

# Association of MTRR 66A>G Polymorphism With Superoxide Dismutase and Disease Activity in Patients With Crohn's Disease

Laurent Peyrin-Biroulet,<sup>1,2</sup> Rosa-Maria Guéant-Rodriguez,<sup>1</sup> Min Chen,<sup>1</sup> Jean-Pierre Bronowicki,<sup>1,2</sup> Marc-André Bigard,<sup>1,2</sup> and Jean-Louis Guéant, M.D., D.Sc.<sup>1,2</sup>

<sup>1</sup>Inserm, U724, Laboratory of Cellular and Molecular Pathology in Nutrition, Faculty of Medicine, Vandoeuvre-les-Nancy, France; and <sup>2</sup>Department of Hepato-Gastroenterology, University Hospital of Nancy, Vandoeuvre-les-Nancy, France

**OBJECTIVES:** The aim of this study was to evaluate the association of nutritional (folate, vitamin B<sub>12</sub>) and genetic (*MTHFR*, *MTR*, *MTRR*, *TCN*) determinants of homocysteine metabolism and of superoxide dismutase with Crohn's disease (CD).

**METHODS:** One hundred forty patients with CD were compared with 248 matched healthy controls.

**RESULTS:** Plasma homocysteine levels were higher in CD patients than controls (11.8 vs 10.4 μmol/L, *P* = 0.0004). Vitamin B<sub>12</sub> and folate levels were lower in CD subjects compared to controls (207 vs 255 pmol/L, *P* = 0.0082, and 8.6 vs 11 nmol/L, *P* = 0.0036, respectively). Patients with a personal history of ileal resection, ileitis, or colectomy had significantly lower vitamin B<sub>12</sub> levels. In multivariate analysis, vitamin B<sub>12</sub> and *MTHFR* 677 *TT* carriers were the two significant independent factors of plasma homocysteine >15 μmol/L in CD patients (*P* = 0.0187 and 0.0048, respectively). The significant association between homocysteine and vitamin B<sub>12</sub> levels remained significant only in patients with the highest superoxide dismutase values (*P* < 0.0001). The *MTRR* *AA* genotype was a significant independent predictor of CD risk (odds ratio 3.7, 95% CI 1.218–11.649, *P* = 0.0213). The level of superoxide dismutase was significantly higher (*P* = 0.0143) and was correlated with Crohn's Disease Activity Index (CDAI) scores (*P* for trend = 0.0276) in patients carrying *MTRR* *AA* genotype.

**CONCLUSIONS:** Vitamin B<sub>12</sub> and *MTHFR* 677 *TT* genotype are the main determinants of hyperhomocysteinemia in CD patients. The association of *MTRR* 66A>G polymorphism with oxidant stress and disease activity provides rationale for screening vitamin deficiencies in these patients.

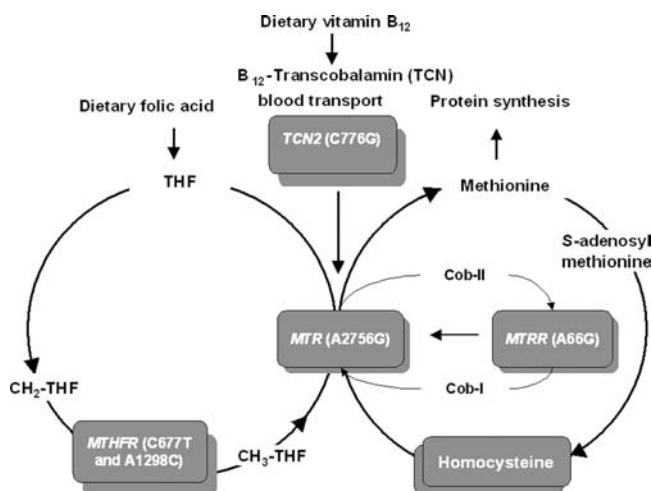
(Am J Gastroenterol 2008;103:399–406)

## INTRODUCTION

Homocysteine is a nonprotein forming, sulphur amino acid that results from the demethylation of the essential amino acid methionine (Fig. 1). Plasma total homocysteine (t-Hcy) is influenced by nutritional factors, mainly folates and vitamin B<sub>12</sub> (cobalamin), as well as genetic polymorphisms of key enzymes of its metabolism: methylenetetrahydrofolate reductase (*MTHFR*) (1), methionine synthase (*MTR*) (2), and methionine synthase reductase (*MTRR*) (3). *MTHFR* catalyses the synthesis of methylenetetrahydrofolate, the methyl donor of homocysteine, and *MTR* and *MTRR* are the two key enzymes of the synthesis of methionine by remethylation of homocysteine. The *C677T* genetic polymorphism in the *MTHFR* gene was found to be associated with a thermo-labile variant enzyme that showed reduced activity (4). A genetic polymorphism in the transcobalamin gene (*TCN2*) is another genetic trait that may influence homocysteine metabolism (5).

Hyperhomocysteinemia is recognized as a risk factor for both venous and arterial thrombosis in the general population (6, 7). In this regard, homocysteine metabolism has received increasing attention over the past decade as a potential contributor to the greater risk of thrombosis in inflammatory bowel disease (IBD) subjects. Mahmud and colleagues were the first to report a higher prevalence of hyperhomocysteinemia and of *MTHFR* 677 *TT* allele in IBD patients, compared to controls (8). Nevertheless a number of subsequent studies have produced conflicting results, suggesting complex interactions between environmental and genetic factors (9). In addition, polymorphisms of other homocysteine metabolism-related enzymes may contribute to high t-Hcy usually found in IBD patients. Overall, the influence of homocysteine on secondary events of IBD, particularly thromboembolic manifestations, remains controversial (10).

Furthermore, genotype-phenotype correlations are poorly documented in most of the already published case-control



**Figure 1.** Metabolic chart of homocysteine cycle and the genetic variants of the enzymes involved in this metabolism. Homocysteine is remethylated into methionine by methionine synthase (MTR) with 5-methyltetrahydrofolate as a methyl donor and vitamin B<sub>12</sub> (cobalamin) as a coenzyme. MTRR is the enzyme needed to maintain vitamin B<sub>12</sub> in a reductive state in the catalytic cycle of MTR dependent remethylation of homocysteine. 5-methyltetrahydrofolate is produced by the FAD-dependent enzyme 5,10-methylenetetrahydrofolate reductase (MTHFR). Methionine is further transformed into S-adenosylmethionine, the universal methyl donor for methylation of nucleic acids, proteins, polysaccharides, phospholipids... etc. After release of the methyl group, S-adenosylmethionine is converted into S-adenosylhomocysteine, which is reversibly hydrolyzed into homocysteine.

studies, and the combined and respective influence of *MTHFR*, *MTR*, *MTRR*, and *TCN* polymorphisms on the primary and secondary risks of IBD has never been evaluated in a same sample population. Finally, growing evidence suggests that hyperhomocysteinemia may promote chronic intestinal mucosa inflammation, mainly through oxidative stress (9, 11, 12). Elevated homocysteine levels were shown to enhance superoxide dismutase (SOD) activity, a well-established marker of oxidative stress (13–15). Recently, plasma levels of SOD activity were found to be increased in patients with IBD (16).

The aims of this study were therefore to evaluate the complex influence of genetic and nutritional determinants of homocysteine metabolism on the primary risk and phenotype of patients with Crohn's disease (CD), and their potential interaction with SOD.

## METHODS

### Study Subjects

Eligible patients were men or women who were at least 18 yr of age, with established CD. One hundred forty patients recruited from the Department of Hepato-Gastroenterology (University Hospital of Nancy, France) were prospectively enrolled in a case-control study. Information regarding clinical characteristics and concomitant medications were collected at study entry. The disease location was defined accord-

ing to the Montreal classification (17). These subjects were compared to 248 healthy volunteers from a center of preventive medicine. Controls matched the individuals of the CD group for age and sex. None of the subjects from the control or the CD patient group received any treatment and/or vitamin B supplementation that could influence vitamin B<sub>12</sub>, folate, or homocysteine metabolism. All individuals were French of European origin. The study protocol was approved by the local ethics committee, and the included subjects gave a written consent.

### Assays and DNA Genotyping Procedures

Fasting venous blood was collected in EDTA-containing tubes, immediately centrifuged, and stored at  $-80^{\circ}\text{C}$  until analysis. Plasma t-Hcy was assayed by FPIA (fluorescence polarization immunoassay) using the Abbott IMX automated Benchtop analyzer system (Abbott Diagnostic, Rome, Italy). Serum folate and vitamin B<sub>12</sub> levels were measured by radio assay (Ciba-Corning, Medfield, MA). The SOD activity assay was carried out with the Ransod kit (Randox, Cruclin, UK) according to the manufacturer's instructions.

DNA was isolated from a lymphocyte-enriched fraction of whole blood with NUCLEON BACC3 for extraction of genomic DNA kit (Amersham Pharmacia Biotech, Milan, Italy). The procedures for detecting the *C677T* and *A1298C* polymorphisms of *MTHFR*, as well as the *A2756G* *MTR* and the *A66G* *MTRR* polymorphisms, were based on polymerase chain reaction amplification, restriction cleavage, and separation of the DNA fragments by 15% nondenaturant polyacrylamide gel electrophoresis (SDS-PAGE), as previously described (18). Genotyping of the *TCN C776G* polymorphism was performed by the amplification-refractory mutation system, as described recently by us (5). DNA samples corresponding to amplified DNA of the *MTHFR*, *MTR*, and *MTRR* genotypes were sequenced and subsequently used as controls in all series of genotype determination.

### Statistical Analysis

Categorical variables are reported as counts and percentages, and continuous variables as mean  $\pm$ SD, median, 25th and 75th interquartiles. For categorical variables, a continuity-corrected  $\chi^2$  test was used to assess differences. For continuous variables, a Mann-Whitney U-test or a Kruskal-Wallis test were employed. A multiple linear regression analysis was used to evaluate the significant independent determinants of t-Hcy and a Cox regression analysis was used for evaluating the association between quartiles of Crohn's Disease Activity Index (CDAI) and SOD activity. The significance, odds ratio, and 95% confidence interval of independent categorical and continuous variables regarding the risk of CD were determined by multivariate stepwise logistic regression analysis using a model that included age, sex, and the variables that had a *P* value  $<0.10$  in univariate analyses. The stepwise model considered only the variables with a *P* value  $<0.10$  as residual independent determinants. A *P* value lower than 0.05 indicated statistical significance. Data were prospectively

**Table 1.** Clinical and Genetic Characteristics of the 140 CD Patients and Their Association With Plasma t-Hcy Level

| Characteristic                          | Number of Cases | Percentage (95% Confidence Interval) | Homocysteine $\mu\text{mol/L}$ : Median (25th–75th Quartiles) |                        | P Value |
|---|-----------------|--------------------------------------|---|------------------------|---------|
|   |                 |                                      | With Characteristic   | Without Characteristic |         |
| Male sex                                | 53              | 37.9 (30.2–46.1)                     | 14.24 (9.8–16.3)  | 11.06 (8.97–14.50)     | 0.0143  |
| Familial history of digestive neoplasia | 15              | 10.7 (6.6–16.9)                      | 12.4 (9.4–16.6)   | 11.4 (9.4–14.6)        | 0.7163  |
| Familial history of thrombosis          | 16              | 11.4 (7.2–17.8)                      | 10.8 (9.1–12.3)   | 11.9 (9.4–14.9)        | 0.3021  |
| Personal history of thrombosis          | 8               | 5.7 (2.9–10.8)                       | 12.3 (8.4–16.2)   | 12.0 (9.5–14.9)        | 0.9579  |
| Current smoker                          | 54              | 38.6 (30.9–46.8)                     | 12.6 (9.7–16.1)   | 11.8 (9.2–14.6)        | 0.2760  |
| Alcohol consumption >20 g/day           | 3               | 2.1 (0.1–6.0)                        | 15.1 (10.6–22.6)  | 11.9 (9.5–14.7)        | 0.4115  |
| Concomitant medications                 |                 |                                      |   |                        |         |
| Infliximab                              | 106             | 75.7 (68.0–82.1)                     | 11.8 (9.4–14.8)   | 14.2 (11.2–15.1)       | 0.3857  |
| Azathioprine                            | 92              | 65.7 (57.5–73.1)                     | 11.7 (9.0–14.6)   | 14.3 (10.8–16.4)       | 0.0505  |
| Methotrexate                            | 7               | 5.0 (2.5–10.0)                       | 16.5 (10.7–17.2)  | 11.9 (9.4–14.6)        | 0.1123  |
| Disease location                        |                 |                                      |   |                        |         |
| Ileal (L1)                              | 22              | 15.7 (10.6–22.7)                     | 11.9 (9.6–16.6)   | 12.0 (9.1–14.6)        | 0.4539  |
| Colonic (L2)                            | 60              | 42.8 (34.9–51.1)                     | 12.4 (9.1–14.6)   | 11.9 (9.9–17.2)        | 0.1649  |
| Ileocolonic (L3)                        | 57              | 40.7 (32.9–49.0)                     | 13.7 (10.8–16.5)  | 11.7 (9.1–14.6)        | 0.1080  |
| Isolated upper disease (L4)             | 0               | –                                    | –   | –                      | –       |
| Perianal disease                        | 45              | 32.1 (25.0–40.3)                     | 11.21 (9.1–15.1)  | 12.3 (9.7–15.1)        | 0.6282  |
| Personal history of surgery             |                 |                                      |   |                        |         |
| Ileal resection                         | 18              | 12.9 (8.3–19.4)                      | 15.8 (11.2–18.2)  | 11.8 (9.2–14.5)        | 0.0133  |
| Colectomy                               | 12              | 8.6 (5.0–14.4)                       | 14.6 (11.3–16.7)  | 11.8 (9.3–14.6)        | 0.0603  |
| Perianal surgery                        | 21              | 15.0 (10.0–21.9)                     | 11.8 (9.5–14.0)   | 12.3 (9.5–15.3)        | 0.4927  |
| Genotypes                               |                 |                                      |   |                        |         |
| <i>MTHFR 677 TT</i>                     | 13              | 9.2 (5.5–15.3)                       | 17.1 (9.0–22.3)   | 11.3 (9.5–14.5)        | 0.0219  |
| <i>MTHFR 1298 CC</i>                    | 12              | 8.6 (5.0–14.4)                       | 10.9 (8.9–13.9)   | 10.7 (9.0–13.5)        | 0.9331  |
| <i>MTR 2756 GG</i>                      | 6               | 4.2 (2.0–9.0)                        | 11.3 (8.0–12.6)   | 10.7 (9.0–13.5)        | 0.1516  |
| <i>MTRR 66 GG</i>                       | 29              | 20.7 (14.8–28.2)                     | 10.2 (8.5–15.5)   | 11.8 (9.6–14.7)        | 0.2629  |
| <i>TCN 776 GG</i>                       | 27              | 19.3 (13.6–26.6)                     | 11.9 (9.4–14.7)   | 11.9 (9.4–12.9)        | 0.5568  |

collected and analyzed using the Statview 5 software for Windows (SAS Institute, Berkeley, CA) and the SPSS 10.0 software for Windows (SPSS, Paris, France).

## RESULTS

### Clinical and Biological Characteristics of CD Patients and Controls

The mean age was 39 (23–45) and 35 (30–45) yr in CD patients and controls, respectively ( $P = 0.1452$ ). Plasma t-Hcy levels were significantly higher in the CD group than in the control group (11.8, 4.05–30.61 vs 10.4, 5.5–21.6  $\mu\text{mol/L}$ , respectively,  $P = 0.0004$ ). In addition, as much as 24% of patients had a moderate hyperhomocysteinemia (>15  $\mu\text{mol/L}$ ), compared with 8% reported in controls. Conversely, vitamin B<sub>12</sub> and folate levels were lower in IBD subjects compared to controls: 207 (79–783) vs 255 (93–1,480) pmol/L, respectively,  $P = 0.0082$  and 8.6 (1.8–48) vs 11 (2.9–34) nmol/L, respectively,  $P = 0.0036$ .

### Predictors of Hyperhomocysteinemia in CD Patients

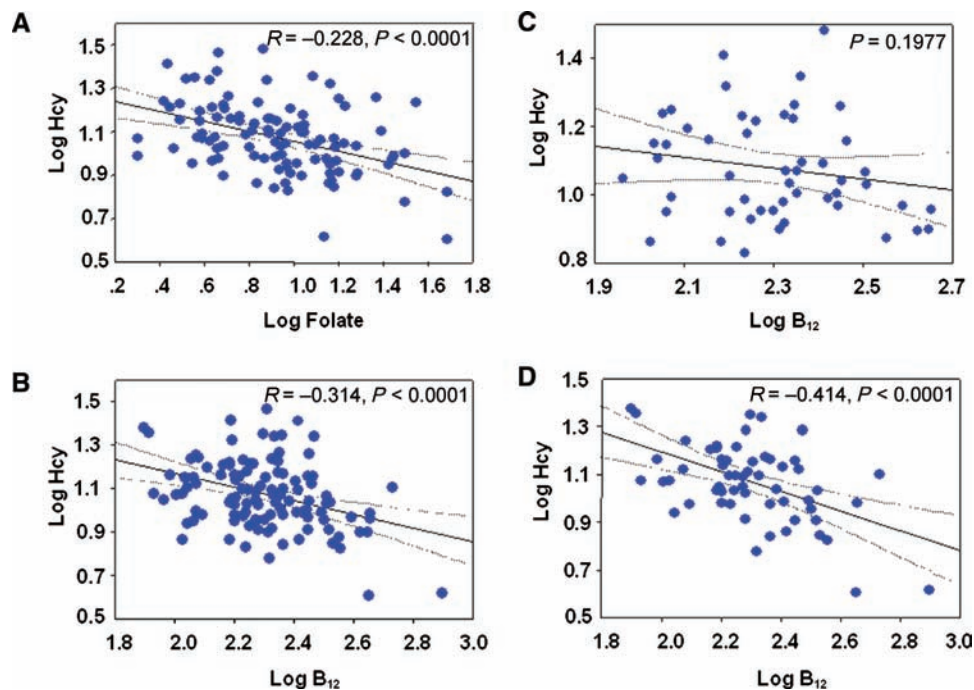
In CD individuals, male subjects had higher homocysteine levels than female subjects ( $P = 0.0143$ ) (Table 1), while plasma t-Hcy levels were not influenced by sex in controls. The plasma t-Hcy level was slightly lower in CD subjects treated with azathioprine than in those not receiving this medication (Table 1). The influence of steroids and aminosaliculates was not evaluable, because, respectively, three and two

CD patients were treated with such medications. Similarly, only one patient had a personal history of digestive neoplasia (colon adenoma). *MTHFR 677 TT* was the only genetic determinant that was significantly associated with an increased t-Hcy in both controls (10.2, 8.6–12.1 vs 12.5, 9.9–17.0,  $P = 0.0030$ ) and CD patients (Table 1). The plasma t-Hcy level was slightly higher in CD subjects carrying the *MTR 2756 AA* genotype, but the difference did not reach statistical significance ( $P = 0.0910$ ).

Vitamin B<sub>12</sub> and folate levels were significantly associated with t-Hcy in univariate analysis (Fig. 2). Age, vitamin B<sub>12</sub>, and folate levels independently influenced plasma t-Hcy, in multiple regression analysis ( $P < 0.0001$ ,  $P = 0.0014$ ,  $P = 0.0005$ , respectively).

We investigated whether the clinical characteristics of the 140 CD patients may influence their serum folate and vitamin B<sub>12</sub> status. CD patients with a personal history of ileal resection (median 156 vs 208 pmol/L,  $P = 0.0389$ ), ileitis (median 159 vs 219 pmol/L,  $P = 0.0261$ ), or colectomy (median 155 vs 206 pmol/L,  $P = 0.0246$ ) had significantly lower vitamin B<sub>12</sub> levels than CD subjects without these characteristics. Active smoking was the single factor negatively associated with serum folate level in CD subjects (median 6.6 vs 9.2 nmol/L in nonsmoking CD patients,  $P = 0.0123$ ).

In multivariate analysis, vitamin B<sub>12</sub> and *MTHFR 677 TT* carriage were the two significant independent predictors of plasma t-Hcy >15  $\mu\text{mol/L}$  (Table 2).



**Figure 2.** Homocysteine concentrations are correlated with folate (A) and vitamin B<sub>12</sub> (B) levels in CD patients. The association between homocysteine and vitamin B<sub>12</sub> remains significant in CD patients with a concentration of superoxide dismutase (SOD) higher than 1,100 UI/g hemoglobin (median) (D) and not in those with a lower SOD activity (C).

**Genetic Determinants of Plasma Homocysteine Level in CD Patients and Controls**

The genotype distributions of *MTHFR*, *MTR*, *MTRR*, and *TCN* polymorphisms were in Hardy-Weinberg equilibrium (data not shown). The allele distributions of *MTHFR*, *MTR*, and *TCN* polymorphisms did not differ between controls and CD patients. In contrast, the frequency of the A allele for *MTRR* genotype was 1.34-fold higher in CD subjects than in controls (Table 3).

The same conclusion was reached when considering the genotypes instead of alleles, with *MTRR 66AA* that was 3-fold more frequent in CD patients (Table 3).

The association of polymorphisms of homocysteine metabolism-related enzymes with the risk of CD was estimated by stepwise logistic regression analysis using a model that retained age, sex, and the variables that had a *P* value <0.10. We confirmed that the only significant independent predictor of CD risk was *MTRR AA* carriers, with an odds ratio for CD risk estimated at 3.77 (Table 4).

**Homocysteine Metabolism, Disease Activity, and Oxidant Stress**

We examined the association of polymorphisms of homocysteine metabolism-related enzymes with SOD level. The

**Table 2.** Factors Associated With Hyperhomocysteinemia (>15 μmol/L) in CD Patients (Multiple Logistic Regression Analysis)

| Variable                | Initial |               |         | Residual |              |         |
|-------------------------|---------|---------------|---------|----------|--------------|---------|
|                         | OR      | 95% CI        | P Value | OR       | 95% CI       | P Value |
| CDAI score              | 0.99    | (0.98–1.00)   | 0.0981  | –        | –            | NS      |
| Serum folate            | 0.95    | (0.87–1.03)   | 0.2263  | –        | –            | NS      |
| Vitamin B <sub>12</sub> | 0.99    | (0.98–0.99)   | 0.0206  | 0.99     | (0.98–0.99)  | 0.0187  |
| SOD                     | 1.00    | (0.99–1.00)   | 0.6353  | –        | –            | NS      |
| Albumin                 | 1.04    | (0.89–1.21)   | 0.5923  | –        | –            | NS      |
| C-reactive protein      | 1.02    | (0.97–1.07)   | 0.4795  | –        | –            | NS      |
| Azathioprine use        | 1.12    | (0.26–4.76)   | 0.8768  | –        | –            | NS      |
| Infliximab use          | 1.96    | (0.27–14.37)  | 0.5092  | –        | –            | NS      |
| Methotrexate use        | 3.26    | (0.14–75.79)  | 0.4609  | –        | –            | NS      |
| <i>MTHFR 677 TT</i>     | 16.35   | (1.09–244.97) | 0.0430  | 6.40     | (1.76–23.31) | 0.0048  |
| <i>MTHFR 1298 CC</i>    | 5.55    | (0.52–59.33)  | 0.1562  | –        | –            | NS      |
| <i>MTRR 66 GG</i>       | 2.46    | (0.36–16.94)  | 0.3591  | –        | –            | NS      |
| <i>MTR 2756 AG/GG</i>   | 1.26    | (0.35–4.56)   | 0.7239  | –        | –            | NS      |

OR = odds ratio; CI = confidence interval; NS = not significant.

**Table 3.** Genotype Frequencies and Minor Allele Frequencies of Genetic Polymorphisms in CD Patients and Controls

|                   | Controls: Percentage<br>(95% CI) | CD Patients: Percentage<br>(95% CI) | P Value |
|-------------------|----------------------------------|-------------------------------------|---------|
| <i>MTHFR 677</i>  |                                  |                                     |         |
| CC                | 0.43 (0.37–0.49)                 | 0.43 (0.35–0.52)                    | 0.3320  |
| CT                | 0.43 (0.37–0.49)                 | 0.47 (0.39–0.55)                    |         |
| TT                | 0.15 (0.11–0.19)                 | 0.09 (0.06–0.15)                    |         |
| Allele T          | 0.36 (0.32–0.40)                 | 0.33 (0.28–0.39)                    | 0.4151  |
| <i>MTHFR 1298</i> |                                  |                                     |         |
| AA                | 0.43 (0.37–0.49)                 | 0.41 (0.34–0.50)                    | 0.7334  |
| AC                | 0.47 (0.41–0.53)                 | 0.50 (0.42–0.58)                    |         |
| CC                | 0.11 (0.07–0.15)                 | 0.09 (0.05–0.14)                    |         |
| Allele C          | 0.34 (0.30–0.38)                 | 0.36 (0.31–0.42)                    | 0.6010  |
| <i>MTR 2756</i>   |                                  |                                     |         |
| AA                | 0.68 (0.62–0.74)                 | 0.61 (0.52–0.68)                    | 0.3042  |
| AG                | 0.29 (0.23–0.35)                 | 0.35 (0.28–0.43)                    |         |
| GG                | 0.03 (0.01–0.06)                 | 0.04 (0.02–0.09)                    |         |
| Allele G          | 0.17 (0.14–0.21)                 | 0.22 (0.17–0.27)                    | 0.1273  |
| <i>MTRR 66</i>    |                                  |                                     |         |
| AA                | 0.08 (0.04–0.13)                 | 0.24 (0.17–0.31)                    | 0.0009  |
| AG                | 0.61 (0.52–0.69)                 | 0.56 (0.47–0.64)                    |         |
| GG                | 0.31 (0.24–0.40)                 | 0.21 (0.15–0.28)                    |         |
| Allele G          | 0.62 (0.56–0.68)                 | 0.49 (0.43–0.55)                    | 0.0019  |
| <i>TCN 776</i>    |                                  |                                     |         |
| CC                | 0.32 (0.27–0.38)                 | 0.30 (0.23–0.38)                    | 0.9146  |
| CG                | 0.49 (0.43–0.55)                 | 0.51 (0.43–0.59)                    |         |
| GG                | 0.19 (0.14–0.24)                 | 0.19 (0.13–0.27)                    |         |
| Allele G          | 0.43 (0.39–0.48)                 | 0.45 (0.39–0.51)                    | 0.3839  |

CI = confidence interval.

latter was significantly higher in CD subjects carrying *MTRR* AA genotype, compared with those carrying *GG* genotype (Fig. 3), while the other polymorphisms, *MTHFR*, *MTR*, *MTRR*, and *TCN2*, had no significant influence on this biological parameter (data not shown).

The relation between oxidant stress and disease severity and *MTRR* polymorphism was next investigated. There was a significant association between the distribution in quartiles of CDAI scores and the level of SOD in patients with the *MTRR* AA genotype (Fig. 3), but not in those with the *AG/GG* genotypes. Finally, we investigated the association between t-Hcy and vitamin B<sub>12</sub> (the substrate of *MTRR*), according to SOD activity, in CD patients. There was a significant association only in patients who had a SOD activity higher than the median (Fig. 3). In contrast, there was no influence of SOD activity on the association between t-Hcy and folate.

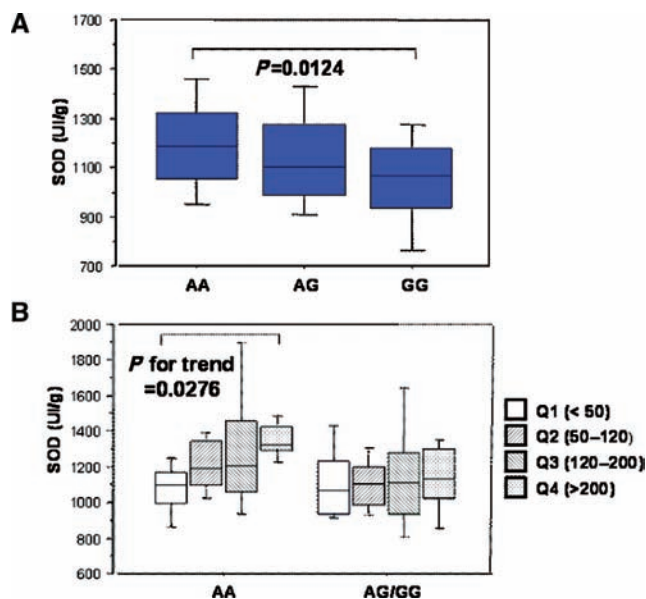
## DISCUSSION

Patients with CD have significantly higher blood levels (9) with one-fourth of them who presented with moderate hyperhomocysteinemia in our study. As previously reported by other groups, folate and vitamin B<sub>12</sub> levels were significantly lower in IBD subjects compared to healthy controls (9). While homocysteine concentration was associated with both vitamin B<sub>12</sub> and folate levels in univariate analysis, the multivariate analysis revealed that only vitamin B<sub>12</sub>, but not folate, was an independent predictor of hyperhomocysteinemia in our population of CD patients. As recently demonstrated by Rogers *et al.* (19), plasma homocysteine may be an accurate predictor of folate deficiency under physiological conditions, but may not reflect folate deficiency in cases of increased oxidative stress. A personal history of ileal

**Table 4.** Stepwise Logistic Regression of the Association of Genetic Determinants of Homocysteine Metabolism With the Risk of CD

| Variable                      | Initial Multivariate Model |                |         | Residual Determinant |              |         |
|-------------------------------|----------------------------|----------------|---------|----------------------|--------------|---------|
|                               | OR                         | 95% CI         | P Value | OR                   | 95% CI       | P Value |
| <i>MTHFR 677 CT</i> carriers  | 1.231                      | (0.491–3.087)  | 0.6576  | –                    | –            | NS      |
| <i>MTHFR 677 TT</i> carriers  | 1.770                      | (0.450–6.970)  | 0.4141  | –                    | –            | NS      |
| <i>MTHFR 1298 AC</i> carriers | 2.159                      | (0.845–5.519)  | 0.1080  | –                    | –            | NS      |
| <i>MTHFR 1298 CC</i> carriers | 2.946                      | (0.676–12.849) | 0.1504  | –                    | –            | NS      |
| <i>MTRR AA</i> carriers       | 4.132                      | (1.299–13.149) | 0.0163  | 3.77                 | (1.22–11.65) | 0.0213  |
| <i>MTR 2756 AG</i> carriers   | 1.401                      | (0.609–3.223)  | 0.4275  | –                    | –            | NS      |
| <i>MTR 2756 GG</i> carriers   | 8.064                      | (1.023–63.593) | 0.475   | –                    | –            | NS      |
| <i>TCN 776 CG</i> carriers    | 1.362                      | (0.463–4.007)  | 0.5742  | –                    | –            | NS      |
| <i>TCN 776 GG</i> carriers    | 1.207                      | (0.466–3.123)  | 0.6984  | –                    | –            | NS      |

OR = odds ratio; CI = confidence interval; NS = not significant.



**Figure 3.** Association between superoxide dismutase (SOD) activity and *MTRR* polymorphism (A). A significant association between SOD activity and the quartile distribution of Crohn's Disease Activity Index (CDAI, Q1–Q4) was found in the patients with *MTRR* AA genotype, but not in those with the AG/GG genotypes (B).

resection or colectomy, but also pure ileitis, were risk factors for B<sub>12</sub> deficiency and hyperhomocysteinemia in this cohort of CD patients, through deficient absorption capacity of the ileum and also probably through bacterial colonization leading to malabsorption of vitamin B<sub>12</sub> (20, 21). Active smoking was associated with significantly lower folate levels, confirming previous reports in non-IBD patients (22). Taken as a whole, these findings underscore the relevance of screening CD patients for folate and vitamin B<sub>12</sub> deficiencies, particularly those with ileal disease, active smoking, and/or history of intestinal resection. Azathioprine was the single drug that appeared to decrease t-Hcy, suggesting interactions between azathioprine and homocysteine metabolisms. Consistently, thiopurine methyltransferase (TMPT) inactivates 6-mercaptopurine, the azathioprine active metabolite, by transferring a methyl group from S-adenosylmethionine to 6-mercaptopurine, forming S-adenosylhomocysteine that is subsequently hydrolyzed into homocysteine (Fig. 1) (23). Genetic variation in the *MTHFR* gene may result in reduced S-adenosylmethionine concentrations, leading to enhanced TPMT enzyme degradation and possibly modulating azathioprine efficacy (23).

We also evaluated the influence of genetic determinants on homocysteine level in CD patients. There was a significant association of *MTHFR* 677 TT genotype with t-Hcy as in the general population, but this polymorphism had no influence on CD risk. These results are in agreement with four studies in Southern Europe (two Italian, one French, one Portuguese) that found no association between *MTHFR* TT genotype and IBD (24–27), whereas two studies in Northern Europe re-

ported a significantly increased frequency of the *MTHFR* TT genotype in IBD patients (8, 28). Ethnic and geographical variations in the distribution of *MTHFR* variants may explain such discrepancy between genetic studies in IBD, since *MTHFR* has a lower phenotypic influence on homocysteine metabolism in white populations from South Europe than in those from North Europe (29). In a small series of IBD patients, no change in frequency of *MTHFR* A1298C genotype was seen when compared to controls (30), while the possible implication of *MTR* and *MTRR* polymorphisms in CD pathogenesis had never been investigated so far. The gene variants *MTHFR* 1298 A>C, *MTR* 2756 A>G, *MTRR* 66A>G, and *TCN2* 677C>G did not influence homocysteine level in the present study. Gender was also a genetic determinant that influenced t-Hcy in CD patients, in univariate analysis. However, it was not retained as an independent determinant of t-Hcy, in multivariate analysis. The difference did not reach statistical significance in controls, probably because of the limited sample size. Indeed, differences between men and women in t-Hcy have been documented in larger samples of healthy populations and may be partially explained by differences in rates of homocysteine remethylation (31).

Moderate hyperhomocysteinemia was not associated with an increased risk of thrombosis in our study, confirming recent reports (32). More interestingly, a growing body of evidence indicates that homocysteine may induce a cellular and vascular stress that could contribute to the maintenance of a chronic mucosal inflammatory state in IBD patients (9, 33). Consistently, homocysteine levels may be correlated with activity, number of flares, and duration of the disease (34), underscoring the potential implication of homocysteine metabolism in IBD pathogenesis.

In this context, the finding that *MTRR* AA was associated with an increased risk of CD as well as an increased activity of SOD may yield a better understanding of the role of homocysteine metabolism in IBD pathogenesis. Extensive experimental data have revealed a central role for oxidative stress in atherogenesis (35). Interestingly, *MTRR* AA genotype was recently found to be an independent predictor of coronary artery disease risk, another chronic inflammatory disease (36). *MTRR* catalyzes the regeneration of methylcobalamin in a reduced form, during the catalytic cycle (Fig. 1). Therefore, the association of the *MTRR* A allele with CD may be considered in function of the increased need for cobalamin reduction in cells challenged by oxidative stress. Indeed, the metabolic function of *MTRR*, e.g., to maintain vitamin B<sub>12</sub> in a reduced form, may be critical in cellular conditions where oxidant stress is increased. This could explain why vitamin B<sub>12</sub> was associated with homocysteine level only in the CD patients who had a high SOD activity (exceeding the median value). Indeed, vitamin B<sub>12</sub> concentration in bowel mucosa could specifically depend on oxidative stress in these patients, and hyperhomocysteinemia viewed as a consequence of vitamin B<sub>12</sub> deficiency. Recently, Rogers *et al.* showed that folate deficiency had no influence on homocysteine concentration in mice with a genetically determined increase of oxidant

stress (19). Consistently with this observation, we found that increased homocysteine plasma level was depending on vitamin B<sub>12</sub>, but not on folate, in the CD patients who had the highest SOD activity. MTRR needs NADPH as a coenzyme, and the cellular oxidant stress (11,12), including the increased production of hydrogen peroxide by increased activity of SOD in intestinal mucosa of IBD patients, may be a condition that increases the cellular consumption in NADPH. An increase of glutathione synthesis by the transsulfuration pathway may be also expected in cases of deficient remethylation of homocysteine, and the reduction of glutathione, in a context of oxidant stress, is catalyzed with NADPH as co-substrate (11, 12). Another condition that may reduce the cellular content in NADPH is the increased activity of NOX1 reported in the intestinal mucosa of IBD patients (37). The link between MTRR and oxidant stress in CD pathogenesis was also illustrated by an association between disease severity (as measured by CDAI scores) and the SOD activity that was restricted to CD patients carrying *MTRR 66AA* genotype. These findings are consistent with experimental data from animal studies showing that SOD transgenic mice exhibit a more severe colitis after dextran sulfate sodium administration than their wild-type littermates (38).

The association of *MTRR A* allele with CD needs to be confirmed in large independent cohorts. The identification of a link between oxidant stress, vitamin B<sub>12</sub> metabolism, and disease severity in patients with CD may open new avenues for intervention and follow-up studies. The relationship between vitamin B<sub>12</sub> metabolism and homocysteine in colonic inflammation is illustrated by the recent finding that homocysteine upregulates *in vitro* the expression levels of transcobalamin (a plasma transporter of vitamin B<sub>12</sub>) and its receptor (39). The authors also showed high expression levels of transcobalamin and its receptor in inflamed colon mucosa of 6-trinitrobenzene sulfonate-treated rats and CD patients (39). For translating these data into clinical management, the benefit of vitamin supplementation in CD patients with evidenced B<sub>12</sub> deficit will need to be assessed in intervention studies, using SOD activity as a marker and *MTRR A* allele as a predictor. B-group vitamin supplementation may have antioxidant and antiinflammatory effects independently of a homocysteine-lowering effect (40), suggesting that these intervention studies will aim at normalizing vitamin B<sub>12</sub> levels instead of homocysteine concentrations.

## STUDY HIGHLIGHTS

### What Is Current Knowledge

- Hyperhomocysteinemia is commonly found in patients with inflammatory bowel disease.
- The implication of genetic determinants other than *MTHFR* remains unknown.
- Hyperhomocysteinemia is associated with increased oxidant stress.

## What Is New Here

- *MTRR AA* genotype may be an independent predictor of Crohn's disease risk.
- *MTRR 66A>G* polymorphism is associated with oxidant stress and disease activity.

**Reprint requests and correspondence:** Professor Jean-Louis Gueant, M.D., D.Sc., Inserm, U724, University Henri Poincaré and CHU of Nancy, Allée du Morvan, 54511 Vandoeuvre-les-Nancy Cedex, France.

Received June 19, 2007; accepted August 21, 2007.

## REFERENCES

1. Frosst P, Blom HJ, Milos R, et al. A candidate genetic risk factor for vascular disease: A common mutation in methylenetetrahydrofolate reductase. *Nat Genet* 1995; 10:111–3.
2. Chen J, Stampfer MJ, Ma J, et al. Influence of a methionine synthase (D919G) polymorphism on plasma homocysteine and folate levels and relation to risk of myocardial infarction. *Atherosclerosis* 2001;154:667–72.
3. Leclerc D, Wilson A, Dumas R, et al. Cloning and mapping of a cDNA for methionine synthase reductase, a flavoprotein defective in patients with homocystinuria. *Proc Natl Acad Sci U S A* 1998;95:3059–64.
4. Goyette P, Christensen B, Rosenblatt DS, et al. Severe and mild mutations in cis for the methylenetetrahydrofolate reductase (*MTHFR*) gene, and description of five novel mutations in *MTHFR*. *Am J Hum Genet* 1996;59:1268–75.
5. Namour F, Olivier J, Abdelmoutaleb I, et al. Transcobalamin codon 259 polymorphism in HT-29 and Caco-2 cells and in Caucasians: Relation to transcobalamin and homocysteine concentration in blood. *Blood* 2001;97:1092–8.
6. den Heijer M, Rosendaal FR, Blom HJ, et al. Hyperhomocysteinemia and venous thrombosis: A meta-analysis. *Thromb Haemost* 1998;80:874–7.
7. Perry IJ, Refsum H, Morris RW, et al. Prospective study of serum total homocysteine concentration and risk of stroke in middle-aged British men. *Lancet* 1995;346:1395–8.
8. Mahmud N, Molloy A, McPartlin J, et al. Increased prevalence of methylenetetrahydrofolate reductase C677T variant in patients with inflammatory bowel disease, and its clinical implications. *Gut* 1999;45:389–94.
9. Peyrin-Biroulet L, Rodriguez-Gueant RM, Chamaillard M, et al. Vascular and cellular stress in inflammatory bowel disease: Revisiting the role of homocysteine. *Am J Gastroenterol* 2007;102:1108–15.
10. Danese S, Papa A, Saibeni S, et al. Inflammation and coagulation in inflammatory bowel disease: The clot thickens. *Am J Gastroenterol* 2007;102:174–86.
11. McKenzie SJ, Baker MS, Buffinton GD, et al. Evidence of oxidant-induced injury to epithelial cells during inflammatory bowel disease. *J Clin Invest* 1996;98:136–41.
12. Middleton SJ, Shorthouse M, Hunter JO. Increased nitric oxide synthesis in ulcerative colitis. *Lancet* 1993;341:465–6.
13. Nishio E, Watanabe Y. Homocysteine as a modulator of platelet-derived growth factor action in vascular smooth muscle cells: A possible role for hydrogen peroxide. *Br J Pharmacol* 1997;122:269–74.

14. Wang XL, Duarte N, Cai H, et al. Relationship between total plasma homocysteine, polymorphisms of homocysteine metabolism related enzymes, risk factors and coronary artery disease in the Australian hospital-based population. *Atherosclerosis* 1999;146:133–40.
15. Moat SJ, Bonham JR, Cragg RA, et al. Elevated plasma homocysteine elicits an increase in antioxidant enzyme activity. *Free Radic Res* 2000;32:171–9.
16. Dincer Y, Erzin Y, Himmetoglu S, et al. Oxidative DNA damage and antioxidant activity in patients with inflammatory bowel disease. *Dig Dis Sci* 2007;52:1636–41.
17. Satsangi J, Silverberg MS, Vermeire S, et al. The Montreal classification of inflammatory bowel disease: Controversies, consensus, and implications. *Gut* 2006;55:749–53.
18. Barbe F, Abdelmoutaleb I, Chango A, et al. Detection of moderate hyperhomocysteinemia: Comparison of the Abbott fluorescence polarization immunoassay with the Bio-Rad and SBD-F high-performance liquid chromatographic assays. *Amino Acids* 2001;20:435–40.
19. Rogers EJ, Chen S, Chan A. Folate deficiency and plasma homocysteine during increased oxidative stress. *N Engl J Med* 2007;357:421–2.
20. Vasilopoulos S, Saiean K, Emmons J, et al. Terminal ileum resection is associated with higher plasma homocysteine levels in Crohn's disease. *J Clin Gastroenterol* 2001;33:132–6.
21. Oostenbrug LE, van Dullemen HM, te Meerman GJ, et al. Clinical outcome of Crohn's disease according to the Vienna classification: Disease location is a useful predictor of disease course. *Eur J Gastroenterol Hepatol* 2006;18:255–61.
22. Gabriel HE, Crott JW, Ghandour H, et al. Chronic cigarette smoking is associated with diminished folate status, altered folate form distribution, and increased genetic damage in the buccal mucosa of healthy adults. *Am J Clin Nutr* 2006;83:835–41.
23. Arenas M, Simpson G, Lewis CM, et al. Genetic variation in the MTHFR gene influences thiopurine methyltransferase activity. *Clin Chem* 2005;51:2371–4.
24. Papa A, De Stefano V, Danese S, et al. Hyperhomocysteinemia and prevalence of polymorphisms of homocysteine metabolism-related enzymes in patients with inflammatory bowel disease. *Am J Gastroenterol* 2001;96:2677–82.
25. Guedon C, Le Cam-Duchez V, Lalaude O, et al. Prothrombotic inherited abnormalities other than factor V Leiden mutation do not play a role in venous thrombosis in inflammatory bowel disease. *Am J Gastroenterol* 2001;96:1448–54.
26. Vecchi M, Sacchi E, Saibeni S, et al. Inflammatory bowel diseases are not associated with major hereditary conditions predisposing to thrombosis. *Dig Dis Sci* 2000;45:1465–9.
27. Magro F, Dinis-Ribeiro M, Araujo FM, et al. High prevalence of combined thrombophilic abnormalities in patients with inflammatory bowel disease. *Eur J Gastroenterol Hepatol* 2003;15:1157–63.
28. Bjerregaard LT, Nederby NJ, Fredholm L, et al. Hyperhomocysteinemia, coagulation pathway activation and thrombophilia in patients with inflammatory bowel disease. *Scand J Gastroenterol* 2002;37:62–7.
29. Gueant-Rodriguez RM, Gueant JL, Debarb R, et al. Prevalence of methylenetetrahydrofolate reductase 677T and 1298C alleles and folate status: A comparative study in Mexican, West African, and European populations. *Am J Clin Nutr* 2006;83:701–7.
30. Fernandez-Miranda C, Martinez Prieto M, Casis Herce B, et al. Hyperhomocysteinemia and methylenetetrahydrofolate reductase 677C→T and 1298A→C mutations in patients with inflammatory bowel disease. *Rev Esp Enferm Dig* 2005;97:497–504.
31. Fukagawa NK, Martin JM, Wurthmann A, et al. Sex-related differences in methionine metabolism and plasma homocysteine concentrations. *Am J Clin Nutr* 2000;72:22–9.
32. Bernstein CN, Sargent M, Vos HL, et al. Mutations in clotting factors and inflammatory bowel disease. *Am J Gastroenterol* 2007;102:338–43.
33. Danese S, Sgambato A, Papa A, et al. Homocysteine triggers mucosal microvascular activation in inflammatory bowel disease. *Am J Gastroenterol* 2005;100:886–95.
34. Drzewoski J, Gasiorowska A, Malecka-Panas E, et al. Plasma total homocysteine in the active stage of ulcerative colitis. *J Gastroenterol Hepatol* 2006;21:739–43.
35. Madamanchi NR, Runge MS. Mitochondrial dysfunction in atherosclerosis. *Circ Res* 2007;100:460–73.
36. Gueant-Rodriguez RM, Juilliere Y, Candito M, et al. Association of MTRRA66G polymorphism (but not of MTHFR C677T and A1298C, MTRRA2756G, TCN C776G) with homocysteine and coronary artery disease in the French population. *Thromb Haemost* 2005;94:510–5.
37. Szanto I, Rubbia-Brandt L, Kiss P, et al. Expression of NOX1, a superoxide-generating NADPH oxidase, in colon cancer and inflammatory bowel disease. *J Pathol* 2005;207:164–76.
38. Krieglstein CF, Cerwinka WH, Laroux FS, et al. Regulation of murine intestinal inflammation by reactive metabolites of oxygen and nitrogen: Divergent roles of superoxide and nitric oxide. *J Exp Med* 2001;194:1207–18.
39. Kalra S, Ahuja R, Binion DG, et al. Upregulation of transcobalamin (TC) and its receptor in colonic inflammation: Effect of homocysteine. *Am J Physiol Gastrointest Liver Physiol* 2007 Jul 19; [Epub ahead of print].
40. Ullegaddi R, Powers HJ, Gariballa SE. B-group vitamin supplementation mitigates oxidative damage after acute ischaemic stroke. *Clin Sci (Lond)* 2004;107:477–84.

---

## CONFLICT OF INTEREST

**Guarantor of the article:** Professor Jean-louis Guéant, M.D., D.Sc.

**Specific author contributions:** Laurent Peyrin-Biroulet and Jean-Louis Guéant, conception and design, recruitment of patients, analysis and interpretation of data, and drafting of the manuscript. Rosa-Maria Guéant-Rodriguez, biological and genetics analyses, interpretation of data, statistical analyses, revising of the manuscript. Marc-André Bigard and Jean-Pierre Bronowicki, recruitment of patients, interpretation of data, and revising of the manuscript. Min Chen, biological and genetics analyses and interpretation of data, statistical analyses. Jean-Louis Guéant, supervision of the study.

**Financial support:** The work was funded by grants from the Institut de Recherche des Maladies de l'Appareil digestif (IRMAD) to Laurent Peyrin-Biroulet.

**Potential competing interests:** None.

---