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Genetic variants of homocysteine/folate metabolism pathway and risk of inflammatory bowel disease: a synopsis and meta-analysis of genetic association studies

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Abstract

A synopsis and meta-analysis of studies that investigated the association between genetic variants involved in the homocysteine/folate metabolism pathway and risk of inflammatory bowel disease (IBD) were conducted. Four variants (*MTHFR* C6TTT, *MTHFR* A1298C, *MTR* A2756G and *MTRR* A66G) showed significant associations in individual studies. In meta-analyses, only the variant *MTR* A2756G indicated an association with the risk of IBD for the allele contrast and the dominant model (odds ratio (OR) 1.48 (1.12–1.97) and OR 1.55 (1.12–2.15), respectively). The effect sizes for Crohn's disease and ulcerative colitis were similar to IBD. Cumulative meta-analysis for C677T indicated a downward trend of association as information accumulates.

Keywords: *Inflammatory bowel disease; Crohn's disease; ulcerative colitis;* MTHFR; MTR; MTRR; TCN2; CBS*; homocysteine; folate; gene; polymorphism; meta-analysis*

Introduction

Inflammatory bowel disease (IBD) is a group of inflammatory conditions of the colon and small intestine. The major types of IBD are Crohn's disease (CD) and ulcerative colitis (UC) (Baumgart & Carding 2007). Although the aetiology of IBD and its pathogenesis is poorly understood, it is thought to have a multifactorial aetiology, being influenced both by genetic and environmental factors (Herrlinger et al. 2005). Hyperhomocysteinemia is one factor of genetic basis that has been implicated in IBD development (Mahmud et al. 1999). Homocysteine is a sulphur-containing amino acid that results from the demethylation of methionine. Homocysteine can be recycled into methionine or converted into cysteine with the aid of B vitamins (Stipanuk 2004). Deficiencies of the vitamins folic acid (B_9), pyridoxine (B_6), or B_{12} (cyanocobalamin) can lead to high homocysteine levels (Miller et al. 1994). Elevations in plasma total homocysteine can be caused by genetic defects in the enzymes involved in homocysteine/folate metabolism (Papa et al. 2001). Genetic polymorphisms of the key enzymes in the homocysteine/folate metabolism include variants in the methylenetetrahydrofolate reductase (*MTHFR*) (Frosst et al. 1995, Weisberg et al. 1998), the methionine synthase (*MTR*) (Ma et al. 1999) and the methionine synthase reductase (*MTRR*) (Leclerc et al. 1998) genes. Transcobalamin (*TCN2*) and cystathionine beta synthase (*CBS*) are another two genes that may influence homocysteine/folate metabolism (Namour et al. 2001, Gaustadnes et al. 1998). Polymorphisms of these genes have been investigated for association with various

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diseases/disorders (Zintzaras & Zdoukopoulos 2009, Zdoukopoulos & Zintzaras 2008, Zintzaras et al. 2008, 2007, 2006a, 2005, Zintzaras 2006a, b, c) on the basis of evidence on functional relevance of these variants (Supplementary Table 1; see online version of this article) and the relatively high frequency of the minor alleles (Supplementary Table 2; see online version of this article) that fits well under the common variant–common disease hypothesis (Kitsios & Zintzaras 2009a).

In order to explore the influence of the genetic determinants of the homocysteine/folate metabolism pathway with the risk of IBD (Peyrin-Biroulet et al. 2007), we systematically searched for all available case–control studies that related the *MTHFR* C677T, *MTHFR* A1298C, *MTR* A2756G, *MTRR* A66G, *TCN2* C776G or *CBS* Ins68 I/N gene polymorphisms with IBD, including CD and UC. Finally, the available data were synthesized using meta-analytic techniques in order to provide better power to detect smaller effect sizes and to decrease the uncertainty of the estimated genetic risk effects (Zintzaras & Lau 2008a). In addition, the heterogeneity between studies and the existence of potential bias were explored. Cumulative and recursive cumulative meta-analyses were also performed to investigate whether the current evidence is sufficient to claim or to deny association (Zintzaras & Lau 2008a, Lau et al. 1992).

Methods

Identification and eligibility of relevant studies

We searched PubMed (until May 2009) for articles using combinations of the following terms: 'methylenetetrahydrofolate reductase', 'MTHFR', 'C677T', 'A1298C', 'methionine synthase', 'MTR', 'methionine synthase reductase' 'MTRR', 'transcobalamin', 'TCN2', 'cystathionine beta synthase', 'CBS', 'inflammatory bowel disease', 'IBD', 'Crohn's disease', 'CD', 'ulcerative colitis', 'UC'. The retrieved studies were then read in their entirety to assess their appropriateness for inclusion in the synopsis and the subsequent meta-analysis. All references cited in the studies were also reviewed to identify additional published work not indexed by the PubMed database.

Genetic association studies that determined the genotype distribution of *MTHFR* C677T, *MTHFR* A1298C, *MTR* A2756G, *MTRR* A66G, *TCN2* C776G or *CBS* Ins68 I/N gene polymorphisms in cases with IBD (CD or UC) and in healthy controls were eligible for inclusion in the meta-analysis. Only studies in human subjects that used validated genotyping methods were considered. Case reports, editorials and review articles were excluded. Non-English articles were also excluded. In studies with overlapping cases or controls, the most recent and/or the largest in size study with extractable data was included in the meta-analysis. Family-based association studies and genome-wide linkage scans were not considered because of different design considerations (Zintzaras & Ioannidis 2008, Zintzaras & Ioannidis 2005).

Data extraction

From each study, the following information was abstracted: first author, journal, year of publication, study area, ethnicity of the study population, demographics, clinical characteristics, matching, genotyping method and the number of cases and controls for each C677T and A1298C genotype. The frequencies of the alleles and the genotypic distributions were extracted or calculated, for both the cases and the controls. In addition, it was recorded whether the genotyping in each study was performed blinded to clinical status. When studies investigated more than one polymorphism, information on linkage disequilibrium (LD) and haplotype estimation (or combined genotypes) was recorded.

Data synthesis and analysis

The meta-analysis examined the association between each polymorphism and the risk of IBD (involving CD and UC), and CD and UC separately for: (1) the allele contrast (mutant type (mt) vs wild type (wt)), (2) the recessive, (3) dominant and (4) additive models of mt (20). The associations were indicated as pooled odds ratios (OR) with the corresponding 95% confidence intervals (CI) (Zintzaras & Lau 2008a, Trikalinos et al. 2008).

The heterogeneity between studies was tested using the Q-statistic (Cochran 1954). If $p_Q < 0.10$ then heterogeneity was considered statistically significant. Heterogeneity was quantified with the I^2 metric, which is independent of the number of studies in the meta-analysis (Zintzaras & Lau 2008a). I^2 takes values between 0% and 100% with higher values denoting a greater degree of heterogeneity. The pooled OR was estimated using random effects (RE) models (DerSimonian & Laird 1986). Random-effects modelling assumes a genuine diversity in the results of various studies, and it incorporates the calculations between study variance. When there is lack of heterogeneity the RE model coincides with the fixed-effects model (Zintzaras & Lau 2008a).

A cumulative and recursive cumulative meta-analysis was carried out for each polymorphism to evaluate the trend of the risk effect (OR) of the recessive model in time for IDB (Zintzaras & Lau 2008a, Lau et al. 1992). In the cumulative meta-analysis, studies were chronologically ordered by publication year, then, the risk effect

was obtained at the end of each year, i.e. at each information step. In the recursive cumulative meta-analysis, the relative change in pooled OR in each information step was calculated. Cumulative and recursive cumulative meta-analysis, provide a frame work for updating a genetic effect from all studies and a measure of how much the genetic effect changes as evidence accumulates (Zintzaras & Lau 2008a, Zintzaras 2007). Thus, the cumulative meta-analysis indicates the trend in estimated risk effect and the recursive cumulative meta-analysis indicates the stability in risk effect. A cumulative meta-analysis was carried out for polymorphisms investigated in more the five studies and three or more information steps.

Whether the OR in the first study versus the pooled OR of the subsequent studies were different beyond chance ($p < 0.05$) was assessed using the z-score, i.e. the difference of the natural logarithm of the ORs divided by the standard error of this difference (Zintzaras & Lau 2008a). In addition, the association between the C677T *MTHFR* gene polymorphism and IBD was assessed with or without the first study. The differential magnitude of effect in large versus small studies was checked for the *MTHFR* C677T recessive model (this is the most commonly investigated gene polymorphism and genetic contrast) in association with IBD using the test proposed by Harbord et al. (2006).

The meta-analysis consisted of the main (overall) analysis which includes all available data concerning IBD. Then, meta-analyses for CD and UC were performed separately. Subgroup analysis by 'racial' descent (Caucasians and East Asians) and sensitivity analysis which examines the effect of excluding specific studies were also performed (Zintzaras & Lau 2008a).

The distribution of the genotypes in the healthy control group was tested for Hardy–Weinberg equilibrium (HWE) using an exact test (Weir 1996, Zintzaras 2008). The meta-analysis was subjected to sensitivity analysis for studies with the controls not in HWE (Zintzaras & Lau 2008a). Analyses were performed using Meta-Analyst V.3 (Evidence-Based Practice Centers, Tufts Medical School, Boston MA, USA) and CUMAGAS (http://biomath.med.uth.gr).

Results

Eligible studies

The literature review identified 40 titles in PubMed that met the search criteria. The full articles of the retrieved studies were read to assess their appropriateness for meta-analysis. Data from 13 articles (i.e. studies) met the inclusion criteria and were included in the synopsis and the meta-analysis. Figure 1 presents a flow chart of retrieved studies and studies excluded, with specification of reasons. The studies were published between 1999 and 2008.

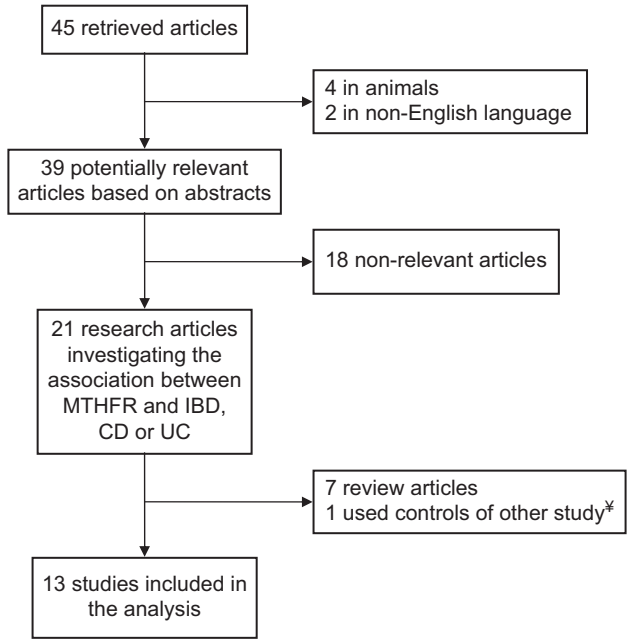

Figure 1. Flow chart of retrieved studies and studies excluded, with specification of reasons. ¥Kader et al. (2002). *J Pediatric Gastroenterol Nutr* 35:629-35.

A list of details abstracted from the studies included in the meta-analysis is provided in Table 1. All 13 studies dealt with the *MTHFR* C677T gene polymorphism in association to IBD (Herrlinger et al. 2005, Mahmud et al. 1999, Papa et al. 2001, Peyrin-Biroulet et al. 2008, Chen et al. 2008, Bernstein et al. 2007, Yasa et al. 2007, Yilmaz et al. 2006, Stocco et al. 2006, Fernández-Miranda et al. 2005, Nakano et al. 2003, Vecchi et al. 2000, Törüner et al. 2004); five studies provided data for IBD by pooling CD and UC data Mahmud et al. 1999, Papa et al. 2001, Yasa et al. 2007, Yilmaz et al. 2006, Vecchi et al. 2000); six studies provided data separately for both CD and UC (Bernstein et al. 2007, Stocco et al. 2006, Herrlinger et al. 2005, Törüner et al. 2004, Vecchi et al. 2000, Mahmud et al. 1999); one study examined only CD (Peyrin-Biroulet et al. 2008) and another study only UC (Chen et al. 2008). Six studies investigated the *MTHFR* A1298C gene polymorphism (Peyrin-Biroulet et al. 2008, Chen et al. 2008, Yilmaz et al. 2006, Stocco et al. 2006, Herrlinger et al. 2005, Fernadez-Miranda et al. 2005), two studies the *MTR* A2756G (Peyrin-Biroulet et al. 2008, Chen et al. 2008), two the *MTRR* A66G (Peyrin-Biroulet et al. 2008, Chen et al. 2008), two the *TCN2* C776G (Peyrin-Biroulet et al. 2008, Chen et al. 2008) and one the *CBS* Ins68 I/N (Papa et al. 2001). Two of the studies (Fernández-Miranda et al. 2005, Nakano et al. 2003) that investigated pooled IBD included patients with indeterminate colitis

Table 1. Characteristics of the studies considered in the meta-analysis.

Author, year of publication	Study area, ethnicity	Polymorphisms	Phenotype studied	Cases			Controls			
				Number	Males (%)	Mean age (years)	Number	Males (%)	Mean age (years)	Matching
Peyrin–Biroulet, 2008	France, Caucasian	*MTHFR* C677T, *MTHFR* A1298C,*MTR* A2756G, *MTRR* A66G, *TCN2* C776G	CD	139	38%	39 (23–45)	251	Nr	35 (30–45)	Age and sex
Chen, 2008	China,E. Asian	*MTHFR* C677T,*MTHFR* A1298C,*MTR* A2756G,*MTRR* A66G,*TCN2* C776G	UC	168	42%	41 ± 12	219	38%	41 ± 15	Age and sex
Bernstein, 2007	Canada, mixed	*MTHFR* C677T	CD and UC separately	486 (CD: 325, UC: 161)	CD: 40%, UC: 51%	CD: 36.0 ± 8.1,UC: 37.2 ± 7.3	409	27%	39.9 ± 5.7	Age, sex and geographically
Yasa, 2007	Turkey, Turkish	*MTHFR* C677T	IBD(CD and UC together)	27 (CD: 2, UC: 22)	74%	CD: 49.8 (26–72),UC: 30.8 (20–49)	47	75%	48.8 ± 7.9	Nr
Yilmaz, 2006	Turkey, Turkish	*MTHFR* C677T, *MTHFR* A1298C	IBD(CD and UC together)	33 (CD: 7, UC: 26)	52%	33.8 ± 14.9	27	63%	34.2 ± 10.5	Age and sex
Stocco, 2006	Italy, Caucasian	*MTHFR* C677T, *MTHFR* A1298C	CD and UC separately	92 (CD: 49, UC: 43)	47%	15 (4–38)	130	Nr	Nr	Nr
Herrlinger, 2005	UK, Caucasian	*MTHFR* C677T, *MTHFR* A1298C	CD and UC separately	407 (CD: 202, UC: 205)	CD: 45%, UC: 48%	CD: 25 (3–82),UC: 32 (2–80)	189	Nr	Nr	Nr
Fernadez–Miranda, 2005	Spain, Caucasian	*MTHFR* C677T, *MTHFR* A1298C	IBD(CD and UC together)	52 (CD: 33, UC: 16,IC: 3)	44%	41.7 ± 11.9	186	38%	41.9 ± 10.1	Not (but similar in age and sex)
Toruner, 2004	Turkey,Turkish	*MTHFR* C677T	CD and UC separately	62 (CD: 28, UC: 34)	48%	37.4 ± 12.1	80	Nr	Nr	Nr
Nakano, 2003	UK,Mixed (84% Caucasians)	*MTHFR* C677T	IBD(CD and UC together)	43(CD: 27, UC: 9,IC: 7)	56%	13.5 (4–16)	47	54%	9.7 (2–15)	Sex
Papa, 2001	Italy, Caucasian	*MTHFR* C677T	IBD(CD and UC together)	64 (CD: 25, UC:39)	CD: 60%, UC: 77%	CD: 40 ± 11,UC: 43 ± 14	195	62%	30 ± 15	Geographically
Vecchi, 2000	Italy, Caucasian	*MTHFR* C677T	CD and UC separately	102 (CD: 51, UC: 51)	CD: 55%, UC: 51%	CD: 40.7 ± 12.8,UC: 43.0 ± 16.4	195	52%	40.9 ± 13.7	Age, sex
Mahmud, 1999	Ireland, Caucasian	*MTHFR* C677T	CD and UC separately	174 (CD: 83, UC: 91)	44%	39 (17–77)	273	Nr	Nr	Nr

nr: not reported

(6% and 16% of the cases). All polymorphisms were meta-analysed, except *CBS* Ins68 I/N.

In all studies, valid genotyping methods were used for the determination of the genetic polymorphisms.

None of the studies provided genotypes according to gender. Controls were reported to be age and/or sex matched in six studies (Peyrin-Biroulet et al. 2008, Chen et al. 2008, Bernstein et al. 2007, Yilmaz et al. 2006, Nakano et al. 2003, Vecchi et al. 2000). Studies were conducted in various populations of 'racial' descent: seven involved Caucasians (Herrlinger et al. 2005, Mahmud et al. 1999, Papa et al. 2001, Peyrin-Biroulet et al. 2008, Stocco et al. 2006, Fernández-Miranda et al. 2005, Vecchi et al. 2000), one East Asians (Chen et al. 2008), three Turkish (Yassa

et al. 2007, Yilmaz et al. 2006, Törüner et al. 2004), and two mixed populations (Bernstein et al. 2007, Nakano et al. 2003).

Summary statistics

Overall, the studies provided 1849/2249 and 892/1002 IBD cases/controls for *MTHFR* C677T and A1298C gene polymorphisms, respectively. Also, the studies provided 308/467 IBD cases/controls for *MTR* 2756, *MTRR* and *TCN2* gene polymorphisms, and 64/195 IBD cases/controls for *CBS*.

Two studies provided data only for 677C carriers (Mahmud et al. 1999, Fernández-Miranda 2005). None of the studies provided data for the combined genotype distribution for C677T and A1298C polymorphisms. The frequency of alleles 677T and 1298C in cases/controls were 0.34/0.35 and 0.32/0.36, respectively. The frequency of genotype 677TC was the highest in both cases (50%) and controls (53%), while that of genotype 677TT was the lowest (13% in both cases and controls). Regarding the A1298C polymorphism, the frequency of genotypes 1298AC and 1298AA were the highest in both cases (46%) and controls (50% and 43%, respectively), while that of genotype 1298CC was the lowest (9% and 8%, respectively). The frequencies of the *MTR* 2756A allele and the *MTRR* 66A allele was the highest in both cases and controls (*MTR* 2756A: 82% and 87%, respectively and *MTRR* 66A: 65% and 56%, respectively). *TCN2* 776G and 776C alleles had similar frequencies in cases (52% and 48%, respectively). The frequency of *CBS* Ins68 normal sequence was 95% and 93% in cases and controls, respectively.

In four studies the distributions of the *MTHFR* C677T (Papa et al. 2001, Törüner et al. 2004), *MTRR* 66 (Peyrin-Biroulet et al. 2008) and *TCN2* (Chen et al. 2008) genotypes in their control groups were not in HWE ($p<0.05$), indicating genotyping errors and/or population stratification and sensitivity analysis was carried out by excluding these studies. None of the studies provided data of combined genotypes or analysis of haplotypes. One study (Chen et al. 2008) reported lack of significant LD between the *MTHFR* C677T and A1298C polymorphisms.

Individual studies' association results

In four individual studies (Mahmud et al. 1999, Peyrin-Biroulet et al. 2008, Chen et al. 2008, Yilmaz et al. 2006), four variants (*MTHFR* C6TTT, *MTHFR* A1298C, *MTR* A2756G and *MTRR* A66G) showed significant associations with IBD, CD or UC risk under any genetic model. Two individual studies showed association for C677T: the one study (Yilmaz et al. 2006) for allele contrast in IBD (OR 2.71, 95% CI 1.04–7.06), and the other study (Mahmud et al. 1999) for the recessive model in IBD, CD and UC (OR 2.64, 95% CI 1.44–4.81; OR 2.57, 95% CI 1.23–5.34; and OR 2.70, 95% CI 1.33–5.47, respectively). One study (Yilmaz et al. 2006) showed association between A1298C and IBD for allele contrast, the additive and dominant models: OR 8.13, 95% CI 2.62–25.20; OR 24.31, 95% CI 1.29–458.3; and OR 7.80, 95% CI 2.20–27.69, respectively. One study (Chen et al. 2008) showed association between *MTR* A2756G and UC for the allele contrast and the dominant model: OR 1.75, 95% CI 1.12–2.73 and OR 1.80, 95% CI 1.10–2.96. One study (Peyrin-Biroulet et al. 2088) showed association between *MTRR* A66G and CD for the allele contrast, the additive and dominant models: OR 0.57, 95% CI 0.42–0.79; OR 0.22, 95% CI 0.11–0.45; and OR 0.20, 95% CI 0.11–0.37, respectively.

Meta-analysis results

Figure 2 and Table 2 show the results for the association between the different gene polymorphisms and the risk of IBD, CD and UC. The overall analysis for investigating the association between the 677T allele and the risk of IBD relative to the allele C, revealed non-significant heterogeneity between the studies ($p=0.34$, $I^2=11\%$) and the risk of IBD on the basis of the current evidence was non-significant (OR 1.01, 95% CI 0.90–1.14). In the subgroup analysis, Caucasians also produced a non-significant association [OR 0.96, 95% CI 0.85–1.07). The sensitivity analysis for HWE did not alter the pattern of results. Regarding the relationship between the C677T polymorphism and CD, overall, the allele contrast showed lack of heterogeneity between studies ($p_Q=0.79$, $I^2=0\%$) with the association being non-significant: OR 0.94, 95% CI 0.82–1.07. In investigating the association between C677T and UC, overall, the allele contrast showed non-significant heterogeneity ($p_Q=0.37$, $I^2=7\%$) and the risk effect was non-significant (OR 0.98, 95% CI 0.84–1.14). Subgroup analysis for Caucasians showed similar results to the overall analysis for both CD and UC. The recessive, dominant and additive models for the effect of allele T produced the same pattern of results with the allele contrast.

Finally, in no case (overall and in Caucasians) was a statistically significant association between the A1298C polymorphism and the risk of IBD, CD or UC for the contrasts under investigation found. The heterogeneity among studies ranged from none (in CD) to high (in IBD).

Concerning the *MTR* A2756G, *MTRR* A66G and *TCN2* C776G gene polymorphisms, significant association was shown only for the allele contrast and the dominant model of *MTR* A2756G (OR 1.48, 95% CI 1.12–1.97 and OR 1.55, 95% CI 1.12–2.15, respectively). However, the information provided for the above polymorphisms was

very limited and therefore, the results should be interpreted with caution. The one study that investigated the *CBS* Ins68 I/N gene polymorphism found non-significant results (for allele contrast OR 0.72, 95% CI 0.31–1.69 and for dominant model OR 0.70, 95% CI 0.29–1.69).

Potential Bias

None of the studies reported that genotyping was blinded to clinical status. In cumulative meta-analysis for C677T in IBD, the recessive model showed a downward trend of association as information accumulates, whereas, A1298C did not show any trend and the association remained non-significant the whole studied period (Figure 3). In the recursive cumulative meta-analysis of the recessive model of C677T, the relative change of OR stabilized around 0.96 indicating that there is sufficient evidence for denying an association (Figure 4). The recursive cumulative meta-analysis for A1298C was based on only three information steps and relative change in 2008/2006 was 0.81 and in 2006/2005 was 1.09, indicating that need of more evidence for denying the association.

Regarding *MTHFR* C677T, the first published study in 1999 (Mahmud et al. 1999) showed an association for the recessive model (OR 2.64, 95% CI 1.39–5.08) whereas the subsequent studies did not replicate the initial finding (only one study published in 2006 (Yilmaz et al. 2006) showed marginal association for the allele contrast). There is no statistical difference between the OR of the first study versus the pooled OR of the subsequent studies ($p = 0.06$). The random effects pooled OR without the first study was OR 0.95, 95% CI 0.78–1.17), and there was lack of heterogeneity between studies ($p_Q = 0.84$, $I^2 = 0\%$). The test by Harbord et al. for C677T in IBD indicated that there is no differential magnitude of effect in large versus small studies ($p = 0.86$).

Discussion

Herein, we presented a comprehensive and systematic assessment of the association between variants involved in the homocysteine pathway and IBD susceptibility. Data from 13 studies provided 1849 IBD cases and 2249 controls were evaluated. The variants included in the synopsis were the *MTHFR* C677T, *MTHFR* A1298C, *MTR* A2756G, *MTRR* A66G, *TCN2* C776G and *CBS* Ins68 I/N gene polymorphisms. All studied polymorphisms (with the exception of *CBS* Ins68 I/N) were meta-analysed providing a decrease of the uncertainty of the estimated genetic risk effects. Overall, no compelling associations between genetic variants and risk of IBD, CD or UC emerged from the analysis, except for the *MTR* A2756G

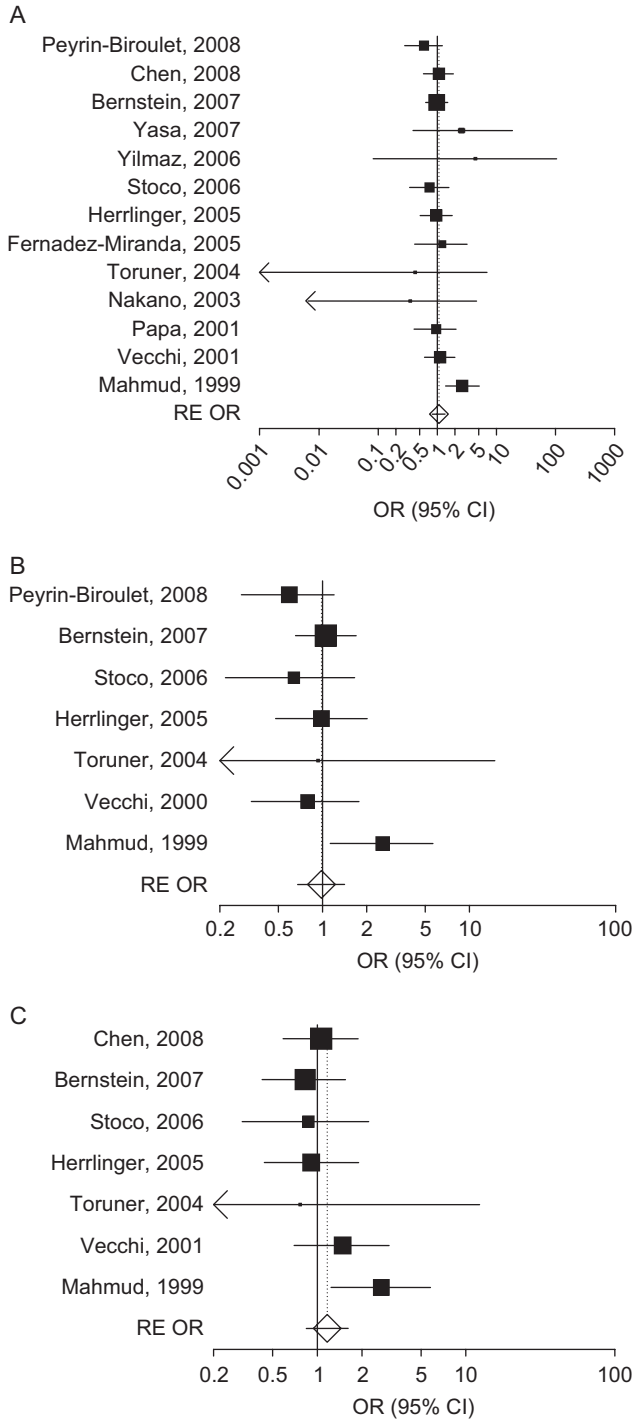

Figure 2. Random effects (RE) odds ratio (OR) estimates with the corresponding 95% confidence interval (CI) for the recessive model of *MTHFR* C677T gene polymorphism and the risk of (A) inflammatory bowel disease (IBD), (B) Crohn's disease (CD) and (C) ulcerative colitis (UC). The OR estimate of each study is marked with a solid black square. The size of the square represents the weight that the corresponding study exerts in the meta-analysis. The CIs of pooled estimates are displayed as a horizontal line through the diamond; this line might be contained within the diamond if the CI is narrow. The arrow indicates a tendency to zero. The horizontal axis is plotted on a log scale.

Table 2. Random effects odds ratios (OR) and heterogeneity results for the genetic contrasts of (a) *MTHFR* C677T, (b) *MTHFR* A1298C and (c) *MTR* A2756G, *MTRR* A66G and *TCN2* C776G gene polymorphisms for inflammatory bowel disease (IBD), Crohn's disease (CD) and ulcerative colitis (UC).

(a)

MTHFR *C677T*		Population	Cases/controls	Studies	OR (95% CI)	I^2, p_Q–value
IBD	Allele contrast	All	1623/1790	11	1.01 (0.90–1.14)	11%, 0.34
		All in HWE	1497/1515	9	1.00 (0.88–1.13)	17%, 0.29
		Caucasians	1290/1369	6	0.96 (0.85–1.07)	0%, 0.62
	Recessive model	All	1849/2249	13	1.06 (0.83–1.36)	27%, 0.18
		All in HWE	1722/1973	11	1.08 (0.82–1.43)	37%, 0.10
		Caucasians	1548/1854	8	1.05 (0.78–1.42)	47%, 0.07
	Dominant model	All	1623/1790	11	1.10 (0.91–1.34)	32%, 0.14
		All in HWE	1497/1515	9	1.05 (0.86–1.28)	32%, 0.17
		Caucasians	1290/1369	6	0.99 (0.80–1.22)	33%, 0.19
	Additive model	All	883/1024	11	0.95 (0.75–1.19)	0%, 0.78
		All in HWE	795/826	9	0.93 (0.73–1.18)	0%, 0.70
		Caucasians	701/765	6	0.89 (0.69–1.15)	0%, 0.85
CD	Allele contrast	All	794/1254	6	0.94 (0.82–1.07)	0%, 0.79
		Caucasians	766/1174	5	0.94 (0.82–1.08)	0%, 0.78
	Recessive model	All	877/1527	7	0.98 (0.68–1.41)	40%, 0.12
		Caucasians	849/1447	6	0.98 (0.66–1.45)	50%, 0.07
	Dominant model	All	794/1254	6	0.95 (0.78–1.15)	0%, 0.51
		Caucasians	766/1174	5	0.95 (0.79–1.15)	0%, 0.43
	Additive model	All	430/699	6	0.86 (0.63–1.17)	0%, 0.87
		Caucasians	403/624	5	0.86 (0.63–1.17)	0%, 0.77
UC	Allele contrast	All	662/1223	6	0.98 (0.84–1.14)	7%, 0.37
		Caucasians	460/923	5	0.96 (0.83–1.12)	0%, 0.47
	Recessive model	All	753/1496	7	1.17 (0.85–1.62)	28%, 0.22
		Caucasians	551/1196	6	1.18 (0.83–1.67)	39%, 0.15
	Dominant model	All	662/1223	6	0.97 (0.74–1.27)	34%, 0.18
		Caucasians	460/923	5	0.93 (0.74–1.17)	17%, 0.31
	Additive model	All	369/680	6	0.97 (0.69–1.29)	0%, 0.89
		Caucasians	249/480	5	0.96 (0.83–1.12)	0%, 0.47

(b)

MTHFR *A1298C*		Population	Cases/controls	Studies	OR (95% CI)	I^2, p_Q–value
IBD	Allele contrast	All	892/1002	5	1.17 (0.84–1.64)	73%, 0.01
		Caucasian	639/570	3	1.09 (0.91–1.30)	0%, 0.85
	Recessive model	All	893/1003	6	1.05 (0.71–1.57)	11%, 0.35
		Caucasians	725/784	4	1.09 (0.74–1.60)	0%, 0.86
	Dominant model	All	893/1003	5	1.19 (0.83–1.72)	62%, 0.03
		Caucasians	639/570	3	1.12 (0.89–1.43)	0%, 0.95
	Additive model	All	483/491	5	1.12 (0.63–1.99)	41%, 0.15
		Caucasians	340/312	3	1.15 (0.75–1.77)	0%, 0.73
CD	Allele contrast	Caucasians	391/570	3	1.02 (0.84–1.25)	0%, 0.69
	Recessive model	Caucasians	391/570	3	0.98 (0.62–1.55)	0%, 0.75
	Dominant model	Caucasians	391/570	3	1.04 (0.80–1.36)	0%, 0.70
	Additive model	Caucasians	210/312	3	1.01 (0.62–1.64)	0%, 0.73
UC	Allele contrast	All	416/538	3	1.09 (0.81–1.47)	44%, 0.17
		Caucasian	248/319	2	1.24 (0.96–1.61)	na, 0.48
	Recessive model	All	416/538	3	1.13 (0.60–2.16)	29%, 0.25
		Caucasians	248/319	2	1.41 (0.79–2.50)	na, 0.48
	Dominant model	All	416/538	3	1.11 (0.82–1.51)	20%, 0.29
		Caucasians	248/319	2	1.30 (0.92–1.84)	na, 0.59
	Additive model	All	251/333	3	1.20 (0.56–2.58)	43%, 0.17
		Caucasians	130/178	2	1.58 (0.86–2.89)	na, 0.44

Table 2. Continued on next page

Table 2. Continued.

(c)

MTR *A2756G*		Population	Cases/controls	Studies	OR (95% CI)	I^2, p_Q–value
IBD	Allele contrast	All	308/467	2	1.48 (1.12–1.97)	na, 0.35
	Recessive model	All	308/467	2	1.76 (0.73–4.25)	na, 0.70
	Dominant model	All	308/467	2	1.55 (1.12–2.15)	na, 0.43
	Additive model	All	220/362	2	1.96 (0.81–4.75)	na, 0.70
MTRR A66G						
IBD	Allele contrast	All	280/467	2	0.82 (0.41–1.63)	na, < 0.01
	Recessive model	All	280/467	2	0.89 (0.58–1.35)	na, 0.33
	Dominant model	All	280/467	2	0.50 (0.09–2.86)	na, < 0.01
	Additive model	All	166/238	2	0.53 (0.09–3.04)	na, < 0.01
TCN2 C776G						
IBD	Allele contrast	All	308/467	2	1.03 (0.84–1.26)	na, 0.87
	Recessive model	All	308/467	2	0.93 (0.67–1.29)	na, 0.67
	Dominant model	All	308/467	2	1.16 (0.83–1.62)	na, 0.71
	Additive model	All	161/260	2	1.09 (0.72–1.64)	na, 0.97

na, non applicable; CI, confidence interval.

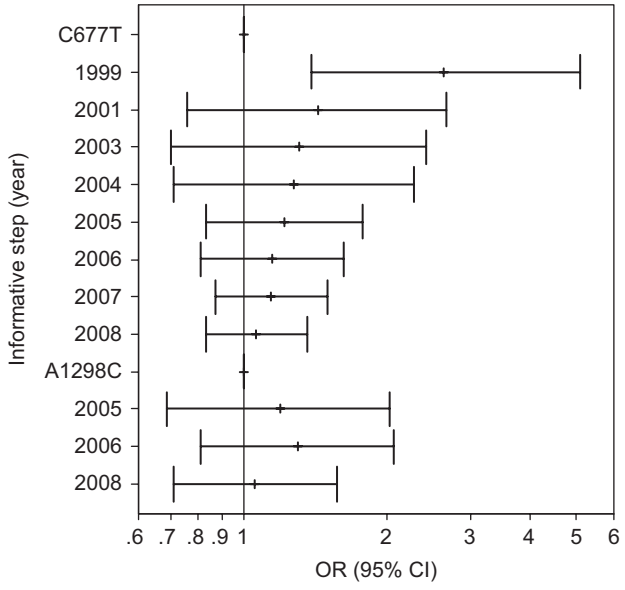

Figure 3. Cumulative meta-analysis for *MTHFR* C677T and A1298C in inflammatory bowel disease (IBD): the pooled odds ratio (OR) with the corresponding 95% confidence interval (CI) at the end of each year-information step is shown.

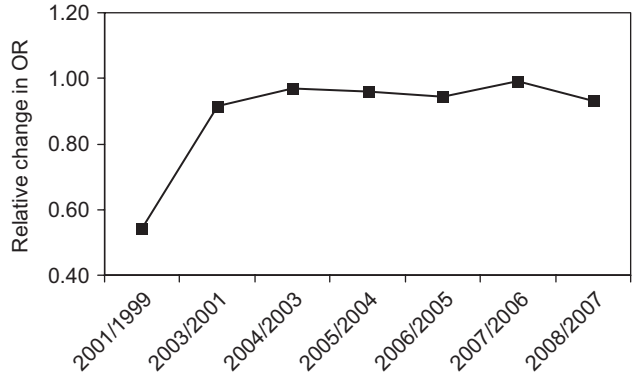

Figure 4. Recursive cumulative meta-analysis for *MTHFR* C677T in inflammatory bowel disease (IBD): the relative change in random effects pooled odds ratio (OR) in each information step (OR in next year/OR in current year) is shown.

gene polymorphism. The analysis indicated that *MTR* 2756G-carriers have 48% higher risk for IBD relative to non-carriers. Regarding the *MTHFR* C677T, the first published study produced significant result but the subsequent studies failed to replicate the initial finding. However, in meta-analysis of genetic association studies, it is frequent that the results of the first study do not correlate with the results of the subsequent studies, and usually the first studies tend to overestimate the magnitude of an association. This can be due to bias or to genuine diversity of the populations involved in the studies (Zintzaras & Lau 2008a). Although the meta-analysis of *MTHFR* C677T produced a non-significant association with IBD, the meta-analysis provided useful information for the uncertainty of the estimated genetic risk, i.e. the pooled OR excluded with 95% certainty that *MTHFR* 677T-carriers would have more than 22% risk increased risk of IBD. The heterogeneity between studies was in general low without indication of differential magnitude of effect in large versus small studies. The conclusions reached in the present analysis were based on relatively small numbers of studies and participants for each gene polymorphism and therefore, any inferences have to be cautious.

In genetic epidemiology, sample sizes of individual genetic association studies tend to be small and a single institution alone will be able to provide a reasonable number of patients (Zintzaras & Lau 2008a). The creation of large databases and consortia that facilitate the sharing and pooling of resources among investigators would be a solution to overcome the requirements for large sample sizes (Zintzaras & Lau 2008b). Synthesis of

data from many studies is expected to improve power and reduce the false discovery rate (Zintzaras & Lau 2008a) in all circumstances and the gain could be considerable, unless there is very large genuine between-study heterogeneity which is not the case in the present meta-analysis. However, power calculations are usually considered inappropriate in meta-analysis as these data are already assembled (Zintzaras & Lau 2008a). In addition to larger sample sizes, the selection cases that are genetically loaded (i.e. cases with strong familial history) may also improve the power to detect small risk effects.

The lack of consistently replicated susceptibility genes for IBD questions selection of the homocysteine/folate pathway genes as candidate genes for elucidating the complex genetic architecture of the disease. The biological plausibility of the investigated candidate genes might have been exaggerated and the selection of genetic variants was possibly unsuccessful. In addition to candidate-gene studies, genome-wide association studies (GWAS) may assist in the selection of candidate polymorphisms by identifying the most active genes involved in the disease pathophysiology (Zintzaras & Lau 2008a). GWAS have provided 'hypothesis-free' evidence for a considerable number of risk variants predisposing to IBD. Genetic variants whose association with IBD has been fairly established include variants of the genes *NOD2/CARD15*, *IL23R* and *ATG16L* (Hugot et al. 2001, Ogura et al. 2001, Hampe et al. 2007, Duerr et al. 2006, Wellcome Trust Case Control Consortium 2007). None of the GWAS has identified variants involved in the homocysteine/folate metabolism pathway. However, the positive hits from the massive genomic scans produced by GWAS still warrant careful replication in independent studies and explain only a fraction of the disease heritability (Kitsios & Zintzaras 2009a). Alternatively, a genomic convergence approach of different data sources (candidate-gene association, hypothesis-free association, linkage or expression profiling data) has the potential to prioritize variants for further research and provide more insights into the mechanisms involved in IBD (Kitsios & Zintzaras 2009a,b).

The overall lack of association between the investigated variants and IBD (or CD and UC) might be due to other variants of the homocysteine/folate metabolic pathway which are in linkage disequilibrium and affect the susceptibility to IBD. As with other complex diseases, the development of IBD is probably determined by multiple epistatic and gene–environment interactions. Because most genetic risk factors are believed to contribute only small influences on disease susceptibility, the analysis of individual gene polymorphism genotypes may not be reliable markers of risk for developing IBD and interactions of gene polymorphisms can be a major determinant of disease risk. Then, a meta-analysis of genotype combinations may provide more reliable information than single gene polymorphisms (Zintzaras et al. 2006b). In the present meta-analysis, there no data were available for performing such an analysis. In addition, the assessment of environmental exposures may affect the genetic risk of IBD, and such parameters should be incorporated in future studies. The present analysis used the available study-level allele and genotype distributions, precluding adjusted analysis for potential gene–gene and gene–environment interactions (Clayton & McKeigue 2001, Cooper 2003, Chapman & Clayton 2007), for which raw genotype data would be required. However, failure to account for these interactions may have reduced the efficiency of the analysis but is unlikely to inflate the number of positive results.

The risk effect may depend on effect modifiers (e.g. age, sex and lifestyle) that influence the estimates of associations and modulate the development of IBD (Mahmud et al. 1999, Papa et al. 2001, Fernández-Miranda et al. 2005). In the meta-analysis possible effect modifiers were not taken into account and only the unadjusted pooled ORs were calculated as data for each level of effect modifier were not provided. Sampling variability and stratification in genetic association studies could be a possible confounding factor on the role of genetic markers. The strict selection criteria ensure a clear case and control definition for meta-analysis because when the possibility of a case being considered as a control is minimized, then the estimation of risk is unbiased (Zintzaras & Lau 2008a). The cases and controls of each study were well defined with similar inclusion criteria, although they unavoidably cover a wide spectrum of disease, in terms of duration, demographics and other clinical manifestations. However, in many studies the controls were not matched by age and a subject may be misclassified as a control, although still at risk for IBD. Then, there is a fundamental risk of bias in these studies. In addition, risk effect may dependent on gene methylation, and thus, gene–environment interaction between the genotypes and dietary intake, and in particular folic acid consumption, is essential to maintain, or alter, the effect of the polymorphic variants (Mahmud et al. 1999, Cooper et al. 2003, Blount et al. 1997).

In three studies the controls did not conform to HWE, the lack of HWE indicates genotyping errors, population stratification and selection bias (Zintzaras 2008). Hence, the validity of the genotyping method and the selection of controls are questionable. Moreover, the absence of reporting blindness to phenotype in genotyping laboratory personnel in all articles and the possible lack of a controlled genotyping procedure could cause potential bias. Non-English, non-indexed and non-published studies were not included in the meta-analysis, thus, bias could be introduced (Egger et al. 2003). However, it has been reported that studies published in non-English journals show lower methodological quality and the

exclusion of published studies in languages other than English may not have an effect on pooled estimates (Egger et al. 2003, Juni et al. 2002).

In summary, the current accumulated evidence has indicated lack of association between genetic variants involved in homocysteine/folate metabolism and risk of IBD, CD or UC. Only the *MTR* A2756G gene polymorphism showed an association. The results of the present analysis were based on relatively small numbers of studies and participants and their interpretation has to be cautious. IBD is a complex disease with multifactorial aetiology and therefore, the minor contributing pathogenetic role of the investigated variants in specific cases, and in combination with other environmental factors, cannot be totally excluded. The results of rigorous studies designed for the investigation of epistatic and gene–environment interactions (Clayton & McKeigue 2001, Zintzaras & Lau 2008a) might produce more conclusive claims about the genetics of IBD.

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