Author’s response to reviews

Title: Factors associated with postprandial lipemia and apolipoprotein A-V levels in individuals with familial combined hyperlipidemia

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Erica Cruz
Journal Editorial Office
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Dear Ms. Cruz,

We thank for the careful review of our article “Factors associated with postprandial lipemia and apolipoprotein A-V levels in individuals with familial combined hiperlipidemia”.

In the following pages the responses to the comments of each of the reviewers are included. The corresponding adjustments have been made to the paper.

We thank you for your attention.

Sincerely yours,

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Reviewer 1

- As stated in “Methods” section (lines 93-94), FCH diagnosis was based on LDLc > 160 mg/dl and/or triglycerides levels > 150 mg/dl. Why these levels have been selected for FCH diagnosis? Is there any published study in this study population with these reference levels? Depending on the sex and age, these levels could be even under percentil 90th. I consider them too low and could overestimate the presence of FCH. Authors have to justify why they have used these limits for diagnosis.

Answer: The diagnostic criteria for FCH remain an area of controversy. Different cholesterol and triglycerides thresholds have been used for the diagnosis, ranging from the 90th percentile for the corresponding population to the National Education Cholesterol Education Program (NCEP) values. Large differences between the highest and the lowest mean cholesterol, triglycerides, HDL-c, and apolipoprotein (apo) B have been reported in affected individuals, and this highlights the variability in the lipid abnormalities associated with FCH [1-4].

We have selected the participants from a cohort of FCH families studied and followed up in our Institution. The diagnosis of these subjects is based on the family history of hyperlipidemia in at least one first-degree family member, apo B levels over the 90th percentile for the Mexican population (in men >108 and women >99 mg/dl) [5] in addition to hypertriglyceridemia (>150 mg/dl) and/or LDL-c >160 mg/dl. Del Rincon et al [6] evaluated the concordance between different diagnostic criteria in Mexican subjects with FCH and concluded that uncertainty in the categorization of these individuals remains, and that it is difficult to identify the ideal diagnostic criteria. However, based on the pathophysiology of FCH, we consider that apo B concentrations in addition to the mentioned criteria support the diagnosis.

- In the same way, authors classify the subjects by presence of hypertriglyceridemia and/or abdominal obesity. Those individuals in Group 3 have triglycerides below 150 mg/dl and, according to Table 3 data, mean cholesterol levels are 206.3 mg/dl (which are among normality). LDLc levels are not provided, so do these patients meet LDLc criteria? Triglycerides > 150 mg/dl criteria is not met so I suppose it but it has to be clarify. Mean apolipoprotein B concentration is also below 120 mg/dl. Do these patients meet FCH criteria?

Answer: All study subjects had apo B levels over the 90th percentile for the Mexican population [5] and hypertriglyceridemia and/or hypercholesterolemia at diagnosis, however, some of them showed inferior levels at the time of the study. FCH is characterized by fluctuations in the lipid profile over time; this would explain your observation. We added this statement in the legend of table 3. In addition, we performed an analysis including only the subjects who showed the full abnormalities at the time of the study (triglycerides ≥150 mg/dl and/or LDL-c ≥160 mg/dl). The main findings of our investigation did not change. We have also included values for LDL-c in table 3 as requested.
Authors make a linear regression to identify those variables associated with triglycerides postprandial response. Triglycerides levels are expressed as median [interquartile range] so I suppose that this is a variable without a normal distribution. To my knowledge, this variable cannot be used for linear regression unless any kind of transformation (i.e.: logarithm, etc.) is made. Has this variable transformed into any kind of variable with a normal distribution to be able to be used in the regression? I have the same comment for apo A-V variable which is also expressed as median so I suppose it has not a normal distribution.

Answer: logarithmic transformation was performed in all variables that did not show a normal distribution before using them in correlations and linear regression analyses. This has been added in the Statistical analysis section line 143 as follows “Following logarithmic transformation of non-normal distributed variables, Pearson correlations were calculated and stepwise multiple linear regression models were constructed to investigate the independent predictors of the iAUC of triglycerides and apo A-V”.

Authors explain statistical analysis they have used to analyse data. They mention ANOVA or Pearson correlation. They are tests to use with variables with normal distribution but the vast of variables have a skewed distribution. In these cases, it should be used Friedman test instead of ANOVA for repeated measures; Spearman correlation should be used instead of Pearson, etc.

Authors also state that they have used “one way ANOVA” but should “t-test” used in this case?

Answer: Due to non-normal distribution Friedman tests (instead of ANOVA for repeated measurements) were performed to examine the triglycerides, apo B-48, and apo A-V postprandial responses. This was changed in the Statistical analysis section lines 141-142 “Friedman tests were performed to examine the triglycerides, apo B-48, and apo A-V postprandial responses”, as well as in figure 1. Logarithmic transformation was made before performing Pearson correlations. We examined differences between the four groups with one way ANOVA or Kruskal-Wallis test, according to the distribution. After that, we evaluated differences between individuals groups using T-test or Mann-Whitney U test as appropriate. This has been clarified in the Statistical analyses section lines 154-156 as follows “One way ANOVA or Kruskal Wallis tests were performed to examine differences between these groups. In case of a P value <0.05 comparisons between individual groups were performed with independent T test or Mann-Whitney U test as appropriate”.

Lines 198-199: Authors state that the model has been adjusted by HOMA-IR and apo B levels. What does this mean? These variables have been included in the model although they did not finally remain because of P > 0.05? Or they are in the model anyway to adjust the model by these variables for any reason? Correlations showed no statically significance. BMI was included in the model but it was not statically significant? Correlation was significant but not the regression model? Indicate in “Methods” section and in the footnote of the regression table those variables that were initially included into the model. If
any of these variables remain despite not statistically significance, specify the reason.

Answer: In the linear regression model only variables that were significantly associated in the bivariate analyses were included. HOMA-IR and apo B levels were included but were not significant (P > 0.05) and therefore did not remain in the final model. The text was changed (lines 203-207) as follows “The linear regression analysis identified the fasting apo B-48 levels (r= 0.40), and the WHR (r= 0.35) as independent parameters determining the triglycerides iAUC, explaining 29% of the variability. The model also included the fasting triglycerides concentration, time of triglycerides peak, HOMA-IR, and the apo B levels, which did not show an independent association with the triglycerides iAUC after adjustment (table 2).”

BMI was not included (we used WHR instead) in the linear regression analysis. In the methods section and in the footnote of table 2 the variables included in the model are listed lines 145-150 “In the first model, the dependent variable was the triglycerides iAUC and the independent variables were the fasting apo B-48, fasting triglycerides, time of triglycerides peak, WHR, HOMA-IR, and the apo B. In the second model, the dependent variable was the apo A-V iAUC. This model included the triglycerides iAUC, apo B-48 iAUC, fasting triglycerides, apo B, and HDL-c as independent variables”.

- I have the same comment regarding independent factors associated with postprandial apo A-V response (line 214).

Answer: In the linear regression model to evaluate independent variables associated with the postprandial apo A-V response, the following variables were included: triglycerides iAUC, apo B-48 iAUC, fasting triglycerides, apo B, and HDL-c. This was included in the Statistical analysis section as mentioned above. The Results section was changed to (lines 220-223): “In the linear regression model, the only variable independently associated with the apo A-V iAUC was the triglycerides iAUC, explaining 54.4% of the variability in this variable (P< 0.001). This model also included the apo B-48 iAUC, fasting triglycerides, apo B, and HDL-c levels as independent variables”.

- Please, review carefully statistical methods. I think it could be useful to consult with a Statistical professional for further assistance.

Answer: The statistical methods were carefully reviewed and discussed with a Statistics expert.

- I think non-HDL cholesterol should be more appropriate in these patients since LDLc is not useful when triglycerides > 300 mg/dl. Besides, the most of international guidelines include non-HDL cholesterol as main factor for clinical diagnosis. So, please, consider to include it in the results (both in tables and regression analysis).

Answer: Non-HDL cholesterol was calculated and included in the results (tables 1 and 3). Bivariate Pearson correlations were examined between non-HDL cholesterol and the triglycerides iAUC and also between non-HDL and the apo A-V iAUC. We did not find any significant correlation r= 0.211, P= 0.081 and r= 0.321,
P= 0.326, respectively; therefore this variable was not included in the linear regression analysis.

- Table 3: Letters indicating statically significance between groups are not completely clear. There are not significant differences between group 2 and groups 3 or 4 or they have not been indicated? I.e: apo B-48 iAUC (no differences between group 2 and 3??)

Answer: For statistical analyses of variables presented in table 3 we first use one way ANOVA or Kruskal-Wallis test in order to identify a statistical difference. In case of a P value <0.05, we proceeded to evaluate differences between individual groups using independent T-test or U Mann-Whitney test, as appropriate. In each case every possible comparison was made (group 1 vs 2, group 1 vs 3, group 1 vs 4, group 2 vs 3, group 2 vs 4, and group 3 vs 4). Significance is annotated when present. In order to clarify this point lines 154-156 have been added on the Statistical analysis section.

- Line 41: Indicate “linear” in “regression model”.
Answer: linear has been added to the text.

- Lines 45-46: Define fasting hypertriglyceridemia and abdominal obesity. Specify the considered levels.
Answer: definition of fasting triglycerides and abdominal obesity have been added as follows (lines 46-48) “The study sample was classified according to the presence of fasting hypertriglyceridemia (≥150 mg/dL) and abdominal obesity (WHR ≥0.92 in men and ≥0.85 in women) to explore differences in parameters”.

- Line 48: WHR abbreviation has not been previously defined.
Answer: definition of WHR was added (line 43)

- Lines 49-50: R2 is usually indicated as “0.22” instead of “22”. Please, consider to modify them here and across the manuscript.
Answer: R2 has been modified

- Line 54: “Fasting triglycerides levels is the main factor” has been indicated. However, this data has not clearly provided in “Results” section of the abstract. Please, indicate the R2 of fasting triglycerides variable (solely) in linear regression for iAUC triglycerides.
The partial correlation of each variable (fasting apo B-48 and WHR) have been added in the Abstract (lines 50) and Results (lines 203-204) sections

- It has to be indicated anywhere in the Abstract that abdominal obesity has been assessed by waist-to hip ratio to avoid confusion.
Answer: This was added as follows in the methods section of the abstract "Abdominal obesity was assessed with the waist to hip ratio (WHR)” (lines 42-43).

- Line 69: “apo” abbreviation has not been previously defined.
Answer: apolipoprotein has been added to indicate the definition of apo.
- Subjects with high alcohol consumption have been excluded? Please, indicate it.
Answer: subjects with high alcohol consumption were excluded. This has been added to the Methods section (line 101)

- Line 112: “Subjects were studied…”. This sentence is confused. Please, explain clearly that patient were 12-hours fasten and meal test was provided after that. I suppose fasting triglycerides were determined in this condition but it is not completely clear.
Answer: To avoid confusion this was modified as follows (lines 114-116) “Subjects were asked to attend the visit after a 12-hour fasting period, and a standardized meal was provided. Blood samples were then obtained at 0 (fasting), 3, 4, 6, and 8 hours after meal ingestion“

- Line 123: “TG levels > 250 mg/dl”. This level is usually “300”. Please, correct or clarify.
Answer: This has been corrected as requested.

- Line 148: Indicate “p” as “P”.
Answer: This has been corrected as requested.

- Lines 153-154: Authors state that 1 subject with high fasting TG levels were excluded. High TG concentration is not an exclusion criterion. Please, clarify.
Answer: In the Methods section we mentioned that a fasting triglyceride concentration >600 mg/dl was an exclusion criteria. The patient fulfilled this criterion; therefore he was excluded from the study.

- Line 162: “p < 0.01” is indicated although in “Figure 2” legend is indicated “p < 0.001”. Please, clarify.
Answer: The correct P value is <0.001. This has been corrected.

- Line 171: “at the beginning and at the end”. Which time point refer to when they state “at the end”? Just finishing the meal test consumption or after 8 hours?
Answer: This time point refers to eight hours following the consumption of the test meal. This has been specified as follows “We calculated the apo B48/apo B index, at baseline and at eight hours following the test meal” (lines 181-182)

Answer: this has been corrected as requested.

- Correlation between apo A-V iAUC and apo B48 iAUC is significant, as stated in lines 206-207) but the model (line 214) has not been adjusted by this variable. If this is statically significant, why the model has not been adjusted by this variable?
Answer: Interestingly we found a bivariate correlation between apo A-V iAUC and apo B48 iAUC. No other studied variable was correlated with the apo B-48 iAUC,
therefore we could not perform a linear regression analysis with apo B-48 iAUC as the dependent variable. We did not include the apo B48 iAUC as an independent variable in the model for explaining the apo A-V iAUC because we consider that apo A-V is a factor that modulates postprandial lipemia and not vice versa.

- Lines 257-258: To my point of view, this affirmation is too speculative. Please, indicate that the “postprandial lipemia is MAINLY determined by fasting…” or specify in a similar way. R2 is 0.22 so these 2 variables explain the 22% of variability of triglycerides iAUC, as stated in lines 278-280. Please, indicate this information together to avoid confusion
Answer: This was modified as follows (lines 260-274) “We found that in patients with FCH postprandial lipemia is mainly determined by the fasting apo B-48 concentration and abdominal obesity. However, the linear regression model explained only a modest proportion of the variability in postprandial lipemia in individuals with FCH; therefore, additional variables related to this phenomenon may be involved”.

- In the Figure 1 and 2 legends, “during” is indicated. Do authors mean “after”?
Answer: During has been changed for after in the figure legends as requested.

- In the same legends, “repeated measures ANOVA” test is indicated. Please, refer to my comment of “Major comments” section for further information. In this case, Friedman test should be performed. If statistical tests used are indicated in Figure legends, they should be also included in the tables footnotes homogenously.
Answer: The correction has been made to indicate that the Friedman and Kruskall Wallis test were used.

- In Figure 2: Please, indicate which each Group means. I.e: High TG and WHR instead of “Group 1”. It would facilitate the comprehension of the results.
Answer: this change has been made as requested

- Table 1: The title is not too clear.
Answer: The title has been changed to “Baseline characteristics of study subjects and of the apo A-V and apo B-48 subgroup”.

- Table 2: In the footnote it is indicated “excluded variables: apo B and HOMA-IR”. I have previously indicated in another comment that it is not clear what “excluded variables” mean. These variables remain in the model despite not being statically significant? Or they have been excluded because they are not significant? In this case, any other variable has been included in the model?
Answer: the footnote has been changed as follows “Dependent variable: triglycerides iAUC; variables included in the model: fasting apo B-48, fasting triglycerides, time of triglycerides peak, apolipoprotein B, HOMA-IR, and waist to hip ratio (WHR)”
- Table 2: “R2” of each variable has to be included in a separate row to clarify the importance of each variable into the model.
Answer: the partial correlation of each variable has been included in table 2 as requested.

- Table 3: The titles “Group 1”, “Group 2”, etc. are not clear. Please, indicate in each row heading what it is each group. It would facilitate the comprehension of the table.
Answer: the titles have been changed to HTG with obesity, HTG without obesity, NTG with obesity, and NTG without obesity to facilitate the understanding of this table.

- Table 3: Triglycerides iAUC, apo B-48 iAUC, apo B-48 peak and apo A-V iAUC differences between groups are not statically significant (P < 0.05)? Data seem very different.
Answer:
1) There was a significant difference in the triglycerides iAUC between the group with HTG with obesity and the group with NTG with obesity and also between the group with HTG with obesity and the group NTG without obesity. This has been indicated.
2) Apo B-48 iAUC was not significantly different between the groups (P= 0.373).
3) There was a significant difference in the Apo B-48 peak between the group with HTG with obesity and the group with NTG with obesity and also between the group with HTG with obesity and the group with NTG without obesity. This has been indicated.
4) There was a significant difference in the apo A-V iAUC between the group with HTG with obesity and the group with NTG with obesity and also between the group with HTG and obesity and NTG without obesity. This has been indicated.

Reviewer 2
- Page 12, it is of great interest that the chylomicron response was independent from triglycerides and other classical parameters. Does this point at an FCH-specific metabolic disturbance? Could this reflect impaired intestinal lipoprotein handling?
Answer: We found this result very interesting and we wonder if this represents a specific metabolic abnormality in FCH perhaps reflecting an abnormal intestinal metabolism of lipoproteins in this group of patients. We recognize that apo B-48 was measured in a small subgroup of patients (n= 44) and further studies are needed to confirm these results.

- Page 13, first line, it seems inadequate to speak of “an abnormal lipid response” when there was no reference group available.
Answer: this was changed to: “In patients with FCH the postprandial lipid response was aggravated by abdominal obesity” (lines 282-283).

- Page 10, line 214: HDL-c instead of c-HDL
Answer: this has been corrected as requested
Reviewer 3

- It is absolutely difficult to understand the explanation at P8 L175-P9L179. In postprandial stage, chylomicron and their remnants are generally observed in many previous reports. ApoA-V is associated with TG-rich lipoprotein and cellular experiments demonstrated that apoA-V binds to VLDL and HDL [reference 22]. However, it is not clear whether apoA-V transfers and binds to chylomicron and their remnants at fasting and postprandial stage in human blood. From the recent reports, it does not have enough evidences to use ApoA-V levels as a representative for VLDL.

Answer: There are publications that have described an increase apo A-V levels in the postprandial period and have associated it with postprandial lipemia (VLDL and chylomicrons) [7]. In addition, O'Brien PJ, et al. studied apo A5 distribution in lipoproteins and found it in VLDL, HDL, and chylomicrons [8]. Therefore, the assessment of the apo AV/triglyceride ratio at fasting and at 8 hours after ingestion of a meal was done to evaluate the change in this relation after the chylomicrons postprandial response. To clarify, the text was modified as follows (lines 185-187) “The fasting and 8-hour apo A-V/triglycerides ratio was estimated to evaluate the change of the association of these parameters in response to the chylomicrons increment after ingestion of a meal”.

- The most important problem is the characters of FCH, meaning that fasting TG and cholesterol levels usually change at sampling as type IIa, IIb, or V hyperlipidemia. Authors should add the explanation for the table 4 and figure 2.

Answer: the following clarification has been added to the legend in table 3 “All study subjects had apo B levels over the 90th percentile for the Mexican population and hypertriglyceridemia and/or hypercholesterolemia at diagnosis; however, some of them showed inferior levels at the time of the study due to the already known variability in the lipid profile associated with FCH”

- In addition, if authors want to state the association of fasting TG levels with postprandial TG levels, authors should demonstrate not only iAUC (TG iAUC), but also AUC with absolute levels (TG AUC). Because subjects are FCH, the fasting TG levels are various.

Answer: the main objective of the study was to evaluate the postprandial triglycerides response. As FCH subjects display a high variability in the fasting triglycerides concentration, we consider that the best way to discern the postprandial increment is utilizing the incremental AUC that does not consider the baseline concentration instead of the total AUC that includes the basal concentration. Using the total triglycerides AUC could make appear the postprandial response of a greater magnitude than it really is due to an elevated baseline triglycerides concentration.

- How is fasting apoB-48 related with TG iAUC or TG AUC?

Answer: We thank you for this observation, fasting apo B-48 was significantly associated with the triglycerides iAUC r= 0.431, P= 0.004. This variable has been added to the multiple regression analysis and was the stronger variable associated
with the postprandial triglyceride response. This finding was modified across the article.

- In addition, why was apoA-V iAUC associated with apoB-48 iAUC? Authors should refer to these questions in the discussion.
Answer: We consider that the association of apo A-V and apo B-48 postprandial responses is due to the previously demonstrated association of apo A-V with chylomicrons [8]. This finding is novel and should be confirmed in future studies. This was added in the Discussion section as follows "Interestingly, we found a significant correlation between the apo B-48 and apo A-V postprandial responses. We consider that this finding could reflect the association of apo A-V with chylomicrons" (lines 296-298)

- P12 L253-258; It is hard to understand these sentences. Authors showed the postprandial significant increase of apoB48 and relation of apoB48 with TG. Many reports already demonstrated that apoB-48 postprandial response may be dependently associated with obesity and hypertriglyceridemia, and fasting B-48 levels is important for postprandial lipemia.
Answer: We agree with the observation that apo B-48 postprandial response is associated with obesity and hypertriglyceridemia in populations with metabolic syndrome and diabetes. However, this association has not been studied specifically in population with FCH. We wonder if this finding represents a specific metabolic abnormality reflecting an abnormal intestinal metabolism of lipoproteins in patients with FCH. We recognize that apo B-48 was measured in a small subgroup (n= 44) and further studies are needed to confirm these results.

- Authors classified 4 groups according to the levels of TG and WHR, but analysis with comparison among 4 groups will lead to confusion. It is well known that FCH is heterogeneous dyslipidemic condition with or without metabolic syndrome and impaired glucose tolerance. For the essential goal that authors want to reveal, authors should separately analysis for fasting and postprandial lipids in each group.
Answer: The classification of the population and description of the subgroups was a secondary and exploratory objective. Our main objective was not to characterize the postprandial lipemia in each of these groups. Table 3 has been organized to separate the fasting and postprandial parameters as requested. We are not performing linear regression analysis in each group due to the low number of subjects in each group after classifying the population.


