Author's response to reviews

Title: Age-of-onset-dependent Influence of NOD2/CARD15 gene variants on Disease behaviour and treatment in Crohn's disease

Authors:

Carsten Posovszky (carsten.posovszky@uniklinik-ulm.de)
Veronika Pfalzer (verop@web.de)
Georgia Lahr (georgia.lahr@uni-ulm.de)
Jan-Hendrik Niess (Jan-Hendrik.Niess@uniklinik-ulm.de)
Jochen Klaus (Jochen.Klaus@uniklinik-ulm.de)
Benjamin Mayer (benjamin.mayer@uni-ulm.de)
Klaus-Michael Debatin (Klaus-Michael.Debatin@uniklinik-ulm.de)
Georg Boyen von (g.vonboyen@klksig.de)

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Author's response to reviews:

Dear Editor,

Thank you for giving us the opportunity to resubmit our paper. We carefully revised our manuscript in accordance to the reviewers’ comments. The current version of the paper is now suitable for publication in BMC Gastroenterology and will find your acceptance.

Sincerely yours,

Carsten POSOVSZKY, MD
Senior Consultant, Gastroenterology
University Medical Center Ulm, Germany

Title: Age-of-onset-dependent Influence of NOD2/CARD15 gene variants on Disease behaviour and treatment in Crohn’s disease Version: 1 Date: 8 November 2012

We would like to thank the reviewers for their critical reading of the manuscript. Specifically we would like to answer to the remaining point as follows:

Response to reviewers' comments

Reviewer #1
Reviewer: Francis Vasseur Reviewer’s report:


#1 Gene names should be typed in italic.

Response

We corrected the notation of the gene Name according to the HGNC as NOD2 italicised.

Changes in the manuscript

Gene names are typed in italic throughout the manuscript.

#2 In the results part of the abstract (page 3) it is mentioned that "Chronic active and moderate to severe course of disease is associated with younger age of onset (p=0.457)". Is it the true p-value or is there a typo error?

Response

It is a typo error!

Changes in the manuscript

Chronic active and moderate to severe course of CD is associated in patients with pediatric-onset (p=0.0001) and NOD2 variant alleles (p=0.001).

#3 In the results part of the abstract (page 3) it is mentioned that "Interestingly, osteoporosis found more frequently in patients carrying NOD2 SNPs than in the wild type group". I should have written "...was found more frequently..."

Response

We followed your proposal and changed the sentence.

Changes in the manuscript

Interestingly, osteoporosis was found more frequently in patients carrying NOD2 variants (p=0.033)....

Page 3

#4 In the abstract the authors first use the term "SNP" to define the genetic
variants and in the abstract conclusion use the term "polymorphism", then "mutation" later in the manuscript. It should be more convenient to use the same term throughout the whole manuscript.

Response

We agree that a more consistent definition would be more convenient for the reader. Therefore we used NOD2 variant allele throughout the manuscript in the context of our results.

Changes in the manuscript

“NOD2 variant allele” was used throughout the manuscript

#5 Page 4 it is mentioned " Polymorphisms in the leucine-rich repeats (LRR) region of the NOD2/CARD15 gene were identified as an independent risk factor". If "polymorphisms" is at the plural, the sentence should be "...as independent risk factors..."

Response

As suggested the plural form was used.

Changes in the manuscript

Polymorphisms in the leucine-rich repeats (LRR) region of the NOD2/CARD15 gene were identified as independent risk factors for CD in Caucasians...

Page 4

#6 Page 4, regarding the severe demineralization and its evaluation using z-scores, the authors should precise what parameter is (are) z-scorized.

Response

We changed the text passage and eliminated Z- and T-score as the subject matter is the high prevalence of severe demineralization which needs no further detailed information regarding the measurement. See also response to point #13 regarding definition of Z- and T-score.

Changes in the manuscript

The prevalence of severe demineralization in children and in adults range from 4.35% to 42% [14]

Page 5

#7 On page 5 the authors wrote " There is increasing evidence that inflammation may significantly contribute to a high-turnover mineral bone loss". This
assumption should be supported by relevant references.

Response

Our statement is supported by several studies in vitro and in vivo and two excellent reviews were cited who summarize the current knowledge. The high incidence of mineral-bone loss in CD independent of the use of corticosteroids suggested that inflammation itself may be a risk factor. There are studies investigating the cell types and mediators involved in the pathophysiology of inflammation-related bone loss reviewed by Tilg et al 2009 in Gut. In addition, the serum level of TNF# in CD patients inversely correlates with bone density (Turk et al 2009). The role of inflammatory cytokines and other mediators in bone disease is reviewed by Agrawal (Curr Osteoporos Rep (2011).

Changes in the manuscript

However, also inflammation may significantly contribute to a high-turnover mineral bone loss in CD [14-16]. Osteoclastogenesis and bone resorption is driven by inflammatory mediators such as tumor necrosis factor (TNF) #, Interleukin (IL)-1# and Il-6 in synergy with receptor activator of nuclear factor kappa B ligand (RANKL). RANKL is released by activated T-cells and binds RANK on osteoclastic precursors and thereby induce and activate osteoclasts [17]

Page 5

#8 On page 5 the authors draw a link between disease behavior and NOD2 genotypes, "as NF-B activation induces TNFa, IL-6 and IL-1". The relationship of cause and effect is far from being evident and would remain to be supported by further explanations and literature.

Response

We agree with the reviewer. Our intention was to hypothesise that there might be a link between osteoporosis and an inflammatory phenotype associated with NOD2 variant alleles, but there is no evidence for that. Therefore we changed the sentence.

Changes in the manuscript

Thus a chronic inflammatory state, such as the gut in CD patients, in addition affects bone remodelling and contributes to high turn-over mineral bone loss. The influence of NOD2 genotype on osteoporosis in CD has not been studied yet.

Page 5

#9 On page 5 at the end of the introduction section as the preterit has been used, the term "hypothesized" should be used.
Response

We corrected the sentence and used the preterit.

Changes in the manuscript

We therefore wished to investigate the potential relationship between the three major NOD2/CARD15 variants in a well characterized, long term follow up dataset of Crohn patients with pediatric- and adult-onset and inflammatory phenotype, response to therapy and lower bone mineral density.

Page 5

#10 As there are 85 pediatric and 116 adult-onset CD patients, the term "large dataset" is perhaps excessive.

Response

Indeed, we analyzed an extensive dataset, resulting from long term follow-up of each patient. The term large does not relate to the number of patients.

Changes in the manuscript

well characterized, long term follow up dataset

Page 5

#11 Page 5 in the material and methods section which criteria have been used, the Montreal or the Vienna classification? Moreover it is not clear if phenotypic parameters were those at diagnosis or those at follow-up or both? And if follow-up was performed what was follow-up duration. Did the authors collected data regarding anoperineal lesions that may be regarded as severe phenotype?

Response

We used the Montreal classification as also stated in the heading of Table 1.

Changes in the manuscript

Parameters including age of diagnosis and duration of disease, sex, disease localization, disease behaviour, family history of IBD, body weight and length, body mass index (BMI), extraintestinal involvement, bone mineral density, medical and surgical therapy and disease activity including hospitalization were updated, analyzed and classified according to the Montreal classification. Disease location and behavior was defined as involvement at any time during disease.

Page 6
#11 The authors defined "underweight" using BMI. It would be more logical to define underweight using weight via the weight Z-score determined using the Cole's method. BMI defines "corpulence" and not weight.

Response

The term underweight is used to describe a person whose body weight is considered too low to be healthy. Using weight charts established for the pediatric populations alone is not appropriate to define underweight as it reflects only weight according to age, sex and ethnic group and neglects the height. Thus to determine the ideal weight the child's height should be considered. The definition of underweight in pediatrics refers to children with a BMI below the 10th percentile for age or a weight 15 to 20% below that normal for their age and height group. In Germany we use the first definition and established pediatric BMI charts (see also www.mybmi.de).

Changes in the manuscript

No changes.

#12 What is the relevance of disease activity indexes that reflect acute phenomena rather than the chronic activity of the disease. In the background of such a study integration of the time activity (i.e. area under the curve) would better reflect what the authors try to link with NOD2 genotypes.

Response

Disease activity indexes were measured at several time points during regular and emergency visits as indicated in the materials and methods section on page 6 and indicate not only acute disease activity as the reviewer stated. The mean activity score of several scores for each patient were given to describe disease activity during the observation period. In our opinion this still reflects disease activity over time quite well. As it is a retrospective study with different time intervals we did not calculate disease activity as area under the curve.

Changes in the manuscript

No changes

#12 Page 6 the authors wrote "a hospitalization of more than two weeks per year was also given to describe the percentage of severe ill patients". As they used the term "also", what were the criteria also used to define severe disease? Were accounted only hospitalizations related with CD?

Response

In other studies disease activity was educed from surgery, disease behaviour,
complications and hospitalization. We registered all this factors. Hospitalization was due to CD and may be an additional indicator for a severe disease.

Changes in the manuscript

In children, a hospitalization of more than two weeks per year due to CD was given to describe disease activity and the percentage of severe ill patients.

Page 6

#13 If the notion of Z-score is well known, for a better readability of the paper, the authors should offer at least limited explanations regarding the "T-score".

Response

We followed the suggestion of the reviewer and explain also shortly the T-score.

Changes in the manuscript

Osteoporosis was defined in children as less than two standard deviations (SD) of BMD adjusted to an age matched population (Z-score)[19] and in adults as BMD compared to a young normal reference mean below 2.5 SD (T-score) [20].

Page 6

#14 Page 7, the term "snp" should be in upper case.

Response

Referring to #4 we use now NOD2 variant throughout the manuscript.

Changes in the manuscript

Genotyping of the three main NOD2 variants p.R702W (rs2066844, exon 4), p.G908R (rs2066847, exon 8), and p.1007fs (rs20066847, exon 11) were performed in 85 pediatric and 117 adult CD patients of Caucasian origin.

Page 7

#15 In the statistical analysis section, what is the chi quadrate test? As may be found in the german version of Wikipedia, it seems that it is indeed the chi2 test. If this is true, the authors should use the chi2 test.

Response

I apologize for this mistake. Here, “chi square test” is the correct English term.

Changes in the manuscript

Differences between frequencies of qualitative characteristics in CD patients
carrying NOD2 SNP variants or wild-type were compared using chi-square test or Fishers exact test, respectively.

Page 7

#16 In the statistical analysis section, why the authors use a parametric test (t-test) to compare two groups and a non parametric one (Wilcoxon test) for three groups?

Response

The reviewer is correct. For consistency we now used the nonparametric tests. Mann Whitney test was used to compare the distributions of two unmatched groups, as this test is most sensitive to changes in median. The non-parametric Kruskal-Wallis test was used two compare more than two groups.

Changes in the manuscript

Differences between frequencies of qualitative characteristics in CD patients carrying NOD2 variants or wild-type were compared using chi-square test or Fishers exact test, respectively. Mann-Whitney or Kruskal-Wallis were applied to compare median and T-test to compare mean, respectively.

Page 7

#17 In the statistical analysis section, for descriptive statistics the authors used mean and SD. If parameters under study were not fitting a normal distribution they better use median and interquartile range for descriptive statistics.

Response

The reviewer is correct. We followed the suggestion.

Changes in the manuscript

Descriptive statistics for quantitative values were given as median and interquartile range in box plot diagrams using Graphpad Prism software.

Page 7

#18 In the statistical analysis section, what do the authors mean with "the descriptive impact of our data-set". What was the definition of "chronic active", of "high active course of the disease".

Response

The definitions of chronic and high active course of disease are made on page 6 in the material and methods section. We deleted the multivariant analysis from our manuscript and therefore also from the material and methods section.
Changes in the manuscript

The sentence was completely removed.

#19 On page 8, results section, "retrospective" should be used instead of "retro-perspective".

Response

We followed the suggestion of the reviewer and used the term retrospective throughout the manuscript. However as we changed the Introduction of this paragraph we did not use the term in this context any more.

Changes in the manuscript

Data regarding NOD2 genotype, age of onset, average disease duration, disease location and behavior were obtained from two hundred and two German patients with adequate follow-up and are presented in Table 1 according to the age of onset of CD.

Page 9

#20 On page 8, results section, why "'s" is appended to SNP? The authors wrote that "NOD2 SNP’s were found". As SNP defines a polymorphism and does not imply the wild type rather than the variant allele, the authors should write "SNP variant alleles were found" or "SNP minor alleles were found".

Response

Referring to #4 and #14 we use now NOD2 variant allele throughout the manuscript.

Changes in the manuscript

NOD2 variant alleles were found in 37 out of 85 pediatric- (44%) and 47 out of 117 adult- (40%) onset CD patients (Table 1).

Page 9

#21 In the supplemental table they report alleles counts in pediatric and adult onset patients. There is a great confusion in presenting this table as it is difficult to understand in the No of variant alleles column, pediatric onset sub column, heterozygous sub column, what is the meaning of 31 heterozygous alleles (or patients?) in a column that reports the number of alleles. Briefly an allele is neither heterozygous. The same confusion is noticed for the adult onset part of the table and the homozygous and compound (compound heterozygote should be better) parts of the table. Moreover including % of patients in this table
reporting the number of alleles add more confusion to this confused table.

Response

This fact is understandable. We therefore deleted the supplemental table and reported the number of variant allele carriers in Table 1.

Changes in the manuscript
Removing supplemental table
Adding informations on allele carriers in Table 1

#22 In the results section why the authors precised that only 14 patients were older than 40 at time of diagnosis as this sentence does not seem to be related neither with what is written before nor what is written after.

Response

We agree with the reviewer and deleted the sentence. However, we described the cohort more precisely by adding median and interquartile range in Table 1 for age and age at onset of disease.

Changes in the manuscript
The sentence in the results section was removed.
More data on the age of the cohort was added in Table 1.

#23 The common allele suggesting the wild type one what do the authors mean with the "common NOD2 allele variants"?

Response

As recommended by the reviewer, we wrote more concisely the statement as followed: “three main NOD2 allele variants”.

Changes in the manuscript
No significant differences were noted in the distribution of the three main NOD2 allele variants (data not shown), ...

Page 9

#24 Did the authors compared allelic frequencies between the two groups of patients?

Response
We compared the allelic frequencies between the two groups using chi-square test and added this information in the text.

Changes in the manuscript

… however the presence of two variant alleles trend to be higher in pediatric-onset than in adult-onset patients (p=0.14) (Table 1).

Page 9

#25 The authors wrote that "7.1% of the pediatric-onset patients are homozygous in contrast to only 2.5% and 9.4% compound heterozygous versus 5.9% in the adult-onset group", did they objectived the reality of these differences with a statistical test?

Response

Referring to #24, we objectify the frequencies using chi-square test and indicated the p-value in the text.

Changes in the manuscript

… however the presence of two variant alleles trend to be higher in pediatric-onset than in adult-onset patients (p=0.14) (Table 1).

Page 9

#26 the authors wrote " There was no statistical difference regarding age at diagnosis, chronological age, average disease duration or gender" and refered to table 1. Even when going to table 1 the reader at last understand that there is no difference according to genotype. This should be mentioned in the text.

Response

As recommended by the reviewer we indicate that this statement refers to NOD2 genotype.

Changes in the manuscript

There was no significant difference regarding age at diagnosis, chronological age, average disease duration or gender according to NOD2 genotype (Table 1).

Page 9

#27 In table 1 how the authors explain there are11 NOD2 variant allele carriers and 14 WT NOD2 genotype patients aged between 17 and 40 years in the pediatric onset group? In this table it is not correct to define the NOD2 genotype by the term "SNP".
Response

In order to define the NOD2 genotype we now used main variants vs. wild-type throughout the manuscript and also in Table 1 (see also response to #4, #14 and #20). The age groups were also defined in the heading according to the age of onset (#18 or >18 years). This definition is further defined in the materials and methods section.

Changes in the manuscript

The pediatric-onset cohort included patients with age of onset until the age of 18 and final diagnosis until the age of 19.

Page 5
See Table 1

Page 10

#28 In table 1 are localizations and behaviors at diagnosis or at maximal follow up? Likewise for the EIMs mentioned on page 9.

Response

As recommended by the reviewer we wrote more concisely that disease behaviour covers the whole observation period and indicated this information in materials and methods and in the title of Table 1.

Changes in the manuscript

Disease location and behavior was defined as involvement at any time during disease.

Page 6

Table 1: Characteristics of 202 patients with pediatric- and adult-onset Crohn’s disease according to the Montreal classification[22] regarding NOD2 genotype at latest follow-up.

Page 10

#29 In the "Disease Localization and Behavior of NOD2 SNP Carriers in the Pediatric- and Adult-onset CD Cohort" section page 8, the sentence "NOD2 SNPs were associated...." is incorrect and should be replaced by "patients with at least one NOD2 variant allele...". In the same sentence what is the significance level of the 68% vs 48%?

Response

We followed the suggestion of the reviewer and inserted “at least one NOD2
variant allele”. In addition, we added the significance of the percentages given by a p-value.

Changes in the manuscript

Disease Localization and Behaviour of NOD2 variant Carriers in Pediatric- and Adult-onset CD:

Carrying at least one NOD2 variant allele is associated with an ileocolonic (L3) localization (68% versus 48%; p=0.048) in pediatric- and a trend to an ileal involvement alone in adult-onset CD patients (38% versus 21%; p=0.06), while pediatric-onset NOD2 wild-type patients more frequently had a colonic involvement alone (p=0.048).

Page 10

#30 Likewise the term "NOD2 positive patients" is unclear, positive for what? Using patients with at least one NOD2 variant allele should be more correct. In the same way two and three lines below are used the incorrect sentence "NOD2 SNP pediatric-onset group". Similar abnormal denominations occur throughout the manuscript and should be corrected accordingly.

Response

Referring to #4, #14 and #27 we use NOD2 variant allele throughout the manuscript.

Changes in the manuscript

However, we found chronic active or high active course of disease in CD patients associated with pediatric-onset of disease (p=0.0001) and any of the main NOD2 variant alleles (p=0.0001). The average PCDAI-Score over time was significantly higher in pediatric-onset CD patients with NOD2 variant than wild-type alleles (23.2 versus 14.1; p=0.0008) (figure 2A),...

Page 11

#31 On page 8 the authors wrote "Extraintestinal manifestation, need for surgery, complications and osteoporosis are more frequent in pediatric-onset than in adult-onset CD patients with NOD2 variants but did not reach significance". What must be understood, a suggestive trend of association (p=0.08?) suggesting a putative but undetected (lack of power?) difference, or a true insignificant difference (p=0.4). This is quite different in terms of discussion and hypotheses that may be drawn. The affirmation by the authors that these are "more frequent" is an over interpretation of the data.

Response

We further clarified the significance of our observation regarding osteoporosis
and indicated the p-value. Osteoporosis was significantly associated with patients carrying NOD2 variant alleles and pediatric-onset but did not achieve significance analyzing pediatric-onset patients with NOD2 variant alleles. From our point of view this suggests a putative, but undetected difference due to the sample size in our study, as we did not have BMD measurements from all patients.

Changes in the manuscript

There is no significant influence of the main NOD2 variants in patients with CD towards extraintestinal manifestation, need for surgery or complications.

Osteoporosis was associated with patients carrying NOD2 variants (p=0.020) and pediatric-onset of CD (p=0.023). There was also a suggestive trend for CD patients with NOD2 variant alleles and pediatric-onset towards osteoporosis (p=0.10) (Figure 1).

Page 11

Interestingly, also CD patients carrying NOD2 variant alleles suffer from osteoporosis more frequent. However, may be due to a lack of power we could not prove our hypothesis that osteoporosis is significant more frequent in pediatric-onset CD patients with NOD2 variant alleles.

Discussion, Page 17

#32 in figure 2 why the authors use "growth delay" on the figure "growth failure" in the legend? This is confusing. Where are the bars for growth failure at diagnosis in figure 2?

Response

Growth failure was defined as inappropriate growth velocity for age. Therefore standard-deviations of height according for sex and age at diagnosis and 1y after follow-up were compared. From this follows that there is only one bar indicating inappropriate growth velocity.

Changes in the manuscript

Figure 2 the term growth delay was replaced by growth failure.

#33 On page 10, what is a "wt carrier"? Referring to what is assumed to be a variant allele carrier: a patient having at least one variant allele, a "wt carrier" should be a patient with at least one wt allele. Thus heterozygous genotypes should be included in this term.

Response

According to the reviewers suggestion we clarified the wild-type carrier as a
Changes in the manuscript

The average PCDAI-Score over time was significantly higher in pediatric-onset CD patients with at least one NOD2 variant than two wild-type alleles (23.2 versus 14.1; p=0.0008) (figure 2A), and a chronic active (PCDAI#10 or CDAI#150; p=0.017) or moderate to severe course (PCDAI # 30 or CDAI#220; p=0.027) of disease significantly more frequent.

Page 11

#34 The authors should add on fig 3 that PCDAI scores refers to pediatric onset patients. Likewise for CDAI scores for adult ones.

Response

As recommended by the reviewer we labelled Figure 3 with headings for pediatric and adult-onset.

Changes in the manuscript

Figure 3

#35 Referring to fig 1A the authors claim that BMD Z-score is lower in the pediatric-onset group of patients with NOD2 variant allele. There is no mention of this BMD parameter in fig 1A and no statistical result to support this affirmation. Only osteoporosis is presented in fig 1A and the difference is unsignificant. Thus this affirmation is an over interpretation of the data. Although the text refers to fig 1A about osteopenia, where is osteopenia in fig1A.

Response

In fact, we presented data only for osteoporosis. Thus we changed the sentence and added the statistical results. However, the definition of osteoporosis is based on the Z-score. A Z-score below -2 indicates osteoporosis.

Changes in the manuscript

Osteoporosis was associated with patients carrying NOD2 variant alleles (p=0.020) and pediatric-onset of CD (p=0.023). There was also a suggestive trend for CD patients with NOD2 variant alleles and pediatric-onset towards osteoporosis (p=0.10) (Figure 1).

Page 11

#36 On page 10, where do the new 78 pediatric onset CD patients come from? And what are the significance levels according to their BMD Z-scores, osteoporosis.... parameters?
Response

As this is a retrospective study, BMD measurements were not available for all patients. Regarding the statistics see also response to #35.

Changes in the manuscript

BMD measured by DEXA was available for 77 pediatric- and 91 adult-onset CD patients.

Page 11

#37 In table 2 it is necessary to precise what is the "Z<-1". We hypothesize it is BMD zscore (?) as compared with the neighbour column. However the authors should demonstrate a minimum of rigor in their presentation of the data.

Response

As suggested by the reviewer we specified low BMD as Z-score < -1 and normal BMD as Z-score > -1

Changes in the manuscript

Table 2

Page 13

#38 On page 10 the authors wrote: "...underweight at diagnosis and during follow up...", what do they mean with "during follow up"? Either they report data and patients phenotypes recorded at maximal follow up or if it is during follow up they should precise after which time they recorded the data.

Response

As indicated in the figure we analyzed a one year follow-up regarding underweight. For short status we used the max. follow-up time.

Changes in the manuscript

In addition, underweight at diagnosis (p=0.045) and after 1 year of follow up (p=0.011) was significant associated with low BMD (Table 2). Thus there was no significant difference regarding short stature at the end of the observation period.

Page 12

#39 In figure 5 the authors have pooled the two groups of patients (pediatric and adult onset patients) in order to compare the age at onset according to the genotype at the NOD2 locus. As presented and handled throughout the manuscript these two groups of patients are quite separated and were
investigated separately. These two groups are significantly different regarding a lot of phenotypes and regarding allele frequencies of NOD2 gene variants. Thus they should not be pooled in an analysis. It is very likely that according to the recruitment of these two groups, the age distribution of the pool displays a bimodal distribution that reflects the two groups and thus would demonstrate that (1) pooling is not possible and (2) the non normal distribution of the parameter makes the parametric t-test misfits to compare means. The results presented in figure 5 panel A may only reflects an higher prevalence of NOD2 gene variants in pediatric than in adult onset CD patients. Thus all comparisons reported on figure 5 that rely on age at onset (for the pooled patients) may not be considered.

Response

According to a consistent presentation of our data we changed figure 5. The text in the manuscript was changed accordingly.

We still think that pooling data from pediatric and adult patients is appropriate and scientifically correct. We therefore show former Figure 5 as supplemental figure 1. As previously shown the age distribution varies not significantly between patients carrying any of the main NOD2 variant alleles or homozygous NOD2 wild-type alleles. In addition, the prevalence of NOD2 gene variants differs not significantly between pediatric and adult-onset. We would consider it entirely feasible to pool the data in this case. The result from the comparisons is quite interesting and should not be neglected, as it shows only a difference regarding therapy in patients carrying NOD2 variant alleles with a young age of onset. According to the recommendation of the reviewer we now used the non-parametric Kruskal-Wallis test.

Changes in the manuscript

Comparing therapy and NOD2 genotype revealed divergent trends between pediatric- and adult onset CD. In pediatric-onset CD patients with NOD2 variants steroid dependent or refractory course of disease was more frequent (p=0.048) (Figure 5). In addition, the use of long-term immunosuppressive drugs such as anti-TNF# agents; azathioprine or methotrexate trend to be higher in patients with NOD2 variants and pediatric onset in contrast to adult-onset CD (Figure 5). This is also confirmed by analyzing the relationship between age of diagnosis, response to therapy and NOD2 genotype. A significant age dependent difference towards therapy was only found in CD patients carrying NOD2 variants. CD patients carrying at least one NOD2 variant allele and young age at diagnosis (median age below 20 years) are at risk for steroid dependent or refractory course of disease (p=0.0022), long-term immunosuppressive therapy with AZA or MTX (p=0.0426) and the use of anti-TNF# agents (p=0.0004) (Supplemental Figure 1).

Page 14

#40 In the discussion section the authors report « high prevalence of
NOD2/CARD15 SNPs with 44% in the pediatric- and 42% in adult-onset CD. Were these frequencies significantly different as later the authors wrote that it «...confirms our hypothesis that pediatric-onset Crohn disease has a greater degree of genetic influence». Moreover do these percentages reflect patients or alleles? It is likely that they describe percentages of patients but the text is ambiguous.

Response
The NOD2 variant allele frequency reflects patients involved and not the alleles.

Changes in the manuscript
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% [Lacher, 2010 #19;de Ridder, 2007 #72;Sun, 2003 #82].

Page 15

#41 The authors report in the discussion section that "...homozygosity and compound heterozygosity for NOD2 SNPs was found more frequent in the pediatric--onset CD group as reported by others". Did the authors tried to ascertain this affirmation using a comparison frequency statistical test?

Response
Indeed, we compared the NOD2 variant allele frequency but we did not found a statistical difference between the two groups.

Changes in the manuscript
In addition, the presence of two NOD2 mutant allele was found more frequent in the pediatric-onset CD patients as reported by others [24, 26] and may predict complicated disease [27].

Page 15

#42 How the authors have taken into account the possible phentotypic bias that relies upon the well known fact that endoscopic explorations are often more complete and more frequent in pediatric rather than in adult onset CD patients. A similar bias may have occured in the frequency of CDAI measure frequency.

Response
Indeed the transition of the patients from pediatrics to internal medicine might influence the frequency of investigations. However, the adult CD patients in our
medical centre are well characterized as they take part in several clinical studies. All adult CD patients of our study had a colonoscopy during the last two years and the patients were regularly monitored by sonography, laboratory and clinics. We therefore think that the reviewers’ concerns play only a minor role.

Changes in the manuscript
No changes

Reviewer #2
Reviewer: Maria Francisca Gonzalez-Escribano

Reviewer's report:
In the manuscript entitled “Age of onset dependent influence of NOD2/CARD15 gene variants on disease behaviour and treatment in Crohn #s disease” Posovszky et al. investigated whether the contribution of mutations in NOD2/CARD15 to the phenotype of the disease and extra-intestinal manifestations (osteoporosis) is different in patients with paediatric and adult onset age. To reach their aims, they study the three mutations in NOD2/CARD15 which have been associated with the disease in a cohort of 201 patients: 85 paediatrics and 116 adults. They collected demographic data of patients and also localization, behaviour, activity and other clinical parameters as well as osteopenia and osteoporosis.

The influence of these NOD2 mutations in paediatric CD has been investigated and some of these reports are not mentioned in this manuscript (Russell RK et al J Pediatr Gastroenterol Nutr. 2004 and Shaoul R et al. Dig Dis Sci. 2009). In my opinion, the weakness of this manuscript lies in two points. One is the sample size which is too low for the successive stratifications made. The other problem is the way in which the results are presented, too long and confusing. The manuscript could improve if the authors show only the most relevant results for their objectives in a clearer way.

Response
We thank the reviewer for the constructive criticism. The manuscript has been carefully revised in order to focus on the main objectives. We also cited the suggested publications. Similar to Shaoul et al. we compared pediatric- and adult-onset CD patients and observed the effect of disease duration between genotype and phenotype. The sample size compared to the study of Shaoul et al. is almost the same 232 versus 202 in our cohort. However, the worth of this study is the duration of follow-up, which is more than adequate and 2-folds longer than in the study of Shaoul et al., in which the aim was to describe the effect of disease duration on disease behaviour. There is no other pediatric study achieving such a long follow-up time.
Changes in the manuscript
The complete manuscript was revised.

Other questions:
1. They need to define how they calculate the onset age and what threshold they use to split paediatric and adult onsets.

Response
According to the reviewers recommendation we defined the pediatric-onset cohort in the material and methods section and also in the Table 1.

Changes in the manuscript
The pediatric-onset cohort included patients with age of onset until the age of 18 and final diagnosis until the age of 19.

Page 5
Table 1
2. There are discrepancies concerning numbers in different sections of the manuscript. For example, according with the heading of Table 1, total number of patients is 203, but in the text and inside the Table 1, the authors register a total of 201 patients. In Table 1 and text they say that they have 48 individuals with NOD2 mutations in the adult onset group, nevertheless, according to data in supplementary information this number is 46. The authors should carefully checked all the data included in Tables and text in order to correct these mistakes.

Response
We apologize for these discrepancies concerning the exact number of patients. We carefully checked all numbers and corrected them throughout the manuscript.

Changes in the manuscript
Manuscript
Tables
Figure legends
3. The authors use the term "NOD2 SNPs" throughout the manuscript to refer to individuals bearing NOD2 mutations This is wrong and could be misinterpreted by readers.
Response

We followed the suggestion of the reviewer and did not use the term “NOD2 SNPs” any more. Instead we used “three main NOD2 CD-associated variants”, “NOD2 variant alleles” and for example “patients carrying NOD2 variants…” to refer to individuals bearing mutations in the NOD2 gene.

Changes in the manuscript

Throughout the manuscript

4. According to Table 1, means of age at diagnosis for paediatric patients were 13.9 (range 7-18) for patients with mutations and 12.1 (range 1.18) for patients without any mutation, nevertheless there are 11 and 14 patient included in the A2 stratum (17-40 y) of these groups. Means this that all these patients were 18 years old at diagnosis? In this case, I suggest establish only two groups older and younger than 18 year at diagnosis.

Response

In order to clarify this issue we used the age of onset at diagnosis as described in the materials and methods section and refer to our response to point 1. The table was revised and we followed the reviewer’s recommendation to use only two groups.

Changes in the manuscript

Table 2

5. In my opinion the Figures are not necessaries. Data of Figure 1 could be included in Table 1, data of Figure 2 in Table 2 and the rest are included or could be included in the text.

Response

We appreciate the opinion of the reviewer. To our opinion, Figure 1 has the advantage to visualize the divergent disease behaviour of pediatric- and adult-onset CD and shows a trend of more severe disease behaviour in patients carrying NOD2 variant alleles. However, we included the p-values for each aspect. Data of Figure 2 are only collected for pediatric-onset CD patients to describe the influence of NOD2 genotype towards growth, underweight and hospitalization and do not fit very well into Table 1. In Table 2 we included these data already regarding BMD.

Changes in the manuscript

No changes in the manuscript.
6. Some data probably interesting are not shown for example data of BMD in patients with homozygous status for NOD2 mutations.

Response

As suggested by the reviewer we added some more information on the distribution of NOD2 variant alleles in Table 2.

Changes in the manuscript

Table 2

7. Table in supplementary information, patients should be split in single dose (heterozygous) and double doses (homozygous and compound heterozygous). These data could be included in Tables 1 and 2. The data relating to the specific mutations could be left as supplementary information.

Response

According to the reviewer’s recommendation we split the information into single and double doses and added this information into table 1. The data relating to the specific mutations are left as supplementary information.

Changes in the manuscript

Table 1

Supplemental Table

Reviewer #3

Reviewer: silvia vidal Reviewer's report:

In the present manuscript, authors analyzed the association between the presence of NOD2 variants and clinical phenotype of pediatric onset Crohn's disease patients. They particularly focused on osteoporosis and growth failure because delayed growth is a well established feature of pediatric onset CD.

NOD2 variants have already been associated with markers of disease severity and specific phenotypes (ileocolitis) in other pediatric-onset CD cohorts. Other authors have reported that NOD2 genotype was not correlated with growth retardation or growth failure. Lower mass index and higher PCDAI have also been reported in patients with NOD2 variants.

Some of the associations have already been published in other cohorts. However, the originality of the manuscript is based on the association between bone mass density/osteoporosis and NOD2 variants. The number of patients was not enough for a complete study of each three NOD2 variants. However,
grouping the three NOD2 variants allowed them to reach significant conclusions.

In order to improve the quality and rigor of the manuscript, I would like to suggest the following comments:

Major compulsory revisions:

1) Authors indicated differences between carriers of NOD2 variants and wild type patients in the text results. However, some of these differences were not statistically significant (underweight at time of diagnosis, longer duration,..).

Response

According to the reviewer’s objection is correct. We revised the text to clarify the statistical differences.

Changes in the manuscript

There is no significant influence of the main NOD2 variants in patients with CD towards extraintestinal manifestation, need for surgery or complications. However, we found chronic active or high active course of disease in CD patients associated with pediatric-onset of disease (p=0.0001) and any of the main NOD2 variant alleles (p=0.0001).

Page 11

2) According to the legend of Figure 2, BMI was only available from 49 of patients. How many were wild type or NOD2 variant? There were visible differences in the graph but they were not statistically significant. A very small number of patients in one of the groups could explain this apparent contradiction.

Response

To refute the argument of the reviewer, the distribution in both groups is very similar to the pediatric-onset group. 39% of the patients carry NOD2 allele variants (20 versus 31). There is a suggestive trend (p=0.15) for underweight at diagnosis, but due to lack of power, this did not reach significance. From our point of view this suggests a putative, but undetected difference due to the sample size regarding this data, as we did not have BMI measurements from all patients at diagnosis.

Changes in the manuscript

BMI at diagnosis and growth delay at one-year follow-up was available from 51 out of 85 patients (39% NOD2 variant allele carriers).

Figure legend 2

3) The authors have not clarified the impact of NOD2 polymorphisms towards
osteoporosis. They have only analyzed the impact of several clinical aspects in BMD, regardless of NOD2 (page 10).

Response
We followed the recommendation of the reviewer and added more information on the impact of NOD2 variant alleles towards osteoporosis and put more focus on this observation throughout the manuscript.

Changes in the manuscript
Interestingly, osteoporosis was found more frequently in patients carrying NOD2 variants (p=0.033), which was more pronounced in pediatric-onset CD (p=0.12).

Abstract
Osteoporosis was associated with patients carrying NOD2 variant alleles (p=0.033) and pediatric-onset of CD (p=0.022). There was also a suggestive trend for CD patients with NOD2 variant alleles and pediatric-onset towards osteoporosis (p=0.12) (Figure 1).

Results section, Page 11
Interestingly, also CD patients carrying NOD2 variant alleles suffer from osteoporosis more frequent. However, may be due to a lack of power we could not prove our hypothesis that osteoporosis is significantly more frequent in pediatric-onset CD patients with NOD2 variant alleles.

Discussion, Page 17
Table 2
4) In Figure 5, response to therapy was evaluated according to age onset. However, the analysis in the other figures (other clinical parameters) was the % of pediatric or adult onset patients. Two different methods of analysis could complicate the interpretation of results. I would suggest a similar evaluation for therapy (separating pediatric and adult onset).

Response
As suggested by the reviewer we presented the data consistently in percent of pediatric- or adult-onset CD. The result from the age of onset comparison is quite interesting and should not be neglected, as it shows only a difference regarding therapy in patients carrying NOD2 variant alleles with a young age of onset. Thus the former figure 5 is now added as supplemental Figure 1.

Changes in the manuscript
Figure 5
Minor essential revisions: Table 1) at the title of the Table, authors indicated 203 patients instead of 201 Table 2) some results were expressed differently. i.e. 22(35%) and 25/34%

Response

We apologize for these discrepancies concerning the exact number of patients. We carefully checked all numbers and corrected them throughout the manuscript. The results are expressed uniformly in Table 1 and 2.

Changes in the manuscript

Throughout the manuscript

Title of table 1

Table 1 and 2

Figure 1) Authors should indicate at the figure (not only legend) which graph is pediatric or adult onset (it would facilitate the comprehension)

Discretionary revisions: Discussion could be reduced

Response

We followed the suggestion of the reviewer and indicated pediatric- or adult-onset in the Figures.

Changes in the manuscript

Figure 1

Figure 5