Genotyping for NOD2 Genetic Variants and Crohn Disease: a Metaanalysis
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BACKGROUND: Arg702Trp, Gly908Arg, and Leu1007fsinsC variants of the NOD2 gene (nucleotide-binding oligomerization domain containing 2; alias, CARD15) influence the risk of Crohn disease. METHODS: We conducted a systematic review to examine whether Arg702Trp, Gly908Arg, and Leu1007fsinsC are equally important risk factors for Crohn disease. In addition, we used studies for which combined information from all genotypes was available to compare risks in simple heterozygotes, compound heterozygotes, and homozygotes. PubMed, EMBASE, and Web of Science were searched. Seventy-five articles (18 727 cases and 17 102 controls) met the inclusion criteria and contributed data to the metaanalyses.

RESULTS: The odds ratios per allele for Crohn disease were 2.2 (95% CI, 2.0–2.5) for Arg702Trp, 2.6 (2.2–2.9) for Gly908Arg, and 3.8 (3.4–4.3) for Leu1007fsinsC (z-test results: Arg702Trp vs Gly908Arg, P = 0.03; Arg702Trp vs Leu1007fsinsC, P < 0.001; Gly908Arg vs Leu1007fsinsC, P < 0.001). When all 3 genotypes were combined, odds ratios for Crohn disease were 2.4 (95% CI, 2.0–2.8) for simple heterozygotes, 9.0 (6.0–13.5) for compound heterozygotes, and 6.7 (4.1–10.9) for homozygotes, compared with noncarriers (z-test results: simple heterozygotes vs compound heterozygotes, P < 0.001; simple heterozygotes vs homozygotes, P < 0.001; compound heterozygotes vs homozygotes, P = 0.18).

CONCLUSIONS: The per-allele risk of Crohn disease was markedly higher for Leu1007fsinsC than for Arg702Trp and Gly908Arg. Combining all genotypes revealed the risks of Crohn disease for compound heterozygotes and homozygotes to be similar and markedly higher than for simple heterozygotes.

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Materials and Methods

See the Supplemental Methods in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol55/issue11.

DATA SOURCES AND SEARCHES
Studies of the NOD2 variants Arg702Trp, Gly908Arg, and Leu1007fsinsC, and the risk of Crohn disease and/or ulcerative colitis published before May 25, 2009, were identified through computer-based searches of PubMed, EMBASE, and Web of Science. The key words and search strategy used were as follows: (NOD2 [All Fields] OR CARD15 [All Fields] OR SNP8 [All Fields] OR Arg702Trp [All Fields] OR 702Trp [All Fields] OR R702W [All Fields] OR SNP12 [All Fields] OR Gly908Arg [All Fields] OR G908R [All Fields] OR SNP13 [All Fields] OR 3020insC [All Fields] OR Leu1007fsinsC [All Fields]) AND (inflammatory bowel diseases [MeSH Terms] OR (crohn disease [MeSH Terms] OR crohn disease [All Fields] OR (colitis, ulcerative [MeSH Terms] OR ulcerative colitis [All Fields]))). The search was limited to humans. There was no language restriction. We used the “related articles” function from PubMed to broaden the search, and we reviewed all abstracts, studies, and citations that were scanned. The computer-based search was supplemented by hand searching the reference lists of articles and review articles identified in the computer-assisted search. Furthermore, if tabular data were insufficient or if we suspected overlap between studies, we attempted to contact the authors by e-mail, fax, and/or phone; we attempted contact up to 3 times if we did not receive answers after the first or second attempt.

STUDY SELECTION
See the flow diagram in Fig. 1 in the online Data Supplement for our method of selecting studies for inclusion.

INCLUSION CRITERIA
We included studies with available allele and/or genotype frequencies for NOD2 variants Arg702Trp, Gly908Arg, and Leu1007fsinsC in both cases and controls. Control individuals were eligible for inclusion if they were free of inflammatory bowel disease, were unrelated to case individuals, and were of the same ethnic origin as the cases. In each meta analysis, each case and control counted only once. When 2 or more studies were reported by the same institution and/or authors, or if cases were recruited from the same hospital/department, we contacted the authors to clarify whether the data in the studies overlapped. Only if there was no overlap among the cases from the different studies were all cases included. To avoid duplication among overlapping studies (according to the authors we contacted), we retained the largest and best-quality study and excluded the others. If 2 or more studies used the same control sample, the controls were partitioned, and each control was considered in only 1 of the studies. All ethnicities were included.

EXCLUSION CRITERIA
We excluded studies if (a) it was impossible to extract or calculate allele or genotype frequencies for NOD2 (alias, CARD15) genetic variants from the published results and attempts at correspondence with the authors failed, or (b) none of the 3 NOD2 genetic variants were found in the cases or controls (in these situations, calculation of a risk estimate is not possible). For more-detailed information, see Table 1 in the online Data Supplement.

DATA EXTRACTION
Two reviewers (S. Yazdanyar and M. Weischer) independently extracted the following from each study: first author; year of publication; journal; study design; familial history of inflammatory bowel disease; genotyping method; numbers of cases and controls; country; ethnicity; recruitment of cases and controls; frequencies of NOD2 Arg702Trp, Gly908Arg, and Leu1007fsinsC alleles and genotypes in cases and controls; sex; age at diagnosis; age at study entry; and numbers of simple heterozygotes (without heterozygosity for the 2 other variants), compound heterozygotes, and homozygotes for cases and controls genotyped for each of the 3 NOD2 variants. In case of disagreement between the 2 reviewers, the third author (B.G. Nordestgaard) conducted a further assessment of the report.

QUALITY ASSESSMENT
The quality of the studies was assessed with the Newcastle–Ottawa Scale (6), with some modifications to match the needs of this study. The quality of the studies was evaluated according to 5 quality criteria: (a) adequacy of case definition (fulfilled if the definition was independently validated according to international criteria for inflammatory bowel disease, partially fulfilled if the definition was based on self-reports that were subsequently evaluated by medical doctors, and not fulfilled if no validation of the diagnosis was performed); (b) representativeness of cases (fulfilled if cases were consecutive, partially fulfilled if cases were only sporadic/familial, and not fulfilled if recruitment was based on selective factors); (c) selection of controls (fulfilled if controls were representative for the general population, partially fulfilled if controls were hospital based or were blood donors, and not fulfilled if controls were derived from a population other than that of
of controls (fulfilled if controls had no history of inflammatory bowel disease and not fulfilled if controls had a history or new occurrence of inflammatory bowel disease); and (e) genotyping method among cases and controls (fulfilled if the same genotyping method was used for both cases and controls, and not fulfilled if 2 different methods were used for cases and controls). Studies achieving 3 or more fulfilled criteria were considered to be of higher quality.

STATISTICAL ANALYSES

The statistical analyses were performed in line with recommendations from the Human Genome Epidemiology Network (HuGENet) guidelines (7). We performed all metaanalyses with STATA statistical software (version 9.2; StataCorp) and used the software’s metan command, which automatically corrects for the zero cell problem (i.e., when 1 arm of a $2 \times 2$ table of a study contains no events) by adding 0.5 to each cell. However, if no $NOD2$ genetic variants occurred in either cases or controls in a study, then any measure of effect summarized as a ratio was undefined, and the study was automatically excluded from the metaanalysis. The Hardy–Weinberg equilibrium for genotype frequencies was checked in the control groups of each study.

We used Mantel–Haentzel statistics to calculate odds ratios as fixed- and random-effects measures. In the report itself, we use only random-effects models for all summary estimates presented in the figures, whereas we show both fixed- and random-effects estimates in the figures in the online Data Supplement. We also calculated 95% CIs for the aggregated estimates of odds ratios. To test whether any 2 aggregated odds ratios were significantly different, we used the $z$-test described by Altman and Bland (8). Use of this test requires that the 2 estimates be independent. Because analyses of the haplotype structure have shown that the 3 $NOD2$ genetic variants are independent and not linked to each other (5), this criterion is reasonably fulfilled for comparisons of these 3 genetic variants.

We graphically tested for publication bias with funnel plots, in which the logarithm of the odds ratio is plotted against the reciprocal of the SE of the odds ratio to produce a simple scatterplot. Asymmetric plots indicate publication bias. We examined whether larger and smaller studies systematically gave different results, and we evaluated whether the magnitudes of the genetic association in the first published studies were overestimated compared with those reported in subsequent studies. Furthermore, for metaanalyses comprising $>10$ studies, we assessed for publication bias with the statistical tests of Begg and Egger (9). In the case of disparate results for the 2 tests, a visual inspection of funnel plots overruled the results of statistical tests. In metaanalyses with $<10$ studies, only visual inspection of funnel plots was performed.

Heterogeneity was assessed with the $I^2$ statistic, which describes the percentage of the total variation across studies due to heterogeneity rather than to chance, and was interpreted as low heterogeneity ($<25\%$), moderate heterogeneity ($25\%$–$50\%$), and high heterogeneity ($>50\%$) (10). As a threshold for investigating sources of heterogeneity, we chose an $I^2$ value of $>50\%$. Between-study heterogeneity was estimated with the Cochran $Q$ $\chi^2$ test (10). To explore possible reasons for heterogeneity, we independently reviewed all studies included in the metaanalyses with high $I^2$ values for any characteristics that could explain the variation between studies. In addition, in an attempt to further explore study heterogeneity, we undertook a sensitivity analysis with the following stratification factors: (a) study size (small studies had $<200$ cases, and larger studies had $\geq200$ cases), (b) Hardy–Weinberg equilibrium/dequilibrium among controls, and (c) study quality (studies with $\geq3$ criteria fulfilled). Such partitioning of data should always lower the $I^2$ value. Therefore, if heterogeneity persisted after these stratifications, we conducted individual post hoc inspections of the studies included in the analyses.

Results

Initially, we found 117 potential hospital- or population-based case–control studies with Crohn disease as cases and identified 39 studies with ulcerative colitis as cases. On the basis of our exclusion criteria, we excluded 42 studies (Table 1 in the online Data Supplement), leaving a total of 79 studies for metaanalyses of Crohn disease [derived from 75 published reports (Table 1; Table 2 in the online Data Supplement)], including 18 727 cases and 17 102 controls and a total of 36 studies for metaanalyses of ulcerative colitis [derived from 36 published reports (Table 1; Table 3 in the online Data Supplement)], including 6 523 cases and 7 981 controls. In all but 2 studies (11, 12), the controls were in Hardy–Weinberg equilibrium.

Because allele frequencies were reported in all studies, but not genotype frequencies, we estimated odds ratios per allele overall and for subgroup analyses. In addition, we estimated odds ratios per genotype for heterozygotes and homozygotes for each of the 3 $NOD2$ variants, and we separately estimated for all 3 genotypes combined the odds ratios for simple heterozygotes (without heterozygosity for the 2 other variants), compound heterozygotes, and homozygotes for the smaller number of studies that had such information available. Only the aggregated data are presented in our published report; results for individual studies.
The odds ratios per allele for Crohn disease were 2.2 (95% CI, 2.0–2.5) for Arg702Trp, 2.6 (95% CI, 2.2–2.9) for Gly908Arg, and 3.8 (95% CI, 3.4–4.3) for Leu1007fsinsC (Fig. 1) (z-test results: Arg702Trp vs Gly908Arg, \(P = 0.03\); Arg702Trp vs Leu1007fsinsC, \(P < 0.001\); Gly908Arg vs Leu1007fsinsC, \(P < 0.001\)). If we included only the 72 studies that had genotype information for all 3 sites, the results were identical for Arg702Trp and Gly908Arg and very similar for Leu1007fsinsC (odds ratio, 3.8; 95% CI, 3.4–4.2). For ulcerative colitis, the corresponding odds ratios and 95% CIs were 1.1 (0.9–1.3) for Arg702Trp, 1.1 (0.9–1.4) for Gly908Arg, and 1.0 (0.9–1.2) for Leu1007fsinsC.

### STUDY QUALITY

No study completely fulfilled all of the evaluated quality criteria, although all studies fulfilled quality measures for the genotyping method among cases and controls (see Table 4 in the online Data Supplement). Of 79 studies, 73 studies fulfilled \(\geq 3\) of the 5 criteria used for quality assessment, 49 studies fulfilled \(\geq 4\) criteria, and 12 studies fulfilled all 5 criteria. If we included only studies with \(\geq 3\), \(\geq 4\), or 5 fulfilled criteria in the metaanalyses, the results were similar to those shown in Fig. 1.

### SUBGROUP ANALYSES

The odds ratios per allele for Crohn disease were similar for the following subgroup comparisons: northern vs southern Europeans, adults vs children, and familial vs sporadic cases (Fig. 2).
HETEROZYGOTES VS HOMOZYGOTES FOR EACH NOD2 VARIANT
Forty-nine studies reported information on heterozygosity and/or homozygosity for each of the 3 NOD2 variants (Table 5 in the online Data Supplement). For Arg702Trp, the odds ratio for Crohn disease was 2.2 (95% CI, 2.0–2.6) for heterozygotes vs noncarriers and 3.2 (95% CI, 2.0–5.1) for homozygotes vs noncarriers (P = 0.13, z-test; Fig. 3). The corresponding odds ratios were 2.3 (95% CI, 2.0–2.8) and 2.4 (95% CI, 1.2–4.8) for Gly908Arg (P = 0.91, z-test), and 3.3 (95% CI, 2.8–3.8) and 9.6 (95% CI, 5.8–15.8) for Leu1007fsinsC (P < 0.001, z-test).

SIMPLE HETEROZYGOTES VS COMPOUND HETEROZYGOTES AND HOMOZYGOTES
Twenty-nine studies reported information on combined NOD2 genotypes, i.e., noncarriers, simple heterozygotes, compound heterozygotes, and homozygotes (Table 6 in the online Data Supplement). Combining all 3 genetic variants, we obtained odds ratio for Crohn disease of 2.4 (95% CI, 2.0–2.8) for simple heterozygotes, 9.0 (95% CI, 6.0–13.5) for compound heterozygotes, and 6.7 (95% CI, 4.1–10.9) for homozygotes, compared with noncarriers (z-test results: simple heterozygotes vs compound heterozygotes, P < 0.001; simple heterozygotes vs homozygotes, P < 0.001; compound heterozygotes vs homozygotes, P = 0.18).

PUBLICATION BIAS AND STUDY HETEROGENEITY
Evidence of publication bias was found in only a single metaanalysis, the analysis for Gly908Arg in individuals with familial Crohn disease. Subgroup analyses indicated that the magnitudes of the genetic association in the first published studies were slightly overestimated compared with subsequent studies (see Fig. 27 in the online Data Supplement).

For 4 of the metaanalyses (Figs. 2 and 3), the I^2 analysis provided evidence of high heterogeneity between studies. The I^2 value was >50% in all 4 analyses, and studies of small size (<200 cases) appeared to have a tendency toward higher odds ratios compared with studies of large size (≥200 cases).

Discussion
The present metaanalysis of 79 hospital- and population-based case-control studies including 18 727 cases and 17 102 controls for Crohn disease provides the most comprehensive assessment thus far for the association between NOD2 genetic variants and the risk of Crohn disease. Four findings were observed in the present metaanalyses.

First, in line with previously reported data for Europeans (12, 13), we observed that the 3 different NOD2 genetic variants are not equally important risk factors for Crohn disease. The per-allele risk of Crohn disease was 2.2 (95% CI, 2.0–2.6) for Arg702Trp, 2.3 (95% CI, 2.0–2.8) for Gly908Arg, and 3.3 (95% CI, 2.8–3.8) for Leu1007fsinsC. For compound heterozygotes, the corresponding odds ratios were 9.0 (95% CI, 6.0–13.5).
A disease was 2.2-fold for Arg702Trp, 2.6-fold for Gly908Arg, and 3.8-fold for Leu1007fsinsC. One possible explanation for the largest odds ratio occurring for Leu1007fsinsC is that Leu1007fsinsC is a frameshift variation followed by a premature stop codon that truncates the protein by 33 amino acid residues, whereas the other 2 variants have less detrimental effects on the protein.

Second, all subgroup analyses produced similar risk estimates for the NOD2 genetic variants. In accordance with results from 2 previous case-control studies (14, 15), we detected no evidence for differences in risk estimates for NOD2 genetic variants for Crohn disease in individuals from northern Europe vs southern Europe. Many individual studies have found an association between early-onset Crohn disease and NOD2 genetic variants (5, 12, 17–22), indirectly suggesting that NOD2 genetic variants could be more frequent in children with Crohn disease than in adults with the disease; however, our metaanalysis provided no support for this hypothesis. Five case-control studies directly compared allele frequencies and risk estimates of NOD2 genetic variants for familial and sporadic cases of Crohn disease (17, 21, 23–25). Three of these studies found no significant difference (17, 21, 24), and the other 2 studies obtained higher risk estimates among familial cases than among sporadic cases (23, 25). Nevertheless, our findings in the present metaanalysis indicating similarly increased odds ratios for Crohn disease for the NOD2 genetic variants in both familial and sporadic cases suggest that NOD2 genetic variants are not involved in the predisposition to familial clustering of Crohn disease.

Third, for the first time in a metaanalysis, we compared the magnitude of the odds ratios for Crohn disease for heterozygosity and homozygosity for each of the 3 genetic variants. In accordance with results from 2 previous case-control studies (14, 15), we detected no evidence for differences in risk estimates for NOD2 genetic variants for Crohn disease in individuals from northern Europe vs southern Europe. Many individual studies have found an association between early-onset Crohn disease and NOD2 genetic variants (5, 12, 17–22), indirectly suggesting that NOD2 genetic variants could be more frequent in children with Crohn disease than in adults with the disease; however, our metaanalysis provided no support for this hypothesis. Five case-control studies directly compared allele frequencies and risk estimates of NOD2 genetic variants for familial and sporadic cases of Crohn disease (17, 21, 23–25). Three of these studies found no significant difference (17, 21, 24), and the other 2 studies obtained higher risk estimates among familial cases than among sporadic cases (23, 25). Nevertheless, our findings in the present metaanalysis indicating similarly increased odds ratios for Crohn disease for the NOD2 genetic variants in both familial and sporadic cases suggest that NOD2 genetic variants are not involved in the predisposition to familial clustering of Crohn disease.

Fourth, when we combined all genotypes in the present study (including 79 studies), the risk of Crohn disease was 2-fold higher for simple heterozygotes and 7-fold to 9-fold higher for compound heterozygotes and homozygotes. A previous metaanalysis by Oostenbrug et al. (12) that included 20 studies found corresponding odds ratios of 3-fold and 12- to 15-fold, and a
metaanalysis by Economou et al. (13) that included 42 studies obtained odds ratios of 2-fold and 17-fold. Our slightly lower risk estimates likely are explained by the 37 additional studies included in our analyses, as well as by our use of stricter inclusion and exclusion criteria, and suggest that the sizes of the overall effect may be somehow less prominent than previously thought. Despite the modest difference in risk estimates between our metaanalysis and previous metaanalyses, it is interesting that each of these risk estimates is smaller than those found in the first published case–control studies among Europeans (3, 4). This fact indicates the importance of using metaanalytical techniques to find the “true” impact of NOD2 genetic variants on the risk of Crohn disease.

In metaanalyses based on tabular data, it is difficult to control for confounding factors. For inflammatory bowel disease, only 2 potential confounders (smoking and appendectomy) are known (2); however, information on these confounders was not available for the majority (95%) of the included studies. Owing to Mendelian randomization, however, confounding by conventional risk factors is rarely a problem in studies of genetic factors (26). The heterogeneity observed in some subgroup metaanalyses may be explained by the many clinical differences in patient selection (differences in disease severity in case–control studies) and by the limited number of studies available.

We performed subgroup analysis within the metaanalysis and searched for interactions; however, to what extent (large) interactions exist is questionable, and whether metaanalysis can help to identify them is probably even more debatable. Subgroup analysis in metaanalysis suffers from the substantial heterogeneity generated by differences in design features, study groups, methods, and other factors. Therefore, the results produced by the present subgroup analyses should be interpreted with caution.

Because all of the included studies were case–control studies, the results of these metaanalyses present effect sizes for people already with a diagnosis for the disease studied, but that fact does not necessarily imply that sizes of the effects on the risk of Crohn disease found for these genotypes will be similar to those in the general population.

An important question is whether NOD2 is the only susceptibility gene for Crohn disease. Recent genome-wide association studies have identified >30 other genetic variants associated with Crohn disease (27); however, many of these new variants have smaller effects on the risk of Crohn disease (27) than those reported for NOD2 (13). Crucial future research may be directed toward investigating these new genetic variants in large case–control and general-population studies.

In conclusion, we found a per-allele risk of Crohn disease of 2.2-fold for Arg702Trp, 2.6-fold for Gly908Arg, and 3.8-fold for Leu1007fsinsC; we obtained similar estimates for subgroups. Combining all genotypes, we obtained a risk of Crohn disease of 2-fold for simple heterozygotes and 7- to 9-fold for compound heterozygotes and homozygotes. These findings are potentially important if genotyping of NOD2 genetic variants is used as a tool to help in the differential diagnosis of Crohn disease vs ulcerative colitis.

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7. Human Genome Epidemiology Network: UK Coor-