Author's response to reviews

Title: Analysis of the contribution of FTO, NPC1, ENPP1, NEGR1, GNPDA2 and MC4R genes to obesity in Mexican children

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Author's response to reviews: see over
1. The classification of BMI by the Centers for Disease Control and Prevention CDC 2000 is not familiar to the non-Mexican readers. Moreover, the criterion of obese children was not clear. How did the authors calculate the 95th percentile?

This question can be combined with the following comment:
2. Why did the authors select children with BMI<85th, not with BMI <75th, percentile as control subjects? The 85th percentile seems to be higher.

**Response:** Eutrophic or obese status was assessed using BMI percentiles according to the ‘Centers for Disease Control and Prevention 2000’ (CDC2000) reference (CDC 2000, Atlanta, GA, USA) (Kuczmarski RJ et al. Vital Health Stat. 2002;11 (246)). Briefly, to calculate the BMI percentile, we used the EPI INFO 3.3.2 software with CDC 2000 growth charts that are based on 5 U.S nationally representative surveys conducted between 1963 and 1994, in which Mexican American children were included. According to those growth charts, for ages 2 to 20 years, overweight was defined as a BMI-for-age between the 85th and 95th percentiles, while obesity was defined when BMI-for-age was higher than the 95th percentile (Kuczmarski RJ et al. Vital Health Stat. 2002;11 (246); Barlow SE et al. Pediatrics. 1998;102(3):e29-e39; Barlow SE. Pediatrics. 2007;120:S164-S192). We have changed the paragraph in the Methods section of the revised manuscript to clarify this point:

“BMI was calculated and classified according to the ‘Centers for Disease Control and Prevention CDC 2000’ (CDC2000) reference [9]. CDC 2000 growth charts are based on 5 U.S nationally representative surveys conducted between 1963 and 1994, in which Mexican American children were included [9]. According to those growth charts, for ages 2 to 20 years, overweight was defined as a BMI-for-age between the 85th and 95th percentiles, while obesity was defined when BMI-for-age was higher than the 95th percentile [9, 10].”

3. In Table 3, metabolic quantitative traits, except BMI, should be adjusted with BMI.

**Response:** We agree with the reviewer that metabolic quantitative traits could be adjusted with BMI. However, we would like to highlight that: i/ we only analyzed metabolic quantitative traits in nonobese children; and more importantly, ii/ the MAGIC consortium that performs large meta-analyses of GWAS for glucose and insulin related traits, does not usually add BMI as a covariate in the linear regression models that assess quantitative metabolic traits (please see: Dupuis J et al. New genetic loci implicated in fasting glucose homeostasis and their impact on type 2 diabetes risk. Nat Genet. 2010;42(2):105-16). The same is true for lipids consortia (please see: Teslovich TM et al. Biological, clinical and population relevance of 95 loci for blood lipids. Nature. 2010;466(7307):707-13). Therefore, in order to be in line with these international consortia and to be able to accurately compare our results with these consortia results (that are publicly available), we have preferred adjusting our regression models for age and gender only.
4. The authors performed case-control study and QTL analysis. Therefore, ‘a case-control study’ should be deleted from title.

**Response:** We agree with the reviewer. Accordingly, we have changed the title.

**Reviewer: Solena Le Scouarnec**

Minor Essential Revisions:
In the results section, rs775456 should be replaced by the correct rs ID (rs7754561).
At the end of the discussion section, “It noteworthy” should be replaced by “It is noteworthy”

**Response:** Many thanks. These two mistakes have been corrected in the revised version of the manuscript.

**Reviewer: Torben Hansen**

- Regarding the selection of study participants: the authors do not provide information on the rounds for excluding ~600 children who were originally recruited. Was the exclusion due to BMI selection?

**Response:** In the present study, we only included participants with informed levels of all metabolic traits that we analyzed, and with a good quality of DNA (the exclusion is not due to BMI selection). In order to clarify this point, we have changed the sentence in the Methods section of the revised manuscript:

“In the present study, we analyzed 1,685 children (aged 6 to 12 years) of Mexican origin, from five different states of Mexico (San Luis Potosí, Queretaro, Tijuana, Guanajuato and Mexico city), who were randomly selected and invited to participate in a cross-sectional study between 2007 and 2011 from public and private schools.”

It should also be clarified how the 1171 non-obese children for QT studies were selected.

**Response:** Sorry for the misunderstanding. As said above and in the manuscript, we analyzed 1,685 children, including a subset of 949 lean children (with BMI < 85th percentile) and 514 obese children (with BMI ≥ 95th percentile) who were involved in the obesity case-control study. Children with BMI < 95th percentile were considered ‘non-obese’ (N = 1,685-514 = 1,171). We have clarified this point in the Results section of the revised manuscript:

“From an initial sample of 1,685 children (between 6 and 12 years old), we extracted 949 lean and 514 obese children (see clinical characteristics in Table 1) for the case-control study, and 1,171 nonobese children (with BMI < 95th percentile) for the study of metabolic quantitative traits including BMI, fasting serum insulin, fasting plasma glucose, total cholesterol and triglycerides.”

Furthermore, are all of the study participants of ‘Mexican origin’/ethnicity?
Response: All participants of the present study are indeed of Mexican origin. This is now clearly written in the Methods section of the revised manuscript (please see above).

- Regarding the selection of obesity-associated genes and SNPs: Aside from a minor allele frequency of >20% in Mexicans, the authors do not provide clear statements as to how these 6 SNPs have been selected. Have the selected genes previously been associated with obesity at a genome-wide significance level? This is relevant, as the authors later state that the association between NPC1 and ENPP1 and obesity/BMI are controversial. Furthermore, have the genes been associated with obesity in individuals of European ethnicity or also in other ethnicities? Also, several SNPs in e.g. the MC4R locus have previously been associated with obesity.

Response: We now highlight in the Introduction and the Results section which references we used for our present study:

“To date, genome wide association studies (GWAS) and linkage studies, mostly performed in European adult populations, have identified more than 50 loci associated with obesity or BMI [5-10].”

“In both studies, we genotyped six SNPs that are known to be associated with risk of obesity in European populations: rs17782313 near MC4R [6]; rs2815752 near NEGR1 [7]; rs7754561 near ENPP1 [8]; rs1805081 in exon 6 of NPC1 [9]; rs10938397 near GNPDA2 [7] and rs1421085 in intron 1 of FTO [10].”

Furthermore, following the Reviewer comments, we now explain more in the Methods section why we selected these SNPs for our association study in Mexican children:

“To achieve a power of at least 80%, we only selected SNPs with minor allele frequencies ≥ 20% in the Mexican population according to the HapMap database. These SNPs were identified by GWAS or meta-analyses of GWAS in European populations [6, 7, 9, 10]. Furthermore, we selected a SNP that was significantly associated with childhood obesity in a French population, according to a linkage association study [8].”

- Have the authors selected SNPs according to lead SNPs or patterns of linkage equilibrium? The authors should include information on these issues.

Response: In the results section, we have added the references of studies for each SNP that we analyzed:

“In both studies, we genotyped six SNPs that are known to be associated with risk of obesity in European populations: rs17782313 near MC4R [6]; rs2815752 near NEGR1 [7]; rs7754561 near ENPP1 [8]; rs1805081 in exon 6 of NPC1 [9]; rs10938397 near GNPDA2 [7] and rs1421085 in intron 1 of FTO [10].”

In other words, we only analyzed SNPs that were referenced in those studies.

- The authors identify an association between the MC4R risk allele and increased fasting plasma glucose levels, however, the authors fail to further discuss this putative association. Does this association remain significant after adjustment for BMI? Furthermore, recently published meta-analyses have in fact found variants near MC4R to associate with type 2 diabetes, both in European and Asian populations. These recent findings should be discussed by the authors. Please see the following references:

  o Large-scale association analysis provides insights into the genetic architecture and pathophysiology of type 2 diabetes. The DIAbetes Genetics Replication And Meta-analysis
Response: Many thanks for this comment. This association remains totally significant after adjustment for BMI. Of note, we missed the two publications as we submitted our manuscript to BMC in mid-July 2012. We have added these references in the revised version of the manuscript and have changed the paragraph in the Discussion accordingly:

"Furthermore, we identified an effect of the risk allele of MC4R variant and increased fasting plasma glucose levels. To our knowledge, no previous study has shown this association. However, two recent large meta-analyses of GWAS identified a significant association between the MC4R locus and type 2 diabetes risk, in European and Asian populations [27, 28]. Of note, our present association with fasting plasma glucose levels remained significant after adjustment for BMI (data not shown). Altogether, these findings would suggest a potential effect of MC4R polymorphisms on decreased pancreatic beta-cell function."

- The authors should provide a clear reference to the calculation and classification of BMI. Are the BMI percentiles based on growth curves from Mexican children?

Response: Many thanks for this comment. Please refer to the answer that we have addressed to Reviewer#1 (Kikuko Hotta) who tackled the same point.

- The authors assess the effect of SNPs using a logistic regression model adjusted for age, gender and Mexican state, however, the term Mexican state is not explained.

Response: Sorry for the misunderstanding. As said in the Methods: “In the present study, we analyzed 1,685 children (aged 6 to 12 years) of Mexican origin, from five different states of Mexico (San Luis Potosí, Queretaro, Tijuana, Guanajuato and Mexico city).” In order to clarify this point, we now re-explain the term “Mexican state” in the “Statistical analysis” section:

“The effect of SNPs on obesity status was assessed using a logistic regression model adjusted for age, gender and Mexican state (1: San Luis Potosí, 2: Queretaro, 3: Tijuana, 4: Guanajuato and 5: Mexico city), under an additive model.”

- It is stated that 45% of the obese children are insulin resistant. Insulin resistance should be defined.

Response: Accordingly, we have added a paragraph in the Methods section of the revised manuscript which defined insulin resistance in our population of Mexican children:

“Insulin resistance was defined as: homeostasis model assessment of insulin resistance (HOMA-IR = [(Fasting glucose (mg/dL) / 110.63) × (Fasting insulin (μU/mL)] / 405) ≥ 3.4 (that is the 90th percentile of HOMA-IR in a population of healthy Mexican children [11,12]).”

- The power of the study and issues related to multiple testing should be discussed

Response: We had already addressed in the manuscript the question of multiple testing: “By applying Bonferroni correction, a significant p-value has been considered when below 1.4×10^-3 (0.05 / 36) and a p-value between 0.05 and 1.4×10^-3 has been considered as nominally
significant.” Furthermore, in the discussion, we had already highlighted that “as we lack some statistical power, additional genetic studies on Mexican children would be needed”.

- The ENPP1 risk allele is found to be protective. Please, discuss this finding.

**Response:** Accordingly, we now discuss more this finding in the discussion of the revised manuscript:

“Of note, the risk alleles for obesity or increased BMI were the same between Europeans and Mexicans, except for the risk allele of ENPP1 rs7754561 (in Europeans) that showed a protective effect in Mexicans. Although the association between both MC4R and NEGR1 and risk of obesity has been confirmed in a plethora of studies and populations, the ENPP1 association signal with obesity is more controversial [21]. To our knowledge, no other studies demonstrate a protective role of the risk allele of ENPP1 rs7754561. Recently, it has been shown that ENPP1 overexpression in human adipocyte cell lines resulted in defective adipocyte maturation [22]. If confirmed in other Mexican populations, the protective effect of the ENPP1 variant may be due to a loss-of-function of the protein.”

- In the results section, the authors provide an odds ratio confidence interval for European populations. It should be elaborated from which studies/populations these estimates have been obtained.

**Response:** In the results section, we have added the references of studies for each SNP:

“In both studies, we genotyped six SNPs that are known to be associated with risk of obesity in European populations: rs17782313 near MC4R [6]; rs2815752 near NEGR1 [7]; rs7754561 near ENPP1 [8]; rs1805081 in exon 6 of NPC1 [9]; rs10938397 near GNPDA2 [7] and rs1421085 in intron 1 of FTO [10].”

Of note, we did not provide an odds ratio and confidence interval for European populations. Actually, we only provided the risk allele in this population.

- OR should be included in the list of abbreviations.

**Response:** Sorry for this oversight. “OR” has been included in the list of abbreviations (as well as β, CI, P, pc, RA, RAF and SE).

**Writing:**
- The authors should ensure that the entire manuscript is written in past tense.

**Response:** We ensure that the entire manuscript is written in past tense.

- The wording: ‘we did not find any significant contribution of both NPC1 … and FTO…’ should be corrected, e.g. to: ‘we did not find any significant contribution of either NPC1 … or FTO…’. Similar corrections should be made throughout the manuscript.

**Response:** Many thanks. We have corrected this wording throughout the manuscript.

- When listing gene variants, the authors should use the same wording throughout the manuscript, e.g. MC4R rs17782313 instead of MC4R SNP rs17782313.

**Response:** Many thanks. We now use the same wording throughout the manuscript.