Is there an association of CARD15 variants with allergy?

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An association of functional CARD 15 polymorphism and allergy has been described recently in this journal [1]. This paper has been widely cited [2] and is promoted as one of the main outcomes of the German National Genome Research Network I (http://www.ngfn.de/22_190.htm). In this study three SNPs previously found to be associated with Crohn´s disease are examined for association with allergy. These are SNP8 (akin 2104C/T, 2023C/T, Arg675Trp, Arg702Trp, R702W, rs2066844), SP12 (akin 2722G/C, 2641G/C, Gly881Arg, Gly908Arg, G908R, rs2066845) and SNP13 (akin 3020-/C deletion, 2936 C insertion, L1007fsinsC, 980/981 frameshift) [3], [4]. While genotyping several CARD15 SNPs in our own family sample we noted several errors, inconsistencies and omissions in the primary report [1].

Laboratory Methods. There is no information available about DNA extraction procedures, re-identification of samples, quality control, pre-amplification, details of PCR reaction, size of restriction fragments and scoring of genotypes.

The SNP8 assay seems to include a MspI restriction site at the forward primer which could interfere with cutting the amplified fragment. The SNP12 assay has identical forward and backward primer and would not lead to any amplification. The assay for SNP13 has a poor design as it does not include any control site to ensure enzyme activity.

Neither results of duplicate sample testing nor results of Hardy-Weinberg equilibrium are being reported. There is also no rationale given why only half of the Dresden samples are being genotyped. According to table I and II only 81% of genotyping attempts were successful while it is known that high genotyping failure rates is leading to false positive associations [5]. Even if the noted errors in the primer sequence may be attributed to simple typing errors, this still raises doubts about the validity of the laboratory procedures.

Analysis. A control group created by the absence of any genetic variant seems to inflate risks if during the following step the presence of a particular genetic variant is being tested against this control group. It may even be argued that the calculation of relative risks would be more appropriate than that of odds ratios (resulting in considerable lower effect estimates). Without any detailed information of phenotypic and genotypic details of the control group, effects are hard to understand and might be also a reason why this definition of controls has been abandoned in further analysis of this population [6]. The 1:2 matching of "supernormal" controls in the consecutive analysis [6] is also questionable as selecting individuals with certain homogenous levels of confounders imposes restrictions of the analysis. With the absence of "normal" controls even a more severe bias may be introduced.

No information is given on the prevalence of Crohn´s disease in this sample although this would have been a unique opportunity to verify the initial findings [7] in an independent patient based cohort.

Results. The method section details the estimation of haplotypes but results are being omitted. Instead of haplotypes associations, the risk of co-occurrence of any two SNPs is reported which increase from 3.16 to 4.64. This result is considerably lower from what is being reported in the Crohn´s literature [8]. Neither results of separate allelic and genotypic analysis are being reported and -as only significant risk estimates are given in table III- it is impossible to judge about any possible dose-response effect.

The risks are also not adjusted for strong confounders like season and there is no assessment of goodness-of-fit of the models used [9] which further undermines the validity of this study.

The numbers in table III are confusing: The legend in table III refers to

a total sample of 1873 children while introduction and methods reports 1872; row numbers in table III do not exceed 1805 and column numbers do not exceed 1765 individuals. Table I, II and III data do not match: For example 8,6% of 1161 genotyped children in table I have atopic rhinitis (may be rounded to N=100) plus 9,2% of 711 genotyped children in table II (may be rounded to N=65) which does not add to N=154 genotyped children with atopic rhinitis (table III). Similar restrictions apply to all other traits tested.

A sample of 1872 children is reported in another paper to originate also from Leipzig and not only from Munich and Dresden [6]. Other reports on the same collection report more than 3,000 samples [10], a number in the same range as here [11], but also less than half of the sample size [12], [13], [14], [15], [16], [17].

The results section of the current paper report that "In Dresden, [….] polymorphism T2104 was also associated with atopic rhinitis to a lesser degree [than in Munich] (16,9% vs 7.6%; OR 2,43; 95% CI 1,24 to 4,78; P<.05)". Results for Munich are not given, but the overall result for C2104T and atopic rhinitis in table III may indicate that the above sentence should read to a *higher* degree.

In the results section the allele frequency of SNP8 is reported to be 5,6%, SNP12 of 2,0% and SNP13 of 3,8%. In contrast percentages calculated from column 1 of table III give 11,2% for SNP8, 4,0% for SNP12 and 7,3% for SNP13.

No significant linkage disequilibrium was observed between the examined SNPs. It is unclear why only homozygous subjects were included for this procedure. As linkage disequilibrium results seems to also unlikely in another paper from the same group [18] this might refer to outstanding problems in the genetics module of SAS version 8.2 used by the authors (SAS notes SN-011039 and SN-008611). Linkage

disequilibrium is not reported correctly if at least one haplotype frequency for a marker pair is estimated to be greater than the frequency of either allele. Another another error may be introduced if there are individuals with all missing alleles as already noted above.

The moderate increase of total IgE probably does not indicate a "higher severity of atopy" [1]. The difference between mean 186,6 IU/ml and 312,1 IU/ml reflect less than the transition between 70th and 80th percentile as may be assumed from another paper [6]. IgE is a laboratory value that may be influenced also by other reasons and is otherwise not used as severity marker of atopy.

Sources of bias. There is no information if the difference observed between the study centres is caused by population stratification (which could have been tested with anonymous marker and been completely avoided by using family-based samples). This is expected to be a particular problem as the authors reported a much lower prevalence of allergy of the Turkish minority in the Munich study center [19] that have a considerable different genetic background (unpublished own observation during the Genetic Analysis Workshop 11/2000). Was ancestry defined by passport or by self-reported affiliation? How were probands with mixed ancestry treated? Mild stratification might exist also in less admixed populations when looking for alleles with modest disease effect [5]. Which steps have been taken to ensure that controls are non-cases [9]?

References. There are numerous referencing errors and misunderstandings: Fig. 1 locates SNP12 in the 6th LRR and SNP13 in the 9th LRR. According to Ogura [7] SNP12 resides in the 7th LRR and SNP13 in the 10th LRR. Table I and II footnotes refers to reference 8 which belongs to another topic. Reference 10 cited in the methods is misleading as it relates to a different skin prick test device. Reference 14 is used to show that impaired LPS recognition by NOD2

polymorphism reduces the capability to interact with bacteria and to develop a T_{reg} reservoir. Unfortunately this is not the content of their reference 14: "NOD" denotes "non-obese diabetic mice" and the review discusses helminth (but not bacterial) effects on T cells. CARD15 gene is also not located in the pericentromeric region of chromosome 16 (which would be q11.1) but on the cytogenetic band q12.1. There exists also a 12 exon isoform of CARD15 [20] (and not only the reported 11 exon form).

Even more important is the omission, that at the time of the study 67 (and not only the reported 13) polymorphism have been known [20]. Linkage disequilibrium with untyped SNPs could therefore confound the current analysis.

Interpretation. It is hard to understand why SNP8 and SNP12 impose the highest risk for atopic rhinitis while the risk for Crohn´s disease comes mainly with SNP13 [8]. Why is the excess risk for atopic rhinitis not found with the underlying biological traits? Although not being discussed in the paper, this could point towards chance effects introduced by multiple testing [21].

The overall number of tests performed is not given in the paper. If we assume, however, 6 traits (total IgE, number of skin prick tests, atopy, atopic dermatitis, atopic rhinitis, asthma), tested in 3 groups (total and 2 subgroups), 4 series (as single SNP plus all combinations of 2 SNPs) and assume their "best" p value from table III to be 0,001 (an exact estimate is not given), the Bonferoni corrected p value would be $p_{corr}= 1-(1-0{,}001)^{6*3*4} = 0{,}0695$ which is above conventional standards.

There seem to be misunderstandings on the role and function of CARD15. In my opinion the main result of CARD15 activation is not so much apoptosis but infection control by activating the adaptive

immune system [22]; its function is also not so much sensing of endotoxin (LPS) from gram negative bacteria but peptidoglycan (PGN) of practically all bacteria [23], [22]. The authors discuss only in part the apparent paradox at the time of submission that lacking the entire LRR region resulted in enhanced NF-κB activity whereas the frameshift mutation by SNP13 resulted in low NF-κB levels [24] (for an updated discussion see [25]). Protein truncation of the most terminal C-terminal LRRs of CARD15 lead to an unresponsiveness to bacterial components but leaves CARD15 still able to activate NF-κB at a level comparable to that of the wild-type protein [26]. An antagonistic effect of these SNPs is therefore possible [23]. It is difficult, however, to follow any further discussion [1] as the authors even mix up the amino acid and genomic positions of SNP8 and SNP12 (which was otherwise correctly denoted in their figure) and assume that SNP8 leads to reduced NF-κB activity.

In a more general view, it does not seem to be adequate to make any conclusions about causal interference from the statistical association in one study as the authors repeatedly do [27]. There are many known fallacies with such an approach [28], [29] and imposes a particular problem in a field where most studies are never reproduced [30]. The main factors accounted so far for non replication are inadequate statistical power, biased analysis and selective reporting [9]. Further criteria for meaningful associations modified after [31] are: (a) functional importance of the tested protein with the trait of interest (b) functional importance of the mutation (c) genetic background and interaction with other genes (d) time of onset of functional change and interaction with relevant pathway (e) interaction with the environment and (f) the existence of alternative pathways. None of these points are being examined in the current study.

The authors conclude in the last sentence of their abstract that “The shared genetic background between Crohn’s disease and atopy may

indicate that an impaired recognition of microbial exposures results in an insufficient downregulation of excessive immune responses, giving rise to either T_H2 dominated allergies or T_H1 related Crohn's disease." This seems to be unwarranted: The authors have not examined the genetic background (genomewide association studies are still out of reach) but association of a few gene variants. They have neither examined any patient with Crohn's disease nor the process how microbes are recognized. Even if we follow their conclusion, how can a shared mutation give rise to either T_H2 dominated allergies or T_H1 related Crohn's disease?

History. A first report of this study published as a poster at the American Thoracic Society Meeting in Atlanta May, 2002 included 528 children and found that there is "No association between polymorphisms in the NOD2 gene and atopic phenotypes". The results changed with the target sample of 1872 children at the NGFN meeting in Berlin, November 2002, where the authors reported "allele C2722 had a more than 3-fold risk to develop allergic rhinitis ($p<0.0001$) and an almost 2-fold risk for atopic dermatitis ($p<0.01$)". The current paper was submitted in Sept 2002 and report a 10-fold higher p-value, e.g. $p < 0.001$ for allergic rhinitis and 5-fold higher p-value, e.g. $p<0.05$ for atopic dermatitis. A third abstract submitted three months after the current paper to the European Respiratory Society in Vienna, Oct 2003, reported again the lower p-values of $p<0.0001$ and $p<0.01$, respectively. Although there was never an association of CARD15 variants with asthma, this study is now being cited by the same group that "mutations in the related gene NOD2 have been shown to predispose to Crohn´s disease (…) as well as to asthma (…)" [6] or again with "asthma, atopy, total Ig E, atopic rhinitis and asthma" [27]. This association now even changes to be "associated with the development and severity of atopic diseases and airway hyper-reactivity" [32] where neither development, nor severity of atopic diseases nor hyperreactivity was tested here.

Omissions. Why do the authors ignore all genomewide linkage studies conducted over the past decade? It would also be interesting to know why the authors omitted the existence of the comprehensive Innate Immunity Net genotyping results of CARD 15 published in the Internet on May 6, 2002 before the submission of their own article (http://innateimmunity.net/IIPGA/IIPGASNPs/IIPGA2/PGAs/InnateImmunity/CARD15/ADsas) and known to the authors [6]. There are no acknowledgments and the list of authors does not match the list of principal investigators (http://medweb.uni-muenster.de/institute/epi/forschung/index.php). Funding sources are also incomplete as the NGFN funding did not start before 2001 (http://www.ngfn.de/15_102.htm).

Editorial problems. There are numerous meaningless and inaccurate statements ("putative amino acid exchange"). Editing errors like double author names in the references and typing errors disrupt the text. The commercial IgE assay "Insulite" which is the main laboratory outcome of this study should probably read "Immulite". This error is particular interesting as it allows to trace this text block to several other papers. The lengthy description of children that never participated in this study is superfluous as well as the discussion of functional properties of CARD 15 that have not been examined here.

Ethics. It is an open question how study methods in 2002 could have been reviewed by an ethics committee more than 7 years before (a paper on CARD 4 variants in the same population reports different ethics committees consulted for this study [6]). As the authors describe variants with a 17-fold risk for Crohn's disease , it would be interesting to know if and how children and parents have been informed on these results.

Post Scriptum. Is there an association of CARD15 with allergy? I don´t know. Unfortunately any criticism of a published paper affects all

collaborators and the scientific network (who is loosing credibility), department heads (who do not establish proper control mechanisms), participating subjects (who would not have consented to such a study), funding agencies (who are loosing their money), journal editors and reviewer (who do not follow accepted standards), colleagues that cite this paper (as it shows that they have not read it) and finally to the whistle blower (who experiences moral pressures). Also others raised doubts about results from this laboratory [18] but I think this is more a general problem of current biomedical research that is centered on impact points [9],[33]. Non-reproducibility of studies is a fundamental problem in this field where genetic association is becoming a dirty word [21]. While in the beginning researchers have directly been accused of having falsified data [34] genetic heterogeneity is now assumed to be responsible for non-reproducibility. I am more inclined to think of a complex mélange where improper study design and poor methods result in unwarranted conclusions. Although there are several checklists available to ensure minimum quality [9],[35] there seems to be an increasing number of reports where the peer-review failed.

References

1. M Kabesch, W Peters, D Carr, W Leupold, SK Weiland, E von Mutius: **Association between polymorphisms in caspase recruitment domain containing protein 15 and allergy in two German populations**. *J Allergy Clin Immunol* 2003, **111**:813-7.

2. W Cookson: **The immunogenetics of asthma and eczema: a new focus on the epithelium**. *Nat Rev Immunol* 2004, **4**:978-88.

3. J Hampe, A Cuthbert, PJ Croucher, MM Mirza, S Mascheretti, S Fisher, H Frenzel, K King, A Hasselmeyer, AJ MacPherson, et al:

Association between insertion mutation in NOD2 gene and Crohn's disease in German and British populations. *Lancet* 2001, **357**:1925-8.

4. J Hampe, J Grebe, S Nikolaus, C Solberg, PJ Croucher, S Mascheretti, J Jahnsen, B Moum, B Klump, M Krawczak, et al: **Association of NOD2 (CARD 15) genotype with clinical course of Crohn's disease: a cohort study**. *Lancet* 2002, **359**:1661-5.

5. JN Hirschhorn, MJ Daly: **Genome-wide association studies for common diseases and complex traits**. *Nat Rev Genet* 2005, **6**:95-108.

6. P Hysi, M Kabesch, MF Moffatt, M Schedel, D Carr, Y Zhang, B Boardman, E von Mutius, SK Weiland, W Leupold, et al: **NOD1 variation, Immunoglobulin E, and asthma**. *Hum Mol Genet; online preview Feb 17, 2005* 2005.

7. Y Ogura, DK Bonen, N Inohara, DL Nicolae, FF Chen, R Ramos, H Britton, T Moran, R Karaliuskas, RH Duerr, et al: **A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease**. *Nature* 2001, **411**:603-6.

8. M Economou, TA Trikalinos, KT Loizou, EV Tsianos, JP Ioannidis: **Differential effects of NOD2 variants on Crohn's disease risk and phenotype in diverse populations: a metaanalysis**. *Am J Gastroenterol* 2004, **99**:2393-404.

9. J Little, L Bradley, MS Bray, M Clyne, J Dorman, DL Ellsworth, J Hanson, M Khoury, J Lau, TR O'Brien, et al: **Reporting, appraising, and integrating data on genotype prevalence and gene-disease associations**. *Am J Epidemiol* 2002, **156**:300-10.

10. M Kabesch, C Hoefler, D Carr, W Leupold, SK Weiland, E von Mutius: **Glutathione S transferase deficiency and passive smoking increase childhood asthma**. *Thorax* 2004, **59**:569-73.

11. M Kabesch, K Hasemann, V Schickinger, I Tzotcheva, A Bohnert, D Carr, M Baldini, H Hackstein, W Leupold, SK Weiland, et al: **A promoter polymorphism in the CD14 gene is associated with elevated levels of soluble CD14 but not with IgE or atopic diseases**. *Allergy* 2004, **59**:520-5.

12. M Kabesch, D Carr, SK Weiland, E von Mutius: **Association between polymorphisms in serine protease inhibitor, kazal type 5 and asthma phenotypes in a large German population sample**. *Clin Exp Allergy* 2004, **34**:340-5.

13. PE Graves, M Kabesch, M Halonen, CJ Holberg, M Baldini, C

Fritzsch, SK Weiland, RP Erickson, E von Mutius, FD Martinez: **A cluster of seven tightly linked polymorphisms in the IL-13 gene is associated with total serum IgE levels in three populations of white children**. *J Allergy Clin Immunol* 2000, **105**:506-13.

14. M Schedel, D Carr, N Klopp, B Woitsch, T Illig, D Stachel, I Schmid, C Fritzsch, SK Weiland, E von Mutius, et al: **A signal transducer and activator of transcription 6 haplotype influences the regulation of serum IgE levels**. *J Allergy Clin Immunol* 2004, **114**:1100-5.

15. B Woitsch, D Carr, D Stachel, I Schmid, SK Weiland, C Fritzsch, E von Mutius, M Kabesch: **A comprehensive analysis of interleukin-4 receptor polymorphisms and their association with atopy and IgE regulation in childhood**. *Int Arch Allergy Immunol* 2004, **135**:319-24.

16. M Kabesch, I Tzotcheva, D Carr, C Hofler, SK Weiland, C Fritzsch, E von Mutius, FD Martinez: **A complete screening of the IL4 gene: novel polymorphisms and their association with asthma and IgE in childhood**. *J Allergy Clin Immunol* 2003, **112**:893-8.

17. M Allen, A Heinzmann, E Noguchi, G Abecasis, J Broxholme, CP Ponting, S Bhattacharyya, J Tinsley, Y Zhang, R Holt, et al: **Positional cloning of a novel gene influencing asthma from chromosome 2q14**. *Nat Genet* 2003, **35**:258-63.

18. MJ Basehore, TD Howard, LA Lange, WC Moore, GA Hawkins, PL Marshik, MS Harkins, DA Meyers, ER Bleecker: **A comprehensive evaluation of IL4 variants in ethnically diverse populations: association of total serum IgE levels and asthma in white subjects**. *J Allergy Clin Immunol* 2004, **114**:80-7.

19. M Kabesch, W Schaal, T Nicolai, E von Mutius: **Lower prevalence of asthma and atopy in Turkish children living in Germany**. *Eur Respir J* 1999, **13**:577-82.

20. S Lesage, H Zouali, JP Cezard, JF Colombel, J Belaiche, S Almer, C Tysk, C O'Morain, M Gassull, V Binder, et al: **CARD15/NOD2 mutational analysis and genotype-phenotype correlation in 612 patients with inflammatory bowel disease**. *Am J Hum Genet* 2002, **70**:845-57.

21. Editorial: **In search of genetic precision**. *The Lancet* 2003, **361**:357.

22. KS Kobayashi, M Chamaillard, Y Ogura, O Henegariu, N Inohara, G Nunez, RA Flavell: **Nod2-dependent regulation of innate and**

adaptive immunity in the intestinal tract. *Science* 2005, **307**:731-4.

23. M Chamaillard, D Philpott, SE Girardin, H Zouali, S Lesage, F Chareyre, TH Bui, M Giovannini, U Zaehringer, V Penard-Lacronique, et al: **Gene-environment interaction modulated by allelic heterogeneity in inflammatory diseases**. *Proc Natl Acad Sci U S A* 2003, **100**:3455-60.

24. DP McGovern, DA van Heel, T Ahmad, DP Jewell: **NOD2 (CARD15), the first susceptibility gene for Crohn's disease**. *Gut* 2001, **49**:752-4.

25. S Maeda, LC Hsu, H Liu, LA Bankston, M Iimura, MF Kagnoff, L Eckmann, M Karin: **Nod2 mutation in Crohn's disease potentiates NF-kappaB activity and IL-1beta processing**. *Science* 2005, **307**:734-8.

26. T Tanabe, M Chamaillard, Y Ogura, L Zhu, S Qiu, J Masumoto, P Ghosh, A Moran, MM Predergast, G Tromp, et al: **Regulatory regions and critical residues of NOD2 involved in muramyl dipeptide recognition**. *Embo J* 2004, **23**:1587-1597.

27. M Kabesch: **Bald Gentests für (potentielle) Allergiker?** *MMW Fortschr Med* 2004, **146**:1017-1020.

28. P Skrabanek, McCormick, J: **Follies and Fallacies in Medicine**. *Prometheus, Buffalo* 1990.

29. S Milloy: **Science without sense**. *Cato Institute, Washington* 1995.

30. JN Hirschhorn, K Lohmueller, E Byrne, K Hirschhorn: **A comprehensive review of genetic association studies**. *Genet Med* 2002, **4**:45-61.

31. P Vineis, P Schulte, AJ McMichael: **Misconceptions about the use of genetic tests in populations**. *Lancet* 2001, **357**:709-12.

32. M Kabesch, RP Lauener: **Why Old McDonald had a farm but no allergies: genes, environments, and the hygiene hypothesis**. *J Leukoc Biol* 2004, **75**:383-7.

33. E Garcia-Berthou, C Alcaraz: **Incongruence between test statistics and P values in medical papers**. *BMC Med Res Methodol* 2004, **4**:13.

34. W Cookson: **Die Jagd nach den Genen**. *VCH Wiley* 2000.

35. ST Weiss: **Association studies in asthma genetics**. *Am J Respir Crit Care Med* 2001, **164**:2014-5.