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ORIGINAL ARTICLE

GST Theta null genotype is associated with an increased risk for ulcerative colitis: a case–control study and meta-analysis of GST Mu and GST Theta polymorphisms in inflammatory bowel disease

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Glutathione *S*-transferases (GSTs) are important in the detoxification of many compounds, including reactive oxygen species. Polymorphisms in GSTs resulting in a decreased enzyme activity might enhance the risk for inflammatory bowel disease by eliciting a state of oxidative stress. Previous case–control studies showed divergent results and were frequently limited in sample size; therefore we conducted a meta-analysis including results from our case–control study. For the case–control study, we genotyped 552 patients with Crohn's disease (CD), 223 patients with ulcerative colitis (UC) and 972 healthy controls by PCR for functional deletions in *GST Mu* and *GST Theta.* Both were not analyzed in recent genome-wide association studies. For the meta-analysis, PubMed, EMBASE and Web of Science were searched. In this meta-analysis, we show an enhanced susceptibility for UC in individuals with the *GSTT1null* genotype (odds ratio (OR) 2.27, 95% confidence interval (CI) 1.31–3.92). In our case–control study, a reduced risk for CD was seen with the *GSTT1null* genotype (OR 0.58, 95% CI 0.43–0.77); however, pooled analysis showed an OR of 1.67, 95% CI 0.81–3.45. In this meta-analysis, we showed an increased risk for UC in individuals with the *GSTT1null* genotype.
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INTRODUCTION

Genetic factors become more and more recognized as contributing factors in the development of inflammatory bowel disease (IBD). Recent genome-wide association studies (GWASs) generated a large set of candidate genes for Crohn's disease (CD), ulcerative colitis (UC) and for IBD in general.[1]

Glutathione *S*-transferases (GSTs) are a family of enzymes that have an essential role within cells, including the conjugation and detoxification of toxic or carcinogenic compounds, such as reactive oxygen species (ROS).[2] Polymorphisms in GSTs can lead to a decreased enzyme function, and an inadequate detoxification of ROS might modulate the susceptibility for IBD.[3] Biopsies of colonic mucosa of IBD patients showed an increased ROS production compared with healthy controls.[4] A reduced enzyme function of GSTs and therewith impaired scavenging of ROS can contribute to a state of oxidative stress, which can trigger the onset of IBD.[5,6]

Human GSTs can be divided in four main classes, GST Alpha (*GSTA*), GST Mu (*GSTM1*), GST Pi (*GSTP1*) and GST Theta (*GSTT1*). For *GSTP1*, several polymorphisms have been described, with *GSTP1 105* (rs1695) and *GSTP1 114* (rs1138272) considered as the most important, because these *GSTP1* polymorphisms result in a reduced enzyme function.[7] Both *GSTP1 105* and *GSTP1 114* were not found to be associated with UC or CD in a GWAS.[1] For *GSTM1*, three alleles have been described of which one, *GSTM1*0*, is characterized by a gene deletion, leading to a non-functional protein. Homozygous presence of the *GSTM1*0* allele (*GSTM1null*) results in no enzyme activity and is found in approximately 50% of Caucasians and Asians.[2,8] The same applies to *GSTT1*, where a gene deletion (*GSTT1*0* allele) is responsible for a failed protein synthesis. The homozygous *GSTT1*0* genotype, *GSTT1null*, which occurs in approximately 10–20% of Caucasians and 16–64% of Asians, leads to the absence of GSTT1 enzyme activity.[2,8] The gene deletions of *GSTM1*0* or *GSTT1*0* were not analyzed in GWASs,[1] so evidence is limited to case–control studies. In these studies, an association between *GSTM1*0, GSTT1*0* or a combination of these polymorphisms and susceptibility for CD or UC was found. However, it has to be stressed that some studies had relatively low patient numbers, and overall results remain inconsistent.[9–12] Therefore we conducted a large

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case–control study and included our results in a meta-analysis of studies addressing the susceptibility for CD and UC with respect to *GSTM1* and *GSTT1 null* polymorphisms.

MATERIALS AND METHODS

Study design and ethics

For the case–control study, IBD patients were recruited at the outpatient clinic of the Radboud University Medical Center, Nijmegen, the Netherlands. Healthy controls were recruited from the Nijmegen area, by advertisement in local papers, as described earlier.[13] Diagnosis of IBD was based on accepted clinical, endoscopic, radiological and histological findings.[14] The investigations were approved by the Medical Ethical Review Committee of the Radboud University Nijmegen Medical Center under the protocol number CWOM-nr 9804-0100.

DNA isolation and genotyping

Peripheral blood samples of IBD patients and healthy controls were collected and genomic DNA was isolated with the ROCHE High Pure PCR template Preparation Kit (Roche Applied Science, Mannheim, Germany). DNA was stored at 4 °C until further use. Polymorphisms in *GSTM1* and *GSTT1* were analyzed using PCR as described previously.[15] The primers 5′-CTGGATTGTA GCAGATCATGC-3′/5′-CTCCTGATTATGACAGAAGCC-3′ and 5′-TCACCGG ATCATGGCCAGCA-3′/5′-TTCCTTACTGGTCCTCACATCTC-3′ were used for *GSTM1* and *GSTT1*, respectively. To detect *GSTM1* and *GSTT1* genotypes, melt curve analysis was applied using the Bio-Rad Precision Melt Analysis Software version 1.0 (Bio-Rad, Hercules, CA, USA) and in this way homozygous and heterozygous most common genotypes could be distinguished from homozygous *GSTM1*0* (null) or *GSTT1*0* (null) genotypes.

Data search

The meta-analysis was conducted according to PRISMA guidelines. Articles for the meta-analyses were retrieved by searching PubMed, EMBASE and Web of Science on 13 October 2013 with the following search terms: GST, glutathione *S*-transferase, detoxification enzymes, inflammatory bowel disease, ulcerative colitis, and Crohn's disease. Case–control studies examining the association of *GST* polymorphisms in patients with IBD in relation to healthy controls were included, provided that absolute numbers of genotypes of patients and controls were given. Studies were excluded when no full text was available or written in a language other than English, German or Dutch. In case of overlapping data, the study with the largest number of cases was included. The reference lists of included articles were screened for potential eligible studies. Two researchers (MB and RM) independently extracted raw data of the genotype distributions of *GSTM1*, *GSTT1* and the combination of *GSTM1* with *GSTT1* from the included studies, and in case of inconsistency, results were discussed until consensus was reached.

Statistical analysis

For the case–control study, baseline continuous variables were compared by independent *t*-test. Categorical data between patients and healthy controls were analyzed with the chi-squared test. Genotypes with predicted high enzyme activity were used as reference to calculate odds ratios (OR) and 95% confidence intervals (95% CI) with logistic regression. The combination *GSTM1null* and *GSTT1null* was compared with all other genotypes. Analyses were conducted with the SPSS for windows version 20.0 (SPSS Inc., Chigaco, IL, USA).

For the meta-analyses a random effect model was used to calculate pooled ORs from studies, including results of our case–control study. ORs with 95% CI were calculated with the Mantel–Haenszel method in Review Manager (RevMan) version 5.2. (The Nordic Cochrane Centre, The Cochrane Collaboration, Copenhagen, Denmark). The *P*-values of significance of the ORs are depicted in the bottom line of the corresponding forest plots. Finally, heterogeneity was assessed with the I^2 test, and publication bias was estimated with a funnel plot.

RESULTS

Case–control study

For the case–control study, we included 552 patients with CD (mean age 44.4 years, s.d. ± 13.8), 233 patients with UC (mean age 45.8 years, s.d. ± 13.7) and 972 healthy controls (mean age 47.2 years, s.d. ± 16.6). Table 1 shows the *GST* genotype distribution in patients with CD and UC in comparison with healthy controls. With the homozygous + heterozygous most common genotypes set as reference, the *GSTM1null* genotype was not associated with a significant increased susceptibility for CD or UC. The *GSTT1null* genotype was more often present in healthy controls compared with patients with CD (OR 0.58; 95% CI 0.43–0.77). Although in UC patients the *GSTT1null* genotype was more frequently seen, difference was not significant (OR 1.14, 95% CI 0.81–1.61). The combination of *GSTM1null* and *GSTT1null* genotypes was significantly seen more in healthy controls compared with patients with CD (OR 0.44, 95% CI 0.29–0.66). The association with the *GSTT1null* genotype and CD and the combination of *GSTT1null* and *GSTM1null* had a *P*-value of <0.001. This was still significant after correction for multiple testing (Bonferroni's correction).

Study characteristics of the meta-analyses

In total, 251 articles were identified. After removing duplicates and screening for title and abstract, 11 articles remained.[9–12,16–22] Three studies[20–22] were excluded because of Chinese language. One study was excluded[18] because of overlapping data with the results of our case–control study. In the study of Karban *et al.*,[10] the results of Jewish, Arab moslems and Druze were merged. No studies had to be excluded because of no full text availability. Furthermore, all studies used genotyping methods that can detect the null genotype with equivalent accuracy, except one study that used a genotype-validated assay based on the GSTMu activity to identify patients with the null genotype.

Results of the meta-analyses

No increased susceptibility was found for CD with the *GSTM1null* (Figure 1) or *GSTT1null* genotype (Figure 2; OR 1.08, 95% CI 0.84–

Table 1 GST genotype distributions and OR with 95% CI of patients with CD and UC compared with controls in our case–control study

GST	*Genotype*	*Predicted enzyme activity*	*Controls (%)*	*CD (%)*	*OR (95% CI)*	*UC (%)*	*OR (95% CI)*
GSTM1	*1*1/*1*0	High–intermediate	442 (45.5)	263 (47.6)	Reference	116 (49.8)	Reference
	*0/*0	Absent	530 (54.5)	289 (52.4)	0.92 (0.74–1.13)	117 (50.2)	0.84 (0.63–1.12)
GSTT1	*1*1/*1*0	High–intermediate	769 (79.1)	479 (86.8)	Reference	179 (76.8)	Reference
	*0/*0	Absent	203 (20.9)	73 (13.2)	0.58 (0.43–0.77)*	54 (23.2)	1.14 (0.81–1.61)
GSTM1 and GSTT1	*1*1/*1*0 and *1*1/*1*0	High–intermediate	850 (87.4)	490 (94)	Reference	209 (89.7)	Reference
	*0/*0 and *0/*0	Absent	122 (12.6)	31 (6.0)	0.44 (0.29–0.66)*	24 (10.3)	0.80 (0.50–1.27)

Abbreviations: CD, Crohn's disease; CI, confidence interval; GST, glutathione *S*-transferase; *GSTM1*, *GST Mu*; *GSTT1*, *GST Theta*; OR, odds ratio; UC, ulcerative colitis. ORs were calculated with the genotypes with high–intermediate enzyme activity set as reference. *$P<0.001$.

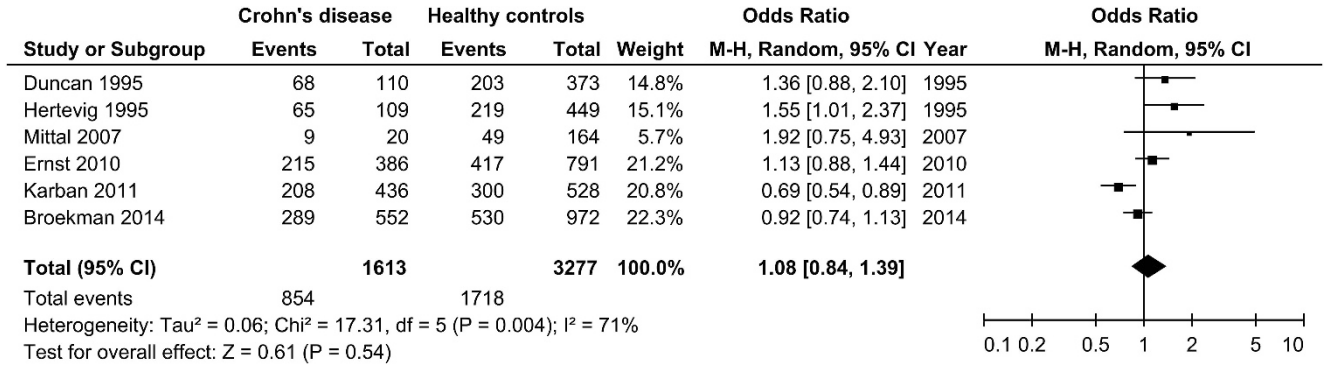

Figure 1 Forest plot of the association of the *GSTM1null* genotype with Crohn's disease (CD). Effect of *GSTM1null* on the risk of CD (odds ratios (ORs) and the 95% confidence intervals (CIs)) for each study is shown. The overall OR is 1.08 (95% CI 0.84–1.39). *GSTM1null*, homozygous presence of the *GSTM1*0* allele.

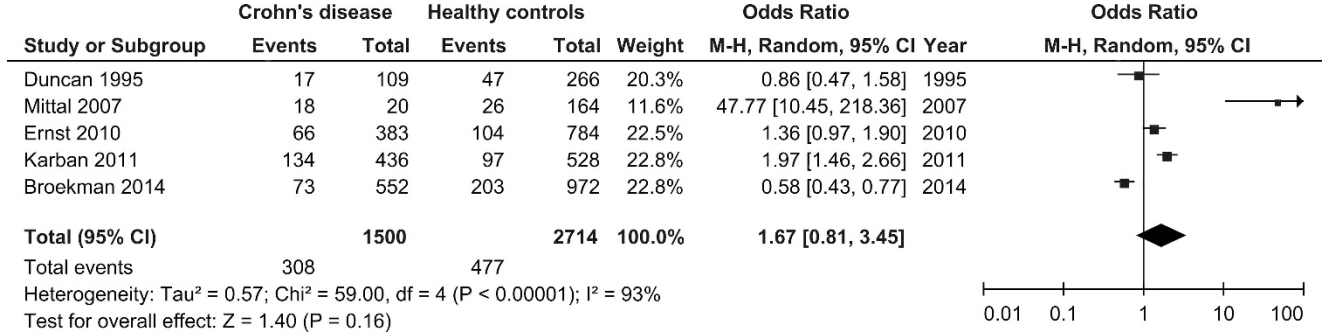

Figure 2 Forest plot of the association of the *GSTT1null* genotype with Crohn's disease (CD). Effect of the *GSTT1null* genotype on the risk of CD (odds ratios (ORs) and the 95% confidence intervals (CIs)) for each study is shown. The overall OR is 1.67 (95% CI 0.81–3.45). *GSTT1null*, homozygous presence of the *GSTT1*0* allele.

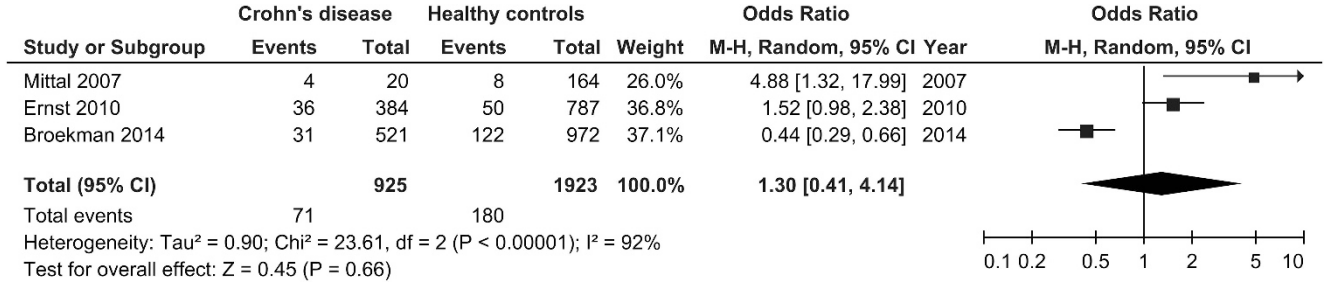

Figure 3 Association of the combination of *GSTM1null* and *GSTT1null* genotypes with Crohn's disease (CD). Risk of CD (odds ratios (ORs) and the 95% confidence intervals (CIs)) with both the *GSTM1null* and *GSTT1null* genotype versus all other variants is shown for each study. The overall OR is 1.30 (95% CI 0.41–4.14). *GSTM1null*, homozygous presence of the *GSTM1*0* allele; *GSTT1null*, homozygous presence of the *GSTT1*0* allele.

1.39 and 1.67, 95% CI 0.81–3.45, respectively). Also the combination of both did not influence disease susceptibility (OR 1.30, 95% CI 0.41–4.14; Figure 3). Pooled analysis revealed an increased risk for UC with the *GSTT1null* genotype (OR 2.27, 95% CI 1.31–3.92; Figure 4), still significant after correction for multiple testing ($P = 0.003$). For *GSTM1null* (OR 1.35, 95% CI 0.89–2.03) and the combination of *GSTT1null* and *GSTM1null* (OR 1.99, 95% CI 0.65–6.12), no significant influence on disease susceptibility was seen (see Figures 5 and 6).

Publication bias

The funnel plot from the pooled analysis of *GSTT1* in UC, the only meta-analysis showing a significant effect, is depicted in Figure 7. This meta-analysis included seven studies, and with the exception of one outlier, the shape of the plot appears symmetrical.

DISCUSSION

The results of the current meta-analyses show a significantly increased risk for UC, but not for CD, when bearing the *GSTT1null* genotype.

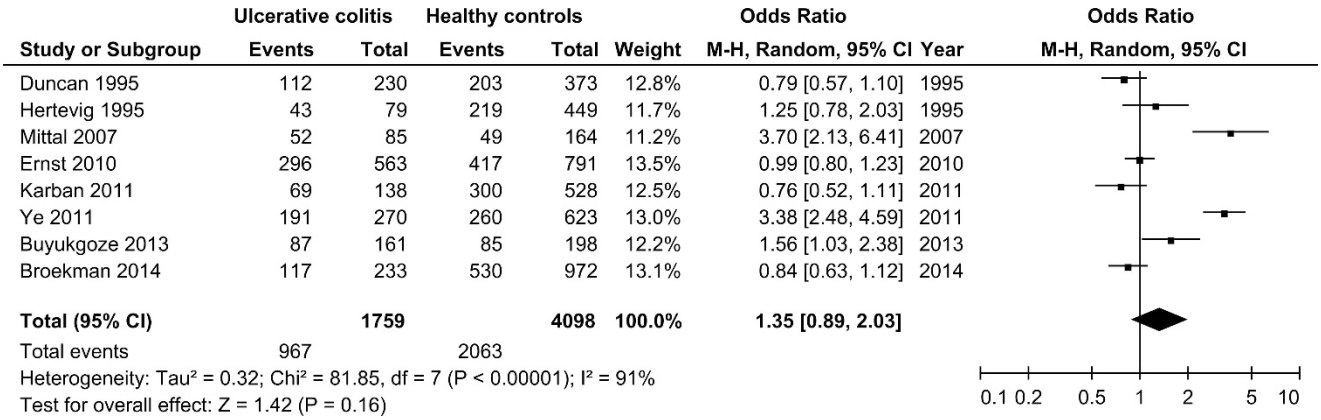

Figure 4 Forest plot of the association of the *GSTT1null* genotype with ulcerative colitis (UC). Effect of the *GSTT1null* genotype on the risk of UC (odds ratios (ORs) and the 95% confidence intervals (CIs)) for each study is shown. The overall OR is 2.27 (95% CI 1.31–3.92). *GSTT1null*, homozygous presence of the *GSTT1*0* allele.

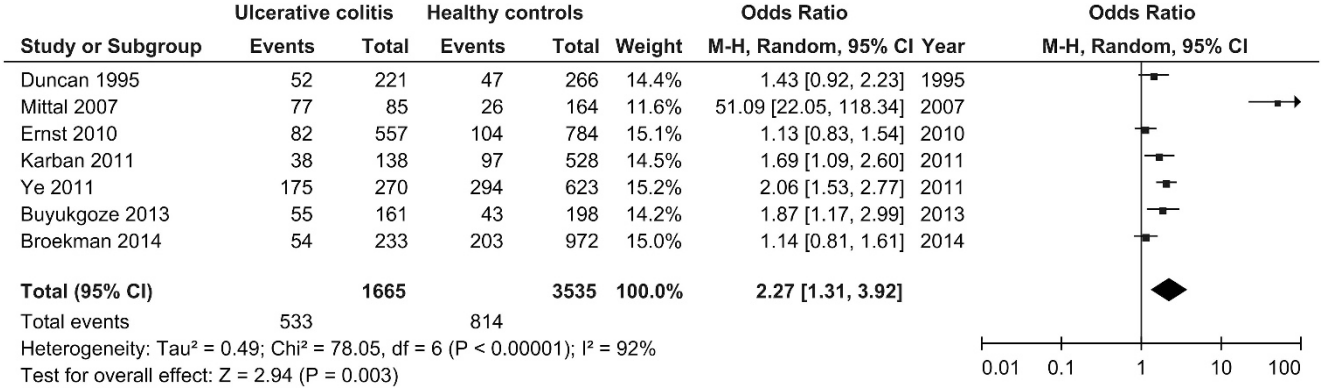

Figure 5 Forest plot of the association of the *GSTM1null* genotype with ulcerative colitis (UC). Effect of the *GSTM1null* genotype on the risk of UC (odds ratios (ORs) and the 95% confidence intervals (CIs)) for each study is shown. The overall OR is 1.35 (95% CI 0.89–2.03). *GSTM1null*, homozygous presence of the *GSTM1*0* allele.

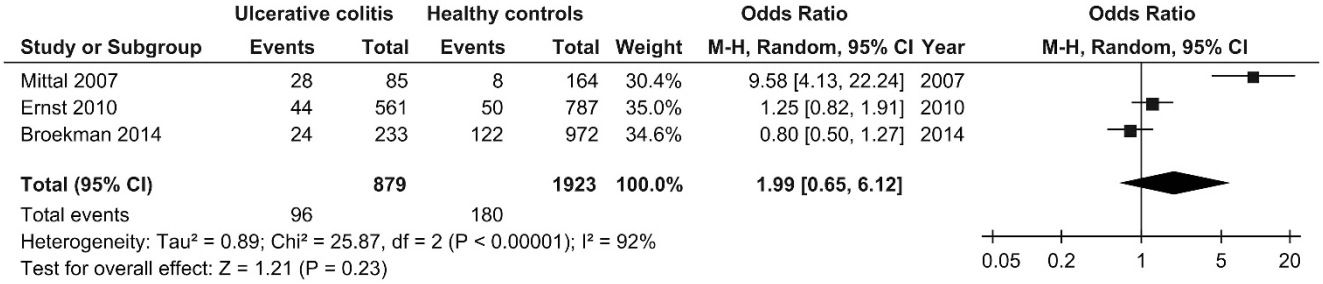

Figure 6 Association of the combination of *GSTT1null* and *GSTM1null* genotypes with ulcerative colitis (UC). Risk of UC (odds ratios (ORs) and 95% confidence intervals (CIs)) with both the *GSTM1null* and *GSTT1null* genotype versus all other variants is shown for each study. The overall OR is 1.99 (95% CI 0.65–6.12). *GSTM1null*, homozygous presence of the *GSTM1*0* allele; *GSTT1null*, homozygous presence of the *GSTT1*0* allele.

The *GSTM1null* genotype was not associated with CD or UC. It is important to mention that the *GSTM1null* and *GSTT1null* polymorphisms were not analyzed in GWASs,[1] because these polymorphisms consist of large deletions rather than single-nucleotide substitutions. With pooling data from case–control studies, supplemented with our data, overall IBD patient numbers exceed 3200, which makes this meta-analyses the largest study so far.

The mechanism that absence of GSTT1 enzyme activity in *GSTT1null* individuals contributes to the risk for UC might be explained by the important role of this enzyme in the detoxification of ROS,[23] which may provide a trigger in the etiology of IBD.[24,25] Also, in other inflammatory-driven diseases, such as asthma or type 2 diabetes mellitus, an increased susceptibility was found with the *GSTT1null* genotype.[26,27] In this meta-analyses, we showed that the

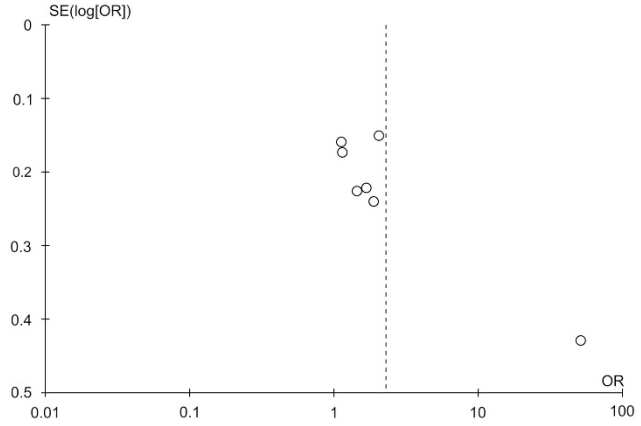

Figure 7 Funnel plot for the meta-analysis of the *GSTT1null* genotype in ulcerative colitis to detect potential publication bias.

GSTT1null genotype is associated with UC but not with CD. This finding is not unique as 53 of the 163 loci associated with IBD were disease specific.[1]

Although the studies conducted in Asia showed a larger association between the *GSTT1null* genotype and UC than European studies, all studies revealed an increased risk with only variation in the magnitude. When the studies of Mittal *et al.*[19] (OR 51.09, 95% CI 22.05–118.34) and Ye *et al.*[9] (OR 2.06, 95% CI 1.53–2.77) were excluded from the pooled analysis, leaving only Caucasians studies, the *GSTT1null* genotype was still significantly seen more in UC patients (OR 1.36, 95% CI 1.11–1.65). The increased, though not significant susceptibility, for CD with the *GSTT1null* genotype is, to a large extent, caused by the study of Mittal *et al.*[19] with an OR of 47.8 (95% CI 10.5–218.4). The likely cause of this large OR is the low number of patients ($n = 20$) with CD included, which is reflected by the broad CI. When this study is excluded from the meta-analysis, the OR is 1.08 (95% CI 0.59–2.00). Furthermore, in the forest plot of *GSTT1* in CD, a large difference was seen between the study of Ernst *et al.*[11] and our results, despite only a small geographical difference. This might be caused by a relatively low number of healthy controls with the *GSTT1null* genotype in the Danish study compared with our study (13.2% vs 20.8%, respectively), where 19.7% is the average for this area.[8]

Also, with the *GSTM1null* genotype, both the two studies conducted in Asia (Mittal *et al.*[19] and Ye *et al.*[9]) revealed the highest association with IBD. However, overall no significant influence was found. It might be that the loss of function of the *GSTM1* enzyme is compensated by other *GST Mu* classes, such as *GSTM2*,[28] this also explains why the combination of both *GSTM1null* and *GSTT1null* did not have a synergistic effect on disease susceptibility. Another explanation could be that the expression of *GSTM1* in the bowel is limited and therefore does not contribute in the pathogenesis of IBD.[2]

The difference between the studies conducted in Asia and Europe support the recent insights in differences in genetic variants associated with IBD between the Caucasian and Asian race.[29,30] Ng *et al.*[29] showed that genetic variants of *NOD2* frequently seen in Caucasian IBD patients were rare in the Asian IBD population and *vice versa*. Therefore it can be hypothesized that for Asians both *GSTT1null* and *GSTM1null* may be involved in the etiology of UC, whereas in Caucasians only the *GSTT1null* genotype is associated with UC. This distinction might result from differences in life style, environmental factors and the microbiome, requiring a different utilization of GST subclasses. Ideally, subgroup analyses for different races are included; however, absolute numbers of Asian IBD patients are insufficient for a reliable estimation. This accentuates the need for larger studies in the Asian IBD population exploring *GSTM1null* as well as *GSTT1null* genotypes.

Single-nucleotide polymorphisms (SNPs) in the neighbor of the deletion polymorphism may be in linkage disequilibrium and subsequently might be used as a tag SNP. However, this method is limited by the inter-population differences in the degree of linkage disequilibrium. Most SNPs in linkage disequilibrium with *GSTM1* and *GSTT1*[31] were not included in the most recent GWAS.[1] This can partly be explained by the fact that some of these tag SNPs such as rs407257 lie in the deletion area.

As previous studies with comparable or smaller sample sizes were able to show significant associations, we assumed to have sufficient power with the patient numbers included in our case–control study. In the present meta-analysis, heterogeneity between studies was observed, which besides differences in race could have been originated from differences in study design. For example, patient enrolment in academic hospitals could be confounded by more severe disease phenotypes included in academic hospitals. Also, the selection of healthy controls might have an impact and could be a possible cause for heterogeneity. Considering these potential differences, we used a random-effect model for computing pooled ORs, with the knowledge that in case of low heterogeneity results of a random model will approach values of a fixed model, but preventing poorly substantiated significant outcomes.[32] The funnel plot of *GSTT1null* in UC contains one outlier (the study of Mittal *et al.*[19] with an OR of 51.09), though, as mentioned above, with exclusion of this study still a significant effect of *GSTT1null* and UC was observed. It has to be mentioned that the number of studies published on this topic is limited, which makes correct interpretation of the funnel plot difficult. Therefore influence of publication bias should be considered, as studies showing a significant influence of specific polymorphisms might be published more often.[33] Furthermore, the PCR techniques used in our case–control study, and the other included studies, can only detect homozygous (null) genotypes of the *GSTM1*0* and *GSTT1*0* alleles, failing to detect heterozygous individuals. One study measured GSTM1 enzyme activity instead of the *GSTM1null* genotype.[12] We decided to include this study in the meta-analyses as this method is validated against the *GSTM1* genotype and their findings showed that 50% of controls had no *GSTM1* activity, which is in line with the *GSTM1null* genotype frequency in that geographical area.[8,34] In addition, this method also lacks the capability to identify *GSTM1null* heterozygotes

In conclusion, in this meta-analysis we show that the *GSTT1null* genotype is associated with an increased the risk for UC but not for CD.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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