lines 17-22).