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Polymorphisms of glutathione S-transferase and methylenetetrahydrofolate reductase genes in Moldavian patients with ulcerative colitis: Genotype-phenotype correlation

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ABSTRACT

Background: Glutathione S-transferases (GSTM1, GSTT1, and GSTP1) and methylenetetrahydrofolate reductase (MTHFR) are important enzymes for protection against oxidative stress. In addition, MTHFR has an essential role in DNA synthesis, repair, and methylation. Their polymorphisms have been implicated in the pathogenesis of ulcerative colitis (UC). The aim of the present study was to investigate the role of selected polymorphisms in these genes in the development of UC in the Moldavian population.
Methods: In a case-control study including 128 UC patients and 136 healthy individuals, *GSTM1* and *GSTT1* genotypes (polymorphic deletions) were determined using multiplex polymerase chain reaction (PCR). The *GSTP1* rs1695 (Ile105Val), *MTHFR* rs1801133 (C677T), and *MTHFR* rs1801131 (A1298C) polymorphisms were studied with restriction fragment length polymorphism (RFLP) analysis. Genotype–phenotype correlations were examined using logistic regression analysis.
Results: None of the genotypes, either alone or in combination, showed a strong association with UC. The case-only sub-phenotypic association analysis showed an association of the *MTHFR* rs1801133 polymorphism with the extent of UC under co-dominant (p *corrected* = 0.040) and recessive (p *corrected* = 0.020; OR = 0.15; CI = 0.04–0.63) genetic models. Also, an association between the *MTHFR* rs1801131 polymorphism and the severity of UC was reported for the over-dominant model (p *corrected* = 0.023; coefficient = 0.32; 95% CI = 0.10–0.54).
Conclusion: The *GST* and *MTHFR* genotypes do not seem to be a relevant risk factor for UC in our sample. There was, however, evidence that variants in *MTHFR* may influence the clinical features in UC patients. Additional larger studies investigating the relationship between *GST* and *MTHFR* polymorphisms and UC are required.

1. Introduction

Ulcerative colitis (UC) is a chronic inflammatory condition of the large intestine, which along with Crohn's disease comprises the major part of the inflammatory bowel diseases (Abraham and Cho, 2009). Inflammatory bowel diseases (IBD) and specifically UC are not evenly distributed throughout the world, with North America and western/northern Europe having the highest rate (100–250 per 100,000 population for UC) (Burisch and Munkholm, 2015). In the Republic of Moldova, as in most other eastern/southeastern European countries, UC appears to be much less common (20–25 per 100,000 population in Moldavia, unpublished data), although continuous increase in the disease incidence over the past years has been reported (Burisch and Munkholm, 2015). The precise etiology of UC remains unclear. Several mechanisms related to immunological, genetic, toxic, and infection abnormalities are implicated in the pathogenesis of UC (Ananthakrishnan, 2015).

Oxidative stress due to overproduction of reactive oxygen species (ROS) and decreased efficiency of antioxidant defenses has been considered to be a common pathogenic factor in UC and its complications. ROS overproduction has been shown in the inflamed mucosa of UC patients and in experimental animal models of IBD (reviewed by Rezaie et al., 2007). Excessive amounts of ROS can destroy biomolecules such as lipids, proteins, and DNA, leading to cellular stress and endothelial dysfunction in UC patients (Valko et al., 2007; Piechota-Polanczyk and Fichna, 2014). Production of oxidants and free radicals can also facilitate activation of signaling events that mediate expression of inflammatory

Abbreviations: UC, Ulcerative colitis; IBD, Inflammatory bowel disease; GST, Glutathione S-transferase; MTHFR, Methylenetetrahydrofolate reductase; SAM, S-adenosyl methionine; n, Total number; SNP, Single nucleotide polymorphism; PCR, Polymerase chain reaction; RFLP, Restriction fragment length polymorphism; HWE, Hardy–Weinberg equilibrium.

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genes as well as genes regulating cell division, differentiation, and apoptosis (Valko et al., 2007). It has been suggested that the impaired prooxidant and antioxidant system in UC patients may contribute to the disease process (Pravda, 2005). The human glutathione S-transferases (GSTs) are well-known oxidative stress-related detoxification enzymes. Located mainly in the cytosol, GST enzymes catalyze the conjugation of electrophilic substrates to glutathione, thus facilitating detoxification and further metabolization and excretion (Hayes et al., 2005). They also play an important role in peroxidase and isomerase activities (Sheehan et al., 2001). Several classes of GSTs have been identified, with GSTT1, M1, and P1 being the most well-characterized forms. Polymorphisms within these genes either decrease or abolish GST enzyme activity (Strange et al., 2000). Thus, a functionally significant A to G transition in exon 5 of the *GSTP1* gene (A313G, rs1695), which results in replacing isoleucine with valine (Ile105Val), substantially diminishes GSTP1 enzyme activity. By contrast, homozygous whole gene deletions of *GSTT1* or *GSTM1* cause a lack of the respective enzyme function. These *GST* genes polymorphisms have been linked to inflammation and immune processes in a number of reports (Bekris et al., 2005; Aguilera et al., 2004; Babushok et al., 2013; Liang et al., 2013; Živković et al., 2013; Ding et al., 2014).

It is known that homocysteine (Hcy) induces oxidative stress (Loscalzo, 1996). Hyperhomocysteinemia is common among UC patients, and elevated Hcy levels are associated with deep vein thrombosis and thromboembolic disease in UC patients (Peyrin-Biroulet et al., 2007; Akbulut et al., 2010). Besides its prooxidant properties, Hcy is also known to play a role in epigenetic gene regulation being directly involved in the DNA methylation process (Peyrin-Biroulet et al., 2007). Noteworthy, excessive DNA methylation is closely associated with the inflammatory state of the colon in UC (Karatzas et al., 2014). The 5,10-methylenetetrahydrofolate reductase (MTHFR) is a key regulatory enzyme in folate and Hcy metabolism (Finkelstein, 1998). It converts 5,10-methylenetetrahydrofolate (a derivative of folic acid) irreversibly to 5-methyltetrahydrofolate, which donates its methyl group to Hcy in the generation of methionine. Methionine is in turn converted to S-adenosylmethionine (SAM), the methyl donor in DNA methylation. Two single nucleotide polymorphisms (SNPs) in the *MTHFR* gene, rs1801133 (C677T, Ala222Val) and rs1801131 (A1298C, Glu429Ala), have been associated with reduced enzyme activity, elevated Hcy levels, and reduced methionine/SAM supply for methylation (Frosst et al., 1995; Weisberg et al., 1998). Numerous epidemiologic and experimental studies have shown that genetic polymorphisms in the *MTHFR* gene may be related to various pathological states, including colorectal cancer and autoimmune diseases (Zhao et al., 2013; Mavragani et al., 2007; Afeltra et al., 2005; Foffa et al., 2009; Mao et al., 2010).

The roles of the *GSTM1*, *GSTT1*, *GSTP1*, and *MTHFR* genotypes in susceptibility to UC have been discussed by some investigators, but no consistent conclusions have yet been made. Their effect on the particular clinical phenotypes also remains to be clarified. Here we explored the role of these genotypes in the development of UC in Moldavian population.

2. Materials and methods

2.1. Samples

This case-control study comprises 128 unrelated UC patients, recruited at the Department of Gastroenterology, Republican Clinical Hospital, Moldova. The diagnosis of UC was based on standard clinical, endoscopic, and histological criteria (Lennard-Jones, 1989). The distribution of UC lesions was defined according to the Montreal classification (Satsangi et al., 2006). The subjects with UC were also classified using three additional clinical categories: (i) disease severity as assessed by the modified Truelove–Witts disease activity index (Dignass et al., 2012); (ii) UC-related outcomes—i.e. strictures, lead pipe colon, malignancy, steroid-dependency, and colectomy: absent *versus* present; and (iii) disease relapse rate: infrequent (≤1/year) *versus* frequent (≥2/year). All patients were Caucasians of European descent. The control population consisted of 136 unrelated and ethnically matched healthy individuals who had no history of autoimmune or oncological disease. Information on smoking habits was collected from both patients and healthy controls. According to smoking habit, cases were divided into 3 groups: current smokers, ex-smokers, and never smokers, and controls were classified as current smokers and current non-smokers. The lack of correspondence between cases and controls in smoking definitions is due to the lack of information on former smoking status in controls. Therefore, ex-smoker and never-smoker UC patients were combined in one group to make them comparable with healthy controls in the case-control study. The combination is further justified by findings that both the never- and ex-smokers have an increased risk of UC compared with current smokers, as reported previously (Lakatos et al., 2013). The demographics and clinical features of the study population are depicted in Table 1. EDTA anti-coagulated venous blood samples were collected from all participants, and genomic DNA was extracted from peripheral blood leukocytes using a standard salting out method (Miller et al., 1988). The local ethics committee approved the study, and informed written consent was received from all subjects.

2.2. Genotyping

GSTM1 and *GSTT1* genotypes were determined using multiplex polymerase chain reaction (PCR). Three sets of primers were used to amplify a 434-bp sequence of the *GSTT1* gene (Zheng et al., 2001), a 267-bp fragment of the *GSTM1* gene (Tujague et al., 2006), and a 212-bp segment of the human albumin gene as an internal amplification control (Tujague et al., 2006). The PCR reactions were carried out in 20 μL containing 5 pmol of each primer, 100 ng genomic DNA, 1.5 mmol/L MgCl2, 200 μmol/L dNTPs, and 0.5 unit of Taq DNA polymerase in the buffer provided by the manufacturer. Amplification was performed in a Bio-Rad T100 Thermal Cycler (Bio-Rad, Hercules, California) for the PCR reaction. The amplification conditions consisted of an initial melting step 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 s, 57 °C for 30 s, and 72 °C for 40 s; and a final elongation step of 72 °C for 5 min. Resulting fragments were visualized using ethidium bromide staining and 3% agarose gel electrophoresis (Fig. 1). *MTHFR* rs1801133 (C677T, Ala222Val), *MTHFR* rs1801131 (A1298C, Glu429Ala), and *GSTP1* rs1695 (A313G,

Table 1
Characteristics of the study population.

Characteristic	UC (n = 128) n (%)	Healthy controls (n = 136) n (%)
Sex		
Female	59 (46.1)	52 (38.2)
Male	69 (53.9)	84 (61.8)
Smoking		
Never	98 (76.6)	NA
Former	16 (12.5)	NA
Current	14 (10.9)	48 (35.3)
Age at recruitment, median ± S.D. (years)	41.4 ± 13.7	45.9 ± 10.8
Age at diagnosis, median ± S.D. (years)	36.7 ± 13.4	
Extent of disease		
Distal colitis	53 (41.4)	
Left-sided colitis	39 (30.5)	
Pancolitis	36 (28.1)	
Severity		
Mild	41 (32.0)	
Intermediate	60 (46.9)	
Severe	27 (21.1)	
Negative UC-related outcomes		
Absent	73 (57.9)	
Present	53 (42.1)	
Relapse rate		
Infrequent	67 (53.2)	
Frequent	59 (46.8)	

NA, not available.

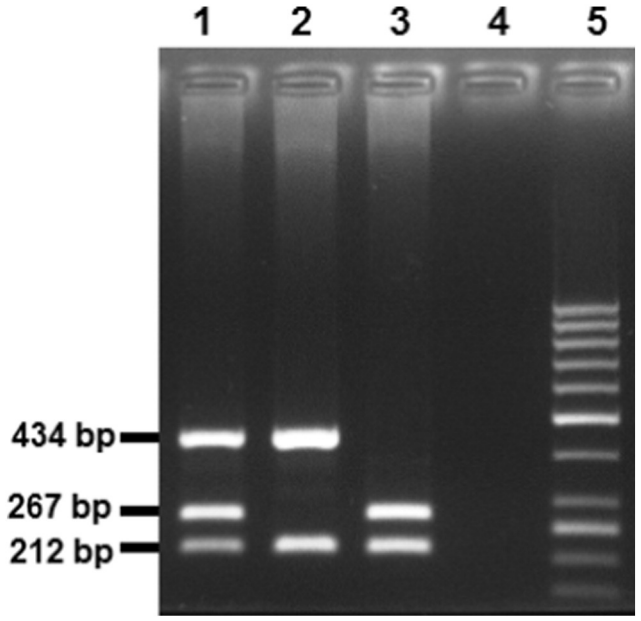

Fig. 1. Electrophoresis of the products of the multiple PCR. Presence of 267 and 434-bp fragments indicates *GSTM1* and *GSTT1* wild-type (non-null) genotypes, respectively. The 212-bp band corresponding to a fragment of the human albumin gene, which serves as a positive control for the PCR. Lane 5: molecular weight marker (GeneRuler 50 bp DNA Ladder, Thermo Fisher Scientific); lane 1: *GSTM1* and *GSTT1* non-null genotype; lane 2: *GSTM1* null/*GSTT1* non-null genotype; lane 3: *GSTM1* non-null/*GSTT1* null genotype; lane 4: negative PCR control.

Ile105Val) were determined by polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) with *Hin*fI, *Mbo*II, and *Alw*26I restriction endonucleases, respectively, as reported elsewhere (Garte et al., 2007; Eloualid et al., 2012). The digestion fragment sizes for the *MTHFR* rs1801133 genotypes were a single 198-bp band for CC; 198, 175, and 23 bp (3 fragments) for CT; and 175 and 23 bp (2 fragments) for TT. For rs1801131 genotypes, the fragments were 56, 31, 30, 28, and 18 bp (5 fragments) for AA; 84, 56, 31, 30, 28, and 18 bp (6 fragments) for AC, and 84, 31, 30 and 18 bp (4 fragments) for CC. For the *GSTP1* rs1695 polymorphism, AA homozygotes had two fragments (329 bp and 104 bp), AG heterozygotes demonstrated four DNA bands (329, 222, 107, and 104 bp) and GG homozygotes showed three fragments (222, 107, and 104 bp). Genotyping errors were excluded by random re-genotyping of the respective loci and therefore, all polymorphisms were included into the association analysis.

2.3. Statistical analysis

Comparison of demographical parameters between cases and controls was performed using Student's t-test for continuous variables and the χ^2 test for categorical data. Deviation from Hardy–Weinberg equilibrium (HWE) was assessed by the Fisher exact test. The linkage disequilibrium between the two polymorphisms in *MTHFR* gene was examined using D′ and r^2 coefficients. Genotype frequencies in the case and control groups were compared by logistic regression with adjustment for sex, age at investigation, and current smoking status under the additive, dominant, recessive, co-dominant, and over-dominant genetic models. Logistic regression model was also constructed to determine the effect of genetic polymorphisms on the clinical phenotypes of UC (disease extent, relapse, severity, complications, and age at onset). The odds ratios (OR) with their corresponding 95% confidence intervals (CI) and p-values were calculated as measures of associations. p-values <0.05 were considered as significant. The Bonferroni correction for multiple comparisons was applied where appropriate, and the threshold was calculated as $0.05/5 = 0.01$. For significant p-values, Fisher exact test was additionally calculated as suggested in (Lettre et al., 2007). The Fisher test is particularly relevant when one of the alleles is rare, and together with pooling the genotypes according to the genetic model, the statistical significance can be estimated more adequately (Lewis, 2002). Combined effects of polymorphisms on the disease risk were analyzed using logistic regression for all possible combinations of loci under three genetic models: dominant, recessive, and over-dominant. In the dominant model, the rare homozygous variant was combined with the heterozygous, while in the recessive model, the rare homozygous genotype was considered alone. In the over-dominant model, the heterozygous and both homozygous genotypes together were encoded using two dummy variables. Therefore, tests for genotype combinations had nine degrees of freedom for pairwise combinations of *MTHFR* rs1801133, rs1801131, and *GSTP1* rs1695 polymorphisms (three for each SNP), six degrees of freedom for combinations of *MTHFR* rs1801133, rs1801131, and *GSTP1* rs1695 polymorphisms with *GSTT1* and *GSTM1* loci, and a four-degree of freedom for combined *GSTT1* and *GSTM1* genotypes. Accordingly, significant p-values were corrected using the Bonferroni method by multiplying by factors 9, 6, or 4. Power of the study was calculated post-hoc assuming the following variables: significance (type 1 error) 0.05, genetic effects (odds ratio [OR]) 1.5 and 2.0, and disease prevalence of 0.00025; allele frequencies were those found in control population. The statistical tests were performed with SNPStats program and E-Views software (IHS Global Inc.).

3. Results

There were significant differences in age and smoking status (current smokers *vs.* non-smokers) distributions between the case and control groups ($p = 0.00322$ and $p = 3.0E\text{-}6$, respectively; Table 1). Although there was no significant difference in sex ratio between the two groups ($p = 0.168462$), their matching on gender was imperfect: the cases had a higher percentage of female (46.1%) than the controls (38.2%) (Table 1). All three of these demographic variables were included as covariates in all subsequent multivariate regression analyses.

Table 2 summarizes the distribution of *MTHFR* and *GST* genotypes and allele frequencies in the cohort of 128 UC patients and in 136 healthy controls. The observed frequencies of the studied polymorphisms were in a range of values observed in other Caucasian–European populations. Allelic distributions of the investigated SNPs were in accordance with Hardy–Weinberg equilibrium (HWE) in both groups except for GSTP1 rs1695, which showed a slight deviation from HWE in controls ($p = 0.026$). Linkage disequilibrium analysis showed strong LD (pairwise $D' = 0.969$) between the two *MTHFR* loci at nucleotide positions 677 (rs1801133) and 1298 (rs1801131). However, due to a weak correlation ($r^2 = 0.205$), the two SNPs cannot substitute each other and were analyzed in an independent manner.

No significant differences were observed in the frequencies of the *MTHFR* and *GST* genotypes and alleles between UC subjects and controls (Table 2). The lack of association persisted after stratification by gender or smoking status (data not shown). In pairwise combined analyses of *MTHFR* rs1801133 and rs1801131, *GSTP1* rs1695, *GSTM1*, and *GSTT1* gene polymorphisms, only five different genotype combinations were significantly associated with UC (Table 3). However, the significance was eliminated after applying a Bonferroni correction (p *corrected* > 0.05). No other combinatorial genotypes reached a nominal significance (data not shown).

In addition, we also performed a detailed genotype–phenotype analysis of *GST* and *MTHFR* variants in UC patients. There was no association of these polymorphisms with age of onset, complications, and relapse rate (data not shown). Also, no association was observed between *GSTM1* and *GSTT1* genes and disease spread or severity (data not shown). We showed that the AC genotype of *MTHFR* rs1801131 was significantly associated with increased severity in the over-dominant genetic model (coefficient = 0.32; 95% CI = 0.10–0.54; $p = 0.0046$; Table 4). Both the co-dominant genetic model ($p = 0.018$) and the

Table 2
Association tests for single polymorphisms.

Polymorphism	Genotype/Allele	Controls n (%)	Cases n (%)	Model	p-value*
MTHFR rs1801133	CC	70 (51.5)	59 (46.1)	Codominant	0.63
	CT	52 (38.2)	55 (43)	Dominant	0.35
	TT	14 (10.3)	14 (10.9)	Recessive	0.87
	C	192 (70.6)	173 (67.6)	Overdominant	0.39
	T	80 (29.4)	83 (32.4)	Additive	0.44
MTHFR rs1801131	AA	66 (48.5)	52 (41.3)	Codominant	0.27
	AC	59 (43.4)	57 (45.2)	Dominant	0.14
	CC	11 (8.1)	17 (13.5)	Recessive	0.27
	A	191 (70.2)	161 (63.9)	Overdominant	0.43
	C	81 (29.8)	91 (36.1)	Additive	0.11
GSTP1 rs1695	AA	60 (44.4)	62 (49.2)	Codominant	0.29
	AG	68 (50.4)	53 (42.1)	Dominant	0.42
	GG	7 (5.2)	11 (8.7)	Recessive	0.27
	A	188 (69.6)	177 (70.2)	Overdominant	0.18
	G	82 (30.4)	75 (29.8)	Additive	0.83
GSTT1	Present	111 (81.6)	108 (84.4)		0.77
	Null	25 (18.4)	20 (15.6)		
GSTM1	Present	61 (44.9)	52 (40.6)		0.65
	Null	75 (55.1)	76 (59.4)		

* The p-values were obtained from logistic regression with co-dominant, dominant, recessive, over-dominant and additive models, and adjusted for sex, age and current smoking status.

dominant genetic model (coefficient = 0.26; 95% CI = 0.04–0.48; p = 0.022) also obtained significant results (Table 4). However, only the p-value under the over-dominant model remained significant after Bonferroni correction for multiple tests was applied (p *corrected* = 0.023). We also report a significant association between *MTHFR* rs1801133 polymorphism and disease extent (co-dominant model: p = 0.008; Table 5). This was primarily due to a significantly higher frequency of the TT genotype in patients with distal colitis than in patients with more extensive UC types (recessive model: OR = 0.15; CI = 0.04–0.63; p = 0.0041). The values were still significant after Bonferroni correction (p *corrected* = 0.040 and p *corrected* = 0.020 for co-dominant and recessive models, respectively; Table 5). Furthermore, the associations were validated by Fisher exact test (p *corrected* = 0.0375 and p *corrected* = 0.0185 for co-dominant and recessive models, respectively; Table 5).

With regard to the power analysis in the combined sample, our sample set was estimated to have enough power (>89%) to detect moderate high-risk alleles (OR = 2) but limited (49–64%) for moderate low-risk alleles (OR = 1.5). The power was even smaller in the stratified and combinatorial analyses and this was a limitation of this study.

4. Discussion

Wide evidence suggests that oxidative stress is involved in UC pathogenesis (Rezaie et al., 2007; Valko et al., 2007; Piechota-Polanczyk and Fichna, 2014). The enzymes glutathione S-transferases T1, M1, and P1, and methylenetetrahydrofolate reductase (MTHFR) are implicated in the antioxidant defenses (Hayes et al., 2005; Raza, 2011; Hoffman, 2011). The analysis of their variation has been widely used in the field of cancer and inflammatory genetics. Their role in the pathogenesis of UC has been also proposed but has not been extensively studied. The present study was designed to investigate the contribution of *GSTT1*, *GSTM1*, *GSTP1*, and *MTHFR* genetic polymorphisms to the risk and pathogenesis of UC in Moldavian population. To the best of our knowledge, this is the first report on *GSTT1*, *GSTM1*, *GSTP1*, and *MTHFR* genes in UC patients from eastern–southeastern Europe.

We did not observe significant associations between any studied polymorphisms and UC. Furthermore, no Bonferroni-corrected significant associations of combined genotypes were found, implying that these combinations are probably not synergistic in their effect on UC risk in Moldavian population. These findings were not totally unexpected. Indeed, previous studies on the association between the same polymorphisms and susceptibility to UC have reported conflicting results. Thus, an association of *MTHFR* rs1801133 genotypes with UC has been reported in Ireland (Mahmud et al., 1999), Denmark (Nielsen et al., 2000), and Portugal (Magro et al., 2003), but not in China (Chen et al., 2005, 2008; Jiang et al., 2012), the UK (Herrlinger et al., 2005), Italy (Vecchi et al., 2000; Papa et al., 2001), Turkey (Yilmaz et al., 2006), or Morocco (Senhaji et al., 2013). For the *MTHFR* rs1801131 polymorphism, positive association results were obtained in studies from Southeast China (Jiang et al., 2012) and Turkey (Yilmaz et al., 2006) but were not confirmed on samples from Central China (Chen et al., 2005, 2008) and the UK (Herrlinger et al., 2005). Regarding *GSTT1*/*M1* genes, both homozygous *GSTT1* and *GSTM1* deletion polymorphisms were shown to be associated with UC in Central China (Ye et al., 2011), northern India (Mittal et al., 2007), and in Turkish population (Buyukgoze et al., 2013). Conversely, studies in Denmark (Ernst et al., 2010) and Holland (Broekman et al., 2014) as well as in Israeli Jews (Karban et al., 2011) failed to demonstrate any relationship between *GSTT1*/*M1* loci and the risk of UC. Likewise, the *GSTP1* rs1695 polymorphism showed significant association with UC in Central China (Ye et al., 2011) and no association in Denmark (Ernst et al., 2010). The observed discrepancy between individual studies may be due to the relatively small number of patients used in different studies. In addition, the impact of these

Table 3
Pairwise genotype - genotype interaction effects on UC risk revealed by logistic regression under dominant, recessive and over-dominant genetic models. Only associations with a nominal p-value p < 0.05 are shown.

1st locus	Genotype	2nd locus	Genotype	Controls n (%)	Cases n (%)	OR (95% CI)[a]	p-value[a]	p-Value corrected
MTHFR rs1801133	CT	*MTHFR rs1801131*	AC	22 (16.2%)	32 (25.4%)	2.18 (1.12–4.21)	0.019	0.171
MTHFR rs1801133	CT + TT	*MTHFR rs1801131*	AC	23 (16.9%)	32 (25.4%)	2.03 (1.06–3.89)	0.03	0.27
MTHFR rs1801131	AA	*GSTP1 rs1695*	AG	32 (23.7%)	17 (13.6%)	0.45 (0.23–0.90)	0.021	0.189
MTHFR rs1801131	AA	*GSTP1 rs1695*	AG + GG	34 (25.2%)	21 (16.8%)	0.51 (0.27–0.98)	0.04	0.36
GSTP1 rs1695	AG	*GSTM1*	Present	34 (25.2%)	19 (15.1%)	0.50 (0.26–0.98)	0.039	0.234

OR, odds ratio; CI, confidence interval.
[a] Adjusted for sex, age at investigation and smoking.

Table 4
Effect of *MTHFR* polymorphism rs1801131 on severity of UC, analyzed by logistic regression.

Genotype	Severity grade			Severity mean (s.e.)	LR coefficient (95% CI)[a]	p-Value[a]	p-Value corrected[a]
	1. (n = 41)	2. (n = 59)	3. (n = 26)				
AA	21 (51.2%)	24 (40.7%)	7 (26.9%)	1.73 (0.1)	0 (Reference)	0.018	0.09
AC	13 (31.7%)	28 (47.5%)	16 (61.5%)	2.05 (0.1)	0.33 (0.10–0.56)		
CC	7 (17.1%)	7 (11.9%)	3 (11.5%)	1.76 (0.18)	0.04 (−0.30–0.38)		
AA	21 (51.2%)	24 (40.7%)	7 (26.9%)	1.73 (0.1)	0 (Reference)	0.022	0.11
AC + CC	20 (48.8%)	35 (59.3%)	19 (73.1%)	1.99 (0.08)	0.26 (0.04–0.48)		
AA + AC				1.9 (0.07)	0 (Reference)	0.43	-
CC	7 (17.1%)	7 (11.9%)	3 (11.5%)	1.76 (0.18)	−0.13 (−0.46–0.19)		
AA + CC	28 (68.3%)	31 (52.6%)	10 (38.4%)	1.74 ± 0.08	0 (Reference)	0.0046	0.023
AC	13 (31.7%)	28 (47.5%)	16 (61.5%)	2.05 ± 0.1	0.32 (0.10–0.54)		

1 = mild, 2 = moderate, 3 = severe.
[a]Adjusted for sex, age at investigation and smoking.
LR, logistic regression; CI, confidence interval.
p-values above 1.0 after Bonferroni correction are not shown.

polymorphisms on the risk of UC may also vary from population to population because of differences in genetic backgrounds as well as environmental and nutritional factors. For example, folate supplementation can efficiently reduce plasma Hcy level and may therefore affect UC susceptibility. A study by Chen et al. (2008) suggested a link between interethnic difference in folate consumption and association of polymorphisms in the *MTHFR* gene with UC. Interestingly, Moldavia, along with Kosovo, is the only European country that mandates fortification of wheat products with folic acid (Food Fortification Initiative, 2015). This background folic acid supplementation could potentially smooth possible effects of *MTHFR* polymorphisms on UC and partially explain the lack of association in the Moldavian population. Consideration of additional information on the dietary habits of participated individuals and controlling for the markers of folate status (i.e. folic acid, Hcy, vitamin B_{12}) would certainly help to clarify the role of *MTHFR* variants in the development of UC and, therefore, are desired for further in-depth studies of UC.

We further investigated whether *GST* and *MTHFR* polymorphisms are associated with certain phenotypic characteristics in UC patients. We found that the *MTHFR* rs1801131 AC heterozygote was more frequently associated with severe clinical subtypes of UC, whereas AA homozygous wild type had an inverse correlation, indicating a possible role of the *MTHFR* gene polymorphism on the severity of the disease. However, no positive correlation has been shown for the homozygous CC genotype, although, given its lowest enzymatic activity (van der Put et al., 1998), a stronger effect for CC than for AC genotype was expected. This lack of association could be due to chance because of small sample size and low occurence of genotype CC in the groups. Yet the possibility of over-dominant inheritance of *MTHFR* rs1801131 polymorphism ('heterozygous advantage') cannot be ruled out. Indeed, although no 'heterozygous advantage' has been reported for *MTHFR* polymorphisms in UC, it has been previously described for other human diseases (Li et al., 2014; Hubacek et al., 2015). Further research is needed to confirm the causal effect of *MTHFR* on the severity of UC.

Finally, we observed a significant association between the *MTHFR* rs1801133 polymorphism and the extent of inflammation. Patients with the TT genotype achieved significantly higher protection against more extensive disease (left-sided UC and pancolitis) than patients with CT or CC genotypes. This finding contradicts the oxidative stress hypothesis of UC and the previous reports on correlation between *MTHFR* rs1801133 and UC extension (Chen et al., 2008; Jiang et al., 2012; Senhaji et al., 2013; Vecchi et al., 2000). The differential results could be due to multifunctional properties of methylenetetrahydrofolate reductase, again modulated by population and nutritional factors. Indeed, the *MTHFR* rs1801133 TT genotype is known to reduce enzyme activity (~30% of normal) (Frosst et al., 1995), and therefore, its carriers may be especially susceptible to Hcy-induced diseases, particularly in the presence of folate deficiency. On the other hand, MTHFR has a crucial role in regulating cellular methylation and gene expression (Chen et al., 2001; Friso et al., 2002; Lu, 2013). A potential biochemical explanation for the inverse association between UC and the *MTHFR* rs1801133 TT genotype is that low MTHFR activity results in lower production of SAM (Schwahn and Rozen, 2001). Eventually, this may lead to diminished spreading of hypermethylation and inflammation. The above scenario on the protective effect of the *MTHFR* rs1801133 TT genotype appears to be more suitable for countries with a sufficient total folate intake, like Moldavia, where the overproduction of SAM conditioned by nutrition has to be balanced by intrinsic regulatory factors. The relevance of the inverse association between the *MTHFR* rs1801133 TT genotype and UC is further supported by association studies on colorectal cancer and Graves' disease (Mao et al., 2010; Zhao et al., 2013) that are etiologically related to UC.

In conclusion, the present study has shown that *GSTT1*, *GSTM1*, *GSTP1*, and *MTHFR* genetic variants, alone or combined, have no

Table 5
Effect of *MTHFR* polymorphism rs1801133 on extent of UC, analyzed by logistic regression and Fisher exact test.

Genotype	Distal colitis (n = 53)	Extended colitis[a] (n = 75)	OR (95% CI)[b]	p-Value log. Reg.[b]	p-value log. reg. corrected[b]	p-Value isher test	p-Value fisher test corrected
CC	24 (45.3%)	35 (46.7%)	1 (Reference)	0.008	0.040	0.0075	0.0375
CT	18 (34%)	37 (49.3%)	1.69 (0.71–4.02)				
TT	11 (20.8)	3 (4%)	0.19 (0.04–0.83)				
CC	24 (45.3%)	35 (46.7%)	1 (Reference)	0.91	-	> 0.9999	-
CT + TT	29 (54.7%)	40 (53.3%)	1.05 (0.48–2.28)				
CC + CT	42 (79.3%)	72 (96%)	1 (Reference)	0.0041	0.020	0.0037	0.0185
TT	11 (20.8)	3 (4%)	0.15 (0.04–0.63)				
CC + TT	35 (66%)	38 (50.7%)	1 (Reference)	0.05	0.25	0.0729	0.3645
CT	18 (34%)	37 (49.3%)	2.28 (1.00–5.21)				

OR, odds ratio; CI, confidence interval.
p-values above 1.0 after Bonferroni correction are not shown.
[a] Left-sided UC + Pancolitis.
[b] Adjusted for sex, age, at investigation and smoking.

significant influence on the primary risk of having UC in Moldavian population. However, our results suggest that *MTHFR* genotypes may affect the spread and severity of UC. The main limitation of our study is its small sample size, which is partly due to the low incidence of UC in Moldavia. It also does not consider the nutritional status of participants as assessed by serum Hcy and folic acid levels. Hence, further work using a larger population and studying additional folate and oxidative stress-related genes taking into account the gene–gene and gene–environment interactions is needed to refine the present results. Such knowledge may have important implications for prevention and management of UC.

Conflict of interest statement

The authors declare no conflicts of interest.

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