Author's response to reviews

Title: Age-of-onset-dependent Influence of NOD2 gene variants on Disease behaviour and treatment in Crohn's disease

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Author's response to reviews: see over
We first like to thank the reviewers again for valuable comments. Specifically we would like to answer to the remaining points as follows:

Response to reviewers' comments

Reviewer #1

Reviewer: Francis Vasseur
Reviewer's report:
The manuscript entitled "Age-of-onset-dependent Influence of NOD2/CARD15 gene variants on Disease behaviour and treatment in Crohn's disease" submitted by Carsten Posovszky et al. aims at evaluating the influence of NOD2 gene variants in disease behavior and treatment of pediatric and adult-onset Crohn’s disease, has been amended by the authors.

#1 In the sentence: “In this retrospective single center study in a tertiary center we report high prevalence of patients with NOD2 variant alleles with 44% in the pediatric- and 42% in adult-onset CD. The frequency of NOD2 variants in our cohort is similar to other European pediatric multicenter cohort studies with a prevalence of 35 upto 45.6%...". The first part of the sentence reports a frequency of patients and the second part of the sentence (beginning with “The frequency NOD2 variants...”) sounds like an allelic frequency although it seems a genotype frequency. This kind of confusion should be corrected.

Response
In order to clarify these sentences and point out that the genotype frequency is meant we changed the manuscript accordingly.

Changes in the manuscript
In this retrospective single center study in a tertiary center we report a high prevalence of CD patients carrying at least one NOD2 variant allele with 44% in the pediatric- and 42% in adult-onset CD similar to other European pediatric multicenter cohort studies with 35 up to 45.6% [12, 24, 25].

Discussion page 14

#2 The previous confusion in mind of the authors as reported in my #1 is evident in the authors response letter to the previous #41 they answered “Indeed, we compared the NOD2 variant allele frequency but we did not found a statistical difference between the two groups.” And refered to allelic frequency although they seem to mean “patients with variant allele?”, indeed in the “change in the manuscript” they now use a correct formulation with “the presence of two NOD2 mutant allele was found more frequent in the pediatric-onset CD patients” which refers to patients and thus to genotype frequencies and not allelic frequencies. The authors must be aware of this mistakes and should not confound between allelic and genotype frequencies.

Response
We are aware of this mistake and do not confound between allelic and genotype frequencies
any more.

Changes in the manuscript
No changes

#3 In the legend of supplemental figure 1, why did the authors do not add the word “allele” to the “patients with NOD2 variant” although they wrote “NOD2 wild-type alleles” just after?

Response
The word “alleles” refers to NOD2 variant or wild-type.

Changes in the manuscript
The age of onset of CD in 202 patients with NOD2 variant (grey bars) or wild-type (white bars) alleles is compared...
Supplemental figure

Reviewer #2

Reviewer: Maria Francisca Gonzalez-Escribano

Reviewer's report:
I have reviewed the revised version of the manuscript entitled “Age of onset dependent influence of NOD2/CARD15 gene variants on disease behaviour and treatment in Crohn’s disease” by Posovszky et al.. I have also read the response of the authors to reviewers.

The authors have made a big effort to meet the different questions raised and to improve the original manuscript. Unfortunately, in my opinion, the main problems persist. Thus, the most original point of the manuscript (BMD variants/ osteoporosis and NOD2) is lost among other results and comparisons.

Response
We appreciate your comments. Indeed this is the first study including influence of NOD2 variant alleles in patients with CD on BMD and therefore representing an important issue. To address this we made an effort in the revision to highlight this issue in the abstract, the introduction, the result section and the discussion. However, by focusing on the age of onset dependent influence of NOD2/CARD15 gene variants in this manuscript we present and discuss also other interesting data e.g. on therapy. In order to compare influence of NOD2 variant alleles in pediatric- and adult-onset CD patients on several items we juxtapose the characteristics of pediatric-onset CD patients regarding BMD. In this revision we stressed our focus in the discussion more on this issue by omitting dispensable discussions on other issues in the discussion.

Changes in the manuscript
Presentation of the results
Reorganization and deletion of several parts in the Discussion section
Other questions:

1. Taking into account the sample size, it is difficult to extract conclusions with the type of analysis performed and the number of variables included. Likely some of the variables are not independent why authors did not performed a multivariate analysis?

   **Response**
   
   The reviewer mentioned that the underlying sample size is “too low” (1st revision) or that “it is difficult to extract conclusions taking into account the sample size and the number of variables included” (2nd revision). However, in our opinion the sample size, with 202 patients in total and 168 with BMD measurement, is quite sufficient to conduct the stratified analyses we made, especially the multivariate analyses we now added. Moreover, this is in accordance with the established practice and respective methodological papers (e.g. Peduzzi, P., Concato, J., Kemper, E., Holford, T.R., Feinstein, A.R. A simulation study of the number of events per variable in logistic regression analysis. Journal of Clinical Epidemiology (1996), 49, 1373-9). Indeed, there should be at least 10 observations per variable included as an independent variable in a regression model. Therefore, it would have been possible to include 15-18 variables (to be conservative) simultaneously in our analyses in one regression model. Even within a subgroup analysis of the pediatric onset patients (n=85/77), for example, there would have been enough sample size for a multivariate analysis with up to 7/6 independent variables simultaneously. Our sensitivity analyses only included up to 5 independent variables.
   
   Also with respect to a stratified analysis reporting frequencies, the underlying sample size is sufficient in principle. To provide even more information, we do not only report the relative frequencies, but also the absolute values to enable the reader to have a more detailed understanding of the distribution of the covariates. Indeed, we are aware of the significance of our analysis and interpreted all statistical tests explorative, as stated in the material and methods section.
   
   In order to satisfactorily show that variables are independent we now provide data from multivariate analysis within the results section, e.g. we analyzed PCDAI-Score as dependent variable and osteoporosis, steroid-dependence or underweight as explaining variables. Using this multivariate regression model we could show that the PCDAI is significant higher in patients with osteoporosis (p 0.03; 5.8 points in PCDAI) or steroid dependency (p 0.0069; 7.5 points in PCDAI) but did not reach significance in patients with underweight.

   **Changes in the manuscript**
   
   Material and methods
   
   Results section

2. In the section “Pediatric-onset CD patients.....” of the Results (page 10) the numbers of the Figures seem to be erroneous.

   **Response**
   
   We apologize for the confusion. The figures are renumbered and appear now in the correct order.

   **Changes in the manuscript**
   
   Figure number
   
   Figure legend

3. Authors suggest a trend of patients with variants of NOD2 towards low BMD but the frequency of patients with two variants is the same among patients with low BMD as among patients with normal BMD.
Response
We thank the reviewer for this note. Indeed, the difference between both groups is not the number of variant alleles but rather the homozygosity of variant alleles. None of the CD patients carrying homozygous NOD2 variant alleles had a normal BMD. We added this information in Table 2.

Changes in the manuscript
Table 2