# Methotrexate-induced apoptosis is enhanced by altered expression of methylenetetrahydrofolate reductase

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**Folates are essential for DNA synthesis and methylation reactions. The antifolate methotrexate (MTX) is a widely used chemotherapeutic drug which inhibits DNA synthesis and induces apoptosis. Changes in activity of a critical folate-metabolizing enzyme, methylenetetrahydrofolate reductase (MTHFR), might alter the chemosensitivity to MTX, as the MTHFR substrate is required for nucleotide synthesis and its product is used in homocysteine remethylation to methionine. Mild MTHFR deficiency is common in many populations due to a polymorphism at bp 677. We previously showed that altered expression of MTHFR enhanced MTX-induced myelosuppression in mice. To determine the cause of the impaired hematopoietic profile in mice with decreased or increased MTHFR expression, we evaluated MTX-induced apoptosis in the major hemolytic organ, spleen, using the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling (TUNEL) staining and caspase-3/7 activity assays, in MTHFR-deficient mice and in MTHFR-overexpressing mice after MTX administration. Decreased or increased expression of MTHFR in mice significantly increased TUNEL-positive cells and caspase-3/7 activities in MTX-treated spleen, compared with that of wild-type littermates. Plasma homocysteine levels correlated with apoptotic index in MTX-treated MTHFR-deficient mice and dUTP/dTTP ratios correlated with apoptotic index in MTX-treated MTHFR-overexpressing mice. The increased apoptosis may therefore relate to hyperhomocysteinemia and deoxyribonucleotide pool imbalances, respectively. Our results suggest that MTHFR underexpression and overexpression enhances MTX-induced apoptosis and myelosuppression, and that genotyping for the MTHFR polymorphism may have therapeutic implications. *Anti-Cancer Drugs* 20:787–793 © 2009 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

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## Introduction

Folates, as methyl donors, are required for DNA synthesis and repair, and the maintenance of DNA integrity and stability. Similarly, apoptosis or programmed cell death is essential for cellular homeostasis and the maintenance of DNA integrity and stability. It is also a protective mechanism against DNA damage induced by chemotherapeutic drugs and critical for their antiproliferative action [1]. Methylenetetrahydrofolate reductase (MTHFR) is a key regulatory enzyme in folate interconversion and stands at a checkpoint that interconnects synthesis of DNA precursors with homocysteine (Hcy) metabolism and methylation reactions. It catalyses the irreversible reduction of 5,10-methylenetetrahydrofolate (5,10-methyleneTHF) to 5-methyltetrahydrofolate (5-methylTHF). The substrate of MTHFR can serve as a source of carbon units for the conversion of dUMP to dTMP by thymidylate synthase or it is converted to 10-formyltetrahydrofolate (10-formylTHF) for *de novo* purine synthesis. The product of MTHFR serves as the methyl donor for the remethylation of Hcy to methionine; methionine is converted to S-adenosylmethionine, a universal methyl donor. Mild MTHFR deficiency, due to homozygosity for a common *MTHFR* polymorphism at bp 677, decreases 5-methylTHF, resulting in hyperhomocysteinemia and global DNA hypomethylation [2,3], and may modify response to methotrexate (MTX) [4,5].

MTX is an antifolate chemotherapeutic drug that inhibits dihydrofolate reductase, the enzyme that converts dihydrofolate to THF. As a consequence of this inhibition, 5,10-methyleneTHF and 10-formylTHF, the essential carbon donors in the biosynthesis of thymidylate and purines, respectively, are depleted resulting in inhibition of DNA and RNA synthesis [6]. MTX induces uracil misincorporation into DNA [7] and, ultimately, DNA strand breaks [8]. MTX-induced nucleotide pool imbalance causes DNA damage and triggers the mitochondrial intrinsic apoptosis pathway [9,10]. In addition, MTX-induced 5-methylTHF depletion results in hyperhomocysteinemia and DNA hypomethylation [11–13].

Hyperhomocysteinemia may induce apoptosis, potentially through a mechanism involving oxidative stress [14].

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We observed increased apoptosis in carcinoma lines and in tumor xenografts following MTHFR inhibition by antisense [15], possibly through hyperhomocysteinemia-induced oxidative stress [16] or through DNA hypomethylation [17] which could affect expression of genes involved in cell survival. We have also reported increased apoptosis in brain of *Mthfr* knockout ($Mthfr^{-/-}$) mice [18].

In more recent work, we showed that MTHFR deficiency in mice enhanced chemosensitivity of hematopoietic cells to MTX. Interestingly, MTHFR overexpression in mice also enhanced chemosensitivity to MTX, but, in contrast to deficient mice, it protected against MTX-induced hyperhomocysteinemia [19]. In this study, we investigated the impact of altered MTHFR expression on MTX-induced apoptosis, as a potential mechanism for the increased myelosuppression after MTX administration.

## Materials and methods

### Animal studies

Animal experimentation received approval from the Animal Care Committee of the Montreal Children's Hospital according to the guidelines of the Canadian Council on Animal Care. *Mthfr*-deficient mice with one or two null alleles [20] and transgenic mice overexpressing *MTHFR* [19], on a C57Bl/6 background, were fed standard rodent chow (Laboratory diet 5001, Agribrands Purina, Woodstock, Ontario, Canada). We recently generated *MTHFR-Tg* mice by construction of an insertion-type targeting vector, in which the human MTHFR cDNA, driven by the ubiquitous CMV promoter, was inserted into the *Hprt* locus. PCR-based genotyping was carried out to identify mice with the transgene. MTHFR overexpression was confirmed in several tissues by semiquantitative reverse transtription-PCR, western blotting, MTHFR enzyme activity assays, and immunohistochemistry [19]. Adult male mice were injected intraperitoneally once every 3 days for 2 weeks (total of four injections) with saline or MTX (Faulding, Kirkland, Quebec, Canada). MTX was administered at a dose of 20 mg/kg (lethal dose 20), a dose which produces toxicity without death. The treatment regimen was identical to that reported in our earlier study [19]. Weight loss and general appearance were monitored regularly. Twenty-four hours after the last injection, mice were killed and tissues were collected.

### Apoptosis measurements

#### *TUNEL staining*

Splenic apoptosis was assessed by the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling (TUNEL) assay using an In Situ Cell Death Detection Kit, POD (Roche Diagnostics, Laval, Quebec, Canada). Briefly, deparaffinized sections were treated with 3% $H_2O_2$ (room temperature, 10 min). After washing with phosphate-buffered saline three times, sections were incubated in TUNEL reaction mixture (terminal deoxynucleotidyl transferase and fluorescein-labeled nucleotide mixture; 37°C, 1 h). Additional phosphate-buffered saline washes were followed by incubation with anti-fluorescein antibody conjugated with horseradish peroxidase (37°C, 30 min). Signal conversion was completed after incubation with 3,3′-diaminobenzidine substrate (Sigma-Aldrich, Oakville, Ontario, Canada) (room temperature, 10 min). Sections were counterstained with a 0.5% methyl green solution for 5 min. Northern Eclipse software (Empix Imaging Inc., Mississauga, Ontario, Canada) was used for quantification of TUNEL-positive cells (cells with brown 3,3′-diaminobenzidine-stained nuclei). A total of five randomly chosen fields ($\times 40$ magnification) in at least three different sections per spleen were assessed in five animals per genotype per treatment group. Apoptotic index (AI) was calculated as the number of TUNEL-positive cells in the defined germinal center area. Counts were confirmed by a second researcher blinded to genotype and treatment status.

#### *Caspase-3/7 activity assays*

The Caspase-Glo 3/7 Assay kit (Promega, Fisher Scientific, Nepean, Ontario, Canada) was used to measure caspase-3/7 activity in frozen spleens. Protein extracts from frozen spleens were prepared as previously described [21]. According to the manufacturer's instructions, 2 µg protein in a total of 20 µl lysis buffer was incubated with 20 µl substrate for 1 h and the signal was detected using a luminometer. Protein extracts from spleens of five animals per genotype per treatment group were assayed in duplicate and the mean of these two values was used for each animal.

### Statistical analysis

Two-factor analysis of variance and independent-sample *t*-tests were performed using SPSS for WINDOWS software, version 11.0 (SPSS Inc., Chicago, Illinois, USA). For analysis of correlations, the Pearson correlation (two-tailed, bivariate) was determined. *P* values of less than 0.05 were considered significant.

## Results

The AI and caspase-3/7 activities in the spleens of MTX-treated mice were significantly higher than those of saline-treated mice (Figs 1 and 2). *Mthfr* genotype also influenced these parameters in both treatment groups. Saline-treated $Mthfr^{-/-}$ mice had higher caspase-3/7 activity compared with saline-treated $Mthfr^{+/-}$ and $Mthfr^{+/+}$ mice (Fig. 2b, $^{\ddagger}P < 0.005$, saline group), although there were no differences due to genotype in the AI (Fig. 2a). The latter finding likely relates to the greater sensitivity of the caspase assay. In the MTX-treated animals, the $Mthfr^{-/-}$ mice had both higher AI and higher caspase-3/7 activities compared with $Mthfr^{+/-}$ and $Mthfr^{+/+}$ mice (Fig. 2a and b,

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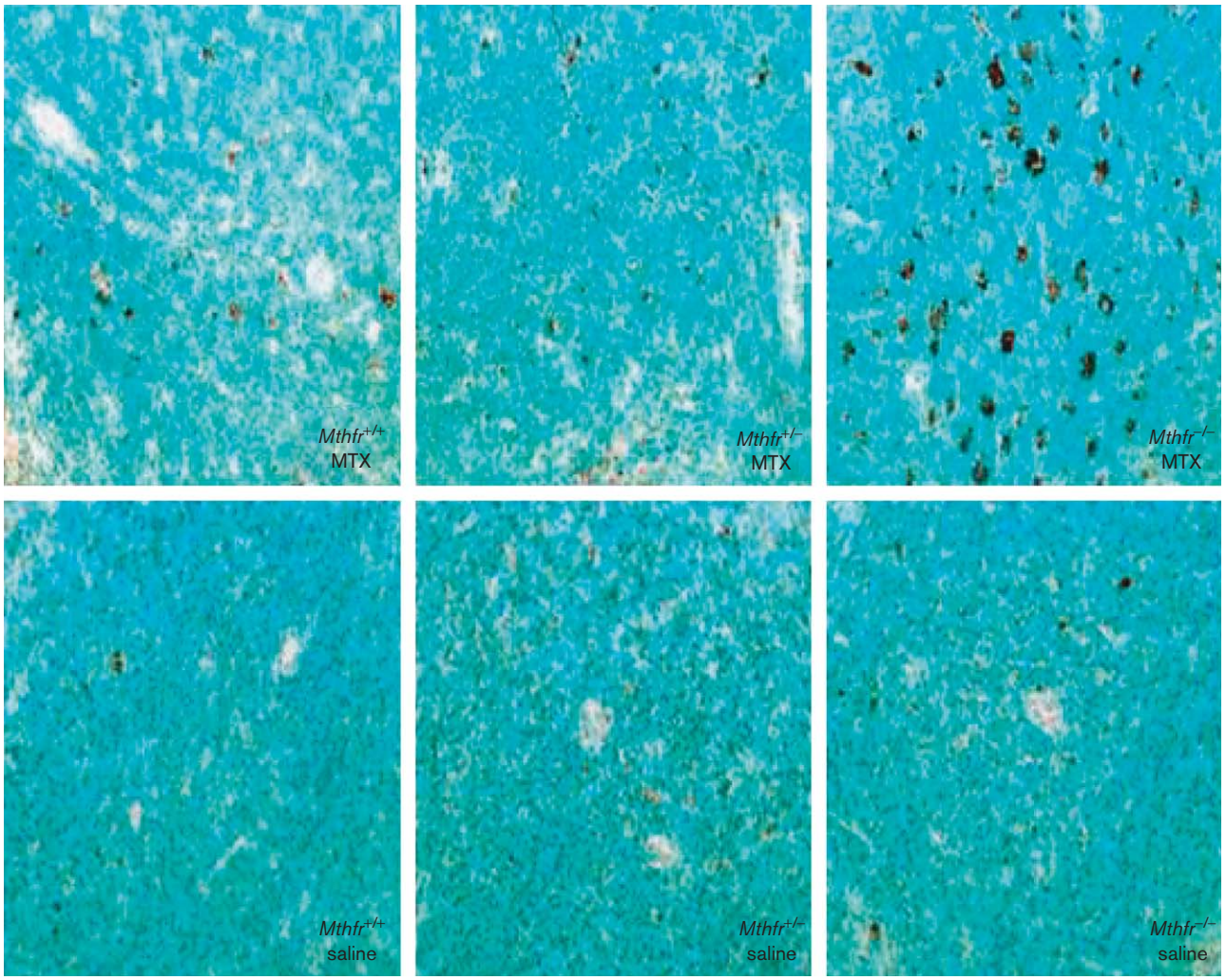
**Fig. 1**




Representative terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling (TUNEL)-stained sections of the germinal centers of spleen of *Mthfr*$^{+/+}$, *Mthfr*$^{+/-}$, and *Mthfr*$^{-/-}$ mice treated with saline or methotrexate (MTX) (20 mg/kg) at $\times 40$ magnification. Apoptotic cells are stained brown.

$^{\ddagger}P < 0.005$, MTX group). Both saline-treated and MTX-treated *Mthfr*$^{+/-}$ mice were observed to have higher caspase-3/7 activity compared with their *Mthfr*$^{+/+}$ littermates (Fig. 2b, $^{\S}P < 0.005$, both groups). MTX-treated *Mthfr*$^{-/-}$, *Mthfr*$^{+/-}$ and *Mthfr*$^{+/+}$ mice had significant increases in each of these parameters compared with their saline-treated counterparts (Fig. 2a and b, $^{\dagger}P < 0.02$). Plasma Hcy levels in MTX-treated mice, reported in our earlier publication [19], were significantly positively correlated with the AI (Fig. 3, $r = 0.75$, $P < 0.001$).

Similar to our observations of *Mthfr*-deficient mice and their wild-type littermates, the AI and caspase-3/7 activities in the spleens of MTX-treated *MTHFR-Tg* and *MTHFR*-Wt mice were significantly higher than those of saline-treated animals (Fig. 4; Fig. 5a and b, $^{\dagger}P < 0.01$). Once again, *MTHFR* genotype influenced both of these parameters in the treated group; MTX-treated *MTHFR-Tg* mice had higher apoptotic indices and caspase-3/7 activities than did MTX-treated *MTHFR*-Wt mice (Fig. 5a and b, $^{\ddagger}P < 0.05$).

A small number of brains from each group were also examined for apoptosis. However, due to the very low level of apoptosis in brain, compared with spleen, and the variability in different brain regions, definitive conclusions could not be reached (data not shown).

In our previous report [19], we found that MTX treatment significantly increased dUTP/dTTP ratios in the spleen of both *MTHFR-Tg* and *MTHFR*-Wt mice. In addition, *MTHFR-Tg* mice had significantly higher dUTP/dTTP ratios compared with *MTHFR*-Wt mice. We

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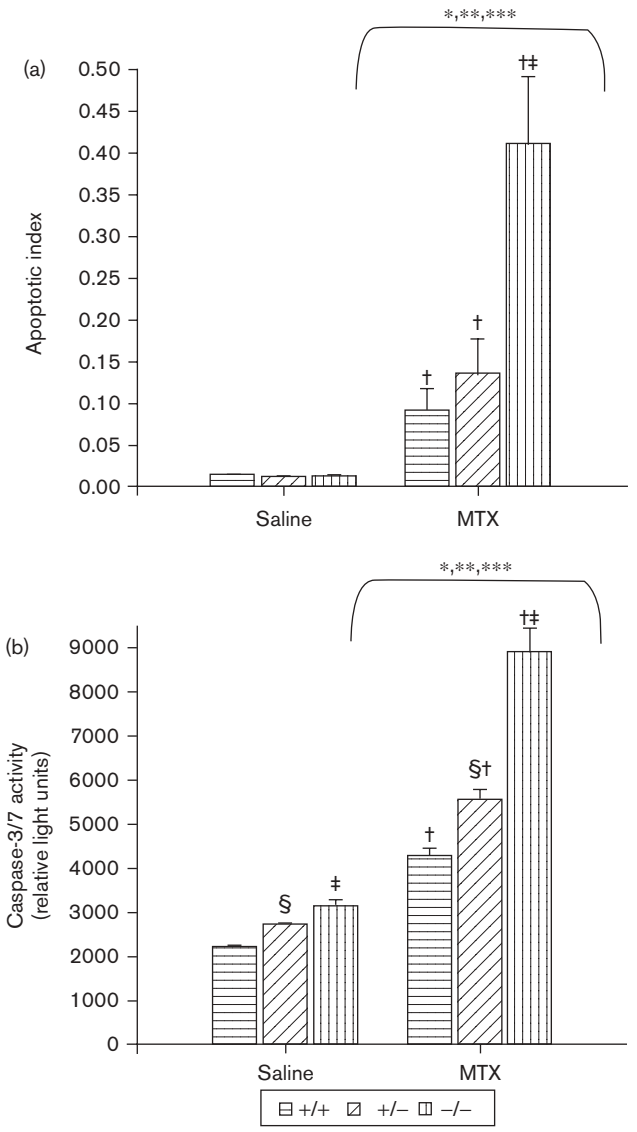
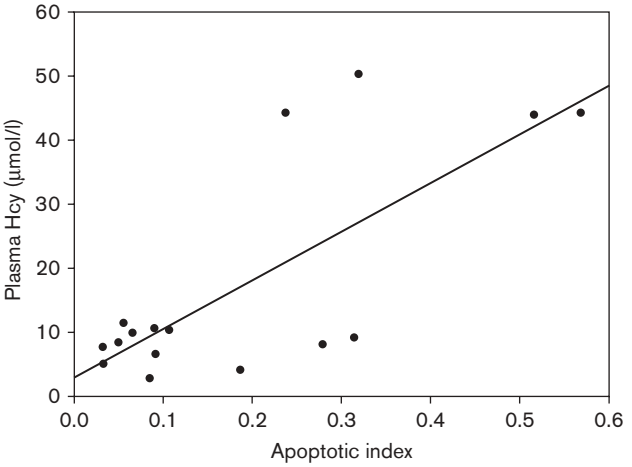
**Fig. 2**




Quantification of terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling (TUNEL) staining in the germinal centers of spleen (a), and caspase-3/7 activity in the spleen (b) of *Mthfr*$^{+/+}$ (horizontal lines), *Mthfr*$^{+/-}$ (diagonal lines) and *Mthfr*$^{-/-}$ (vertical lines) mice treated with saline or methotrexate (MTX) (20 mg/kg). Values represent the mean (± SEM) of four to seven animals per group. *, **, ***$P \leq 0.001$, two-factor analysis of variance for treatment, genotype, and interaction between treatment and genotype, respectively. MTX-treated mice had significantly higher apoptotic index (TUNEL-positive cells/area) and caspase-3/7 activity compared with saline-treated mice. †$P \leq 0.02$, *t*-test (MTX versus saline treatment, genotype constant). Apoptotic index (TUNEL-positive cells/area) and caspase-3/7 activity in MTX-treated *Mthfr*$^{+/+}$, *Mthfr*$^{+/-}$, and *Mthfr*$^{-/-}$ mice were adversely affected compared with their saline-treated littermates. ‡$P < 0.005$, *t*-test (*Mthfr*$^{-/-}$ vs. *Mthfr*$^{+/+}$, within treatment group). After MTX treatment, *Mthfr*$^{-/-}$ mice have higher apoptotic index (TUNEL-positive cells/area) and caspase-3/7 activity than *Mthfr*$^{+/+}$ mice. Saline-treated *Mthfr*$^{-/-}$ mice also have higher caspase-3/7 activity than *Mthfr*$^{+/+}$ mice. §$P < 0.005$, *t*-test (*Mthfr*$^{+/-}$ vs. *Mthfr*$^{+/+}$, within treatment group) *Mthfr*$^{+/-}$ mice have higher caspase-3/7 activity than *Mthfr*$^{+/+}$ mice in both treatment groups.

**Fig. 3**




Correlation of plasma homocysteine (Hcy) levels with apoptotic index [terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling (TUNEL)-positive cells/area] in the spleen of methotrexate-treated *Mthfr*$^{+/+}$, *Mthfr*$^{+/-}$, and *Mthfr*$^{-/-}$ mice. $r = 0.75$, $P < 0.001$.

examined the relationship between the nucleotide ratios and apoptosis, and found that the dUTP/dTTP ratios in MTX-treated *MTHFR-Tg* and *MTHFR*-Wt mice were significantly positively correlated with increased AI in spleen (Fig. 6, $r = 0.74$, $P = 0.001$).

## Discussion

Pharmacogenetics, the investigation of how genetic variation interacts with drug response, holds the promise of individualizing treatment to maximize efficacy and minimize toxicity. Several pharmacogenetic studies of folate metabolism indicate that polymorphisms in a critical folate-metabolizing enzyme, MTHFR, may modify the chemosensitivity to the antifolate drug, MTX [4,5]. We have approached this issue by carrying out controlled experiments using mouse strains expressing varying levels of MTHFR.

In our previous report, we showed that following MTX treatment, both *Mthfr*-deficient and *MTHFR-Tg* (overexpressing) mice displayed enhanced MTX-induced myelosuppression (greater reductions in numbers of hematopoietic cells) compared with wild-type littermates [19]. To investigate a possible role for apoptosis in this response, we proceeded to assess apoptosis in the spleen, a major hemolytic organ. We show in this study that both MTHFR underexpression and overexpression are associated with enhanced MTX-induced apoptosis, a pattern which mirrors our observations in terms of myelosuppression.

As we suggested in our previous study, although the end result may be similar in both groups of mice, the etiology

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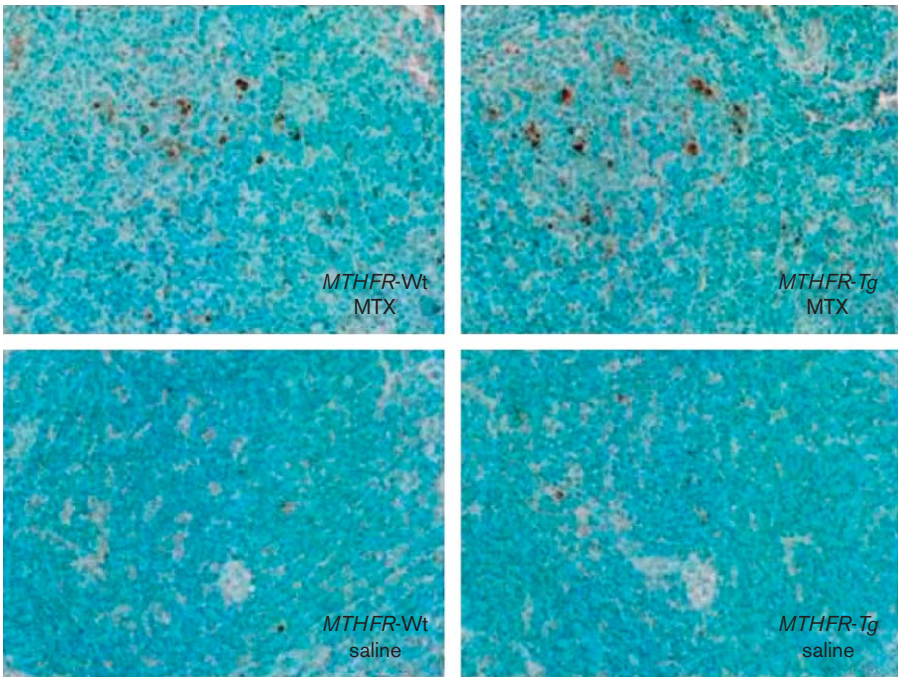
**Fig. 4**




Representative terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling (TUNEL)-stained sections of the germinal centers of spleen of wild-type mice and transgenic littermates treated with saline or methotrexate (MTX) (20 mg/kg) at $\times 40$ magnification. Apoptotic cells are stained brown.

may be distinct. Compared with wild-type mice, *Mthfr*$^{-/-}$ mice have a higher proportion of formyl-folates in their total folate pool, increased levels of DNA hypomethylation and hyperhomocysteinemia [20] as well as more pronounced MTX-induced hyperhomocysteinemia [19]. These metabolic disturbances are due to the increased availability of the MTHFR substrate, 5,10-methyleneTHF, for interconversion to other folate derivatives, and the decreased availability of the MTHFR product, 5-methylTHF, for Hcy lowering and for methylation reactions. The high levels of MTX-induced apoptosis in the spleen of *Mthfr*$^{-/-}$ mice may be a consequence of the additive Hcy-elevating effects of MTX and *Mthfr* deficiency; indeed, we found that plasma Hcy concentrations and splenic AI were positively correlated in MTX-treated mice. Several studies have described a proapoptotic effect of high levels of Hcy in different cell types [16,22]. Furthermore, the Hcy metabolite Hcy thiolactone, which has been identified in both hyperhomocysteinemic humans and mice [23], may be more efficient than Hcy in promoting apoptosis [22,24]. There may also be an additive hypomethylation effect of MTX and *Mthfr* deficiency which could affect the expression or function of cell survival-related proteins.

*MTHFR-Tg* mice have decreased 10-formylTHF levels and elevated dUTP/dTTP ratios suggesting that they have reduced DNA synthesis and repair, and increased DNA damage through their deoxyribonucleotide pool imbalances [19]. This biochemical disturbance is due to the diversion of 5,10-methyleneTHF away from folate interconversion and from the synthesis of dTMP from dUMP, because of the increased activity of MTHFR. Here, we show that there is a significant positive correlation between dUTP/dTTP ratios and AI in the spleen of *MTHFR-Tg* mice; these observations suggest that the increased MTX-induced apoptosis may be due to the deoxyribonucleotide pool imbalances with consequent DNA damage. Imbalances in deoxyribonucleotide pools have been shown to stimulate caspase activation (intrinsic pathway), which initiates apoptosis [9,25]. Depending on the number of double-strand breaks caused by uracil misincorporation, p53 can activate proapoptotic genes [26]. Increased dUTP/dTTP levels were not observed and would not be expected in *Mthfr*-deficient mice (data not shown), as decreased activity of MTHFR would lead to increased substrate for the thymidylate synthase reaction.

The effect of dietary folate intake on MTX efficacy/toxicity and the interaction with *MTHFR* genotype, although of considerable interest, was not addressed in this study. High dietary folate levels may abrogate the antifolate action of MTX; our studies were conducted only on folate-replete mice. Folate can also stabilize the thermolabile MTHFR that is produced from the

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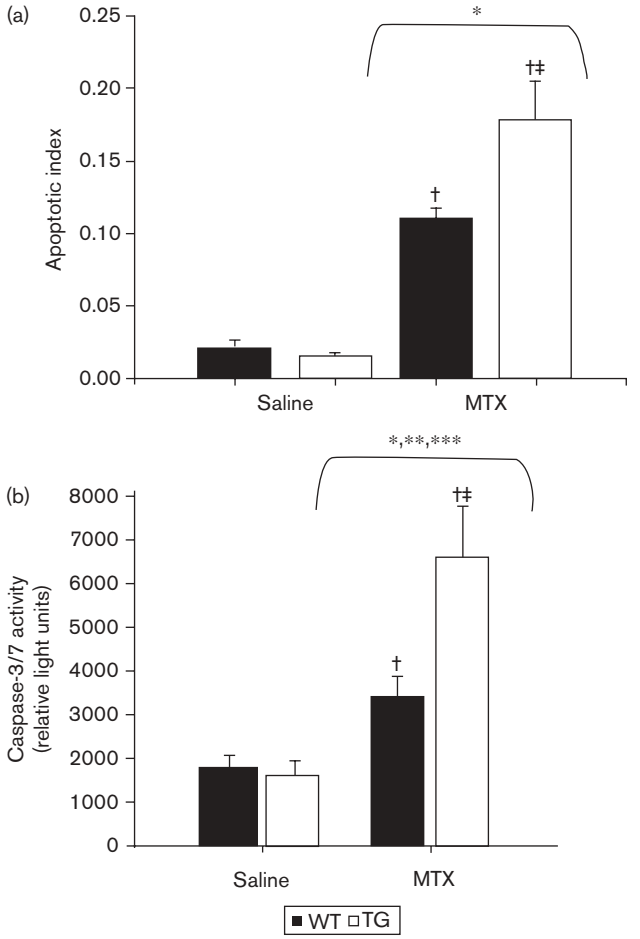
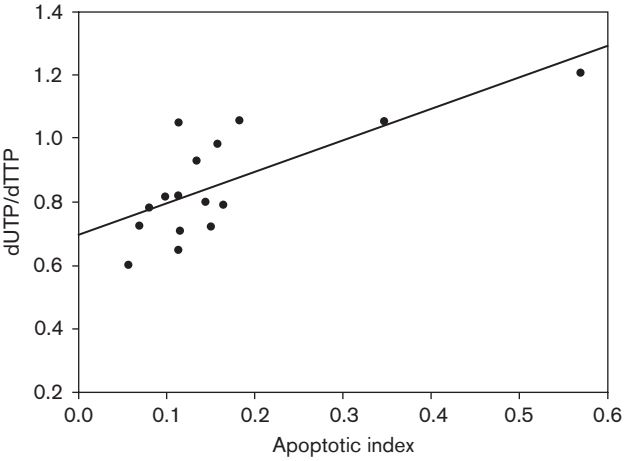
**Fig. 5**




Quantification of terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling (TUNEL) staining in the germinal centers of spleen (a), and caspase-3/7 activity in the spleen (b) of wild-type (WT) mice (filled bars) and transgenic (TG) littermates (unfilled bars) treated with saline or methotrexate (MTX) (20 mg/kg). Values represent the mean (± SEM) of eight to 10 animals per group. *, **, ***$P \leq 0.02$, two-factor analysis of variance for treatment, genotype, and interaction between treatment and genotype, respectively. MTX-treated mice had significantly higher apoptotic index (TUNEL-positive cells/area) and caspase-3/7 activity compared with saline-treated mice. †$P<0.01$, *t*-test (MTX versus saline treatment, genotype constant). Apoptotic index (TUNEL-positive cells/area) and caspase-3/7 activity in MTX-treated *MTHFR-Tg* and *MTHFR*-Wt mice were adversely affected compared with their saline-treated littermates. ‡$P<0.05$, *t*-test (TG versus WT, within MTX group). After MTX treatment, *MTHFR-Tg* mice have higher apoptotic index (TUNEL-positive cells/area) and caspase-3/7 activity than *MTHFR*-Wt mice.

**Fig. 6**




Correlation of dUTP/dTTP ratios with apoptotic index [terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling (TUNEL)-positive cells/area] in the spleen of methotrexate-treated *MTHFR-Tg* and *MTHFR*-Wt mice. $r=0.74$, $P=0.001$.

677C→T allele; our mouse model has lower levels of the wild-type enzyme rather than reduced activity due to an unstable enzyme. Another relevant issue that could be examined is the potential impact of *MTHFR* genotype on the response to MTX and folinic acid rescue. Folinic acid (5-formylTHF) is administered in high-dose MTX chemotherapy protocols to treat severe toxicity. The effect of folinic acid could be modified by *MTHFR* genotype as 5-formylTHF is a precursor of 5,10-methyleneTHF, the substrate for MTHFR. *MTHFR* 677TT individuals may have higher levels of 5,10-methyleneTHF and formylated folates; consequently, the dose of folinic acid in clinical practice might require modulation in these individuals. MTX was administered by the intraperitoneal route in this study; the degree of toxicity to *MTHFR* 677TT individuals might also depend on the route of administration.

In summary, we have demonstrated that decreased or increased MTHFR expression enhances apoptosis in the spleen of MTX-treated mice. These results are consistent with our previous findings which have shown that apoptosis is enhanced in the cerebella of *Mthfr*$^{-/-}$ mice [18] and in tumors treated with *MTHFR*-inhibiting oligonucleotides [15]. Our results also complement several recent association studies in humans. In patients with primary central nervous system lymphoma, the occurrence of confluent central nervous system white matter changes was significantly predicted by the *MTHFR* 677TT genotype [27]. Studies of MTX-treated ovarian cancer [5] and bone marrow transplantation patients [4] also found that the *MTHFR* 677TT genotype was associated with increased MTX-induced toxicity. Our study points to the modulatory effects of MTHFR on MTX response and to the utility of screening for genetic variations in MTHFR or other folate-metabolizing enzymes before MTX therapy, for predicting better therapeutic outcomes and preventing MTX-induced toxicity.

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## References


1 Da Silva CP, de Oliveira CR, da Conceicao M, de Lima P. Apoptosis as a mechanism of cell death induced by different chemotherapeutic drugs in human leukemic T-lymphocytes. *Biochem Pharmacol* 1996; **51**:1331–1340.

2 Castro R, Rivera I, Ravasco P, Camilo ME, Jakobs C, Blom HJ, de Almeida IT. 5,10-methylenetetrahydrofolate reductase (MTHFR) 677C→T and 1298A→C mutations are associated with DNA hypomethylation. *J Med Genet* 2004; **41**:454–458.

3 Frosst P, Blom HJ, Milos R, Goyette P, Sheppard CA, Matthews RG, *et al*. A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. *Nat Genet* 1995; **10**:111–113.

4 Ulrich CM, Yasui Y, Storb R, Schubert MM, Wagner JL, Bigler J, *et al*. Pharmacogenetics of methotrexate: toxicity among marrow transplantation patients varies with the methylenetetrahydrofolate reductase C677T polymorphism. *Blood* 2001; **98**:231–234.

5 Toffoli G, Russo A, Innocenti F, Corona G, Tumolo S, Sartor F, *et al*. Effect of methylenetetrahydrofolate reductase 677C→T polymorphism on toxicity and homocysteine plasma level after chronic methotrexate treatment of ovarian cancer patients. *Int J Cancer* 2003; **103**:294–299.

6 Longo-Sorbello GS, Bertino JR. Current understanding of methotrexate pharmacology and efficacy in acute leukemias. Use of newer antifolates in clinical trials. *Haematologica* 2001; **86**:121–127.

7 Goulian M, Bleile B, Tseng BY. Methotrexate-induced misincorporation of uracil into DNA. *Proc Natl Acad Sci U S A* 1980; **77**:1956–1960.

8 Li JC, Kaminskas E. Accumulation of DNA strand breaks and methotrexate cytotoxicity. *Proc Natl Acad Sci U S A* 1984; **81**:5694–5698.

9 Oliver FJ, Collins MK, Lopez-Rivas A. dNTP pools imbalance as a signal to initiate apoptosis. *Experientia* 1996; **52**:995–1000.

10 Papaconstantinou HT, Xie C, Zhang W, Ansari NH, Hellmich MR, Townsend CM Jr, Ko TC. The role of caspases in methotrexate-induced gastrointestinal toxicity. *Surgery* 2001; **130**:859–865.

11 Refsum H, Ueland PM, Kvinnsland S. Acute and long-term effects of high-dose methotrexate treatment on homocysteine in plasma and urine. *Cancer Res* 1986; **46**:5385–5391.

12 Fiskerstrand T, Ueland PM, Refsum H. Folate depletion induced by methotrexate affects methionine synthase activity and its susceptibility to inactivation by nitrous oxide. *J Pharmacol Exp Ther* 1997; **282**:1305–1311.

13 Winter-Vann AM, Kamen BA, Bergo MO, Young SG, Melnyk S, James SJ, Casey PJ. Targeting Ras signaling through inhibition of carboxyl methylation: an unexpected property of methotrexate. *Proc Natl Acad Sci U S A* 2003; **100**:6529–6534.

14 Wilson KM, Lentz SR. Mechanisms of the atherogenic effects of elevated homocysteine in experimental models. *Semin Vasc Med* 2005; **5**:163–171.

15 Stankova J, Shang J, Rozen R. Antisense inhibition of methylenetetrahydrofolate reductase reduces cancer cell survival in vitro and tumor growth in vivo. *Clin Cancer Res* 2005; **11**:2047–2052.

16 Kruman II, Culmsee C, Chan SL, Kruman Y, Guo Z, Penix L, Mattson MP. Homocysteine elicits a DNA damage response in neurons that promotes apoptosis and hypersensitivity to excitotoxicity. *J Neurosci* 2000; **20**:6920–6926.

17 Friso S, Choi SW, Girelli D, Mason JB, Dolnikowski GG, Bagley PJ, *et al*. A common mutation in the 5,10-methylenetetrahydrofolate reductase gene affects genomic DNA methylation through an interaction with folate status. *Proc Natl Acad Sci U S A* 2002; **99**:5606–5611.

18 Chen Z, Schwahn BC, Wu Q, He X, Rozen R. Postnatal cerebellar defects in mice deficient in methylenetetrahydrofolate reductase. *Int J Dev Neurosci* 2005; **23**:465–474.

19 Celtikci B, Leclerc D, Lawrance AK, Deng L, Friedman HC, Krupenko NI, *et al*. Altered expression of methylenetetrahydrofolate reductase modifies response to methotrexate in mice. *Pharmacogenet Genomics* 2008; **18**:577–589.

20 Chen Z, Karaplis AC, Ackerman SL, Pogribny IP, Melnyk S, Lussier-Cacan S, *et al*. Mice deficient in methylenetetrahydrofolate reductase exhibit hyperhomocysteinemia and decreased methylation capacity, with neuropathology and aortic lipid deposition. *Hum Mol Genet* 2001; **10**:433–443.

21 Lawrance AK, Deng L, Brody LC, Finnell RH, Shane B, Rozen R. Genetic and nutritional deficiencies in folate metabolism influence tumorigenicity in Apcmin/+ mice. *J Nutr Biochem* 2007; **18**:305–312.

22 Kerkeni M, Tnani M, Chuniaud L, Miled A, Maaroufi K, Trivin F. Comparative study on in vitro effects of homocysteine thiolactone and homocysteine on HUVEC cells: evidence for a stronger proapoptotic and proinflammative homocysteine thiolactone. *Mol Cell Biochem* 2006; **291**:119–126.

23 Chwatko G, Boers GH, Strauss KA, Shih DM, Jakubowski H. Mutations in methylenetetrahydrofolate reductase or cystathionine beta-synthase gene, or a high-methionine diet, increase homocysteine thiolactone levels in humans and mice. *FASEB J* 2007; **21**:1707–1713.

24 Jakubowski H. The molecular basis of homocysteine thiolactone-mediated vascular disease. *Clin Chem Lab Med* 2007; **45**:1704–1716.

25 Riedl SJ, Shi Y. Molecular mechanisms of caspase regulation during apoptosis. *Nat Rev Mol Cell Biol* 2004; **5**:897–907.

26 Lane DP. Cancer p53, guardian of the genome. *Nature* 1992; **358**:15–16.

27 Linnebank M, Moskau S, Jurgens A, Simon M, Semmler A, Orlopp K, *et al*. Association of genetic variants of methionine metabolism with methotrexate-induced CNS white matter changes in patients with primary CNS lymphoma. *Neuro Oncol* 2009; **11**:2–8.


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