Homocysteine-betaine interactions in a murine model of 5,10-methylenetetrahydrofolate reductase deficiency

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ABSTRACT

Hyperhomocysteinemia, a proposed risk factor for cardiovascular disease, is also observed in other common disorders. The most frequent genetic cause of hyperhomocysteinemia is a mutated methylenetetrahydrofolate reductase (MTHFR), predominantly when folate status is impaired. MTHFR synthesizes a major methyl donor for homocysteine remethylation to methionine. We administered the alternate choline-derived methyl donor, betaine, to wild-type mice and to littermates with mild or severe hyperhomocysteinemia due to hetero- or homozygosity for a disruption of the *Mthfr* gene. On control diets, plasma homocysteine and liver choline metabolite levels were strongly dependent on the *Mthfr* genotype. Betaine supplementation decreased homocysteine in all three genotypes, restored liver betaine and phosphocholine pools, and prevented severe steatosis in *Mthfr*-deficient mice. Increasing betaine intake did not further decrease homocysteine. In humans with cardiovascular disease, we found a significant negative correlation between plasma betaine and homocysteine concentrations. Our results emphasize the strong interrelationship between homocysteine, folate, and choline metabolism. Hyperhomocysteinemic *Mthfr*-compromised mice appear to be much more sensitive to changes of choline/betaine intake than do wild-type animals. Hyperhomocysteinemia, in the range of that associated with folate deficiency or with homozygosity for the 677T *MTHFR* variant, may be associated with disturbed choline metabolism.

Key words: homocystinuria • hyperhomocysteinemia • folate • methyl donors • choline metabolism

Homocysteine metabolism has become a new focus of biomedical interest after the discovery of epidemiological associations between moderately elevated plasma homocysteine levels and various multifactorial diseases, such as stroke, myocardial

infarction, venous thromboembolism (1), renal failure (2), neural tube defects (3), pregnancy complications (4), and some neuropsychiatric diseases (5, 6).

Homocysteine in body fluids is increased when homocysteine removal through transsulfuration or remethylation becomes insufficient relative to homocysteine production. Nutritional deficiency of cofactors for homocysteine-metabolizing enzymes, such as folate or cobalamin, is a frequent cause of hyperhomocysteinemia, whereas severe genetic defects of these enzymes occur less frequently. These rare genetic defects cause severe hyperhomocysteinemia and homocystinuria and are associated with damage mainly to the nervous and vascular systems (7, 8). The most frequent genetic cause for moderate hyperhomocysteinemia is homozygosity for the thermolabile variant (677C$\rightarrow$T) of 5,10-methylenetetrahydrofolate reductase (MTHFR, E.C. 1.5.1.20), which leads to functional impairment of methionine synthase (MS) and altered risk for several multifactorial diseases (reviewed in ref 9).

Homocysteine is the endogenous product of all transmethylation reactions that use S-adenosylmethionine (SAM) as methyl donor. Methionine intake and transmethylation activity determine the input of homocysteine into the system. A certain amount of that homocysteine is catabolically eliminated by transsulfuration to cysteine, but ~30–50% in humans (10, 11) and 50% in male rats (12) are conserved by remethylation to methionine, using two independent remethylation pathways. Homocysteine can be remethylated to methionine by the cobalamin-dependent enzyme MS, using 5-methylfolate as cosubstrate, which is supplied by MTHFR. Alternatively, betaine:homocysteine methyltransferase (BHMT, EC 2.1.1.5) catalyzes a cofactor-independent methyl transfer from betaine to homocysteine, yielding methionine and N,N-dimethylglycine (DMG). Betaine, or N,N,N-trimethylglycine, is a product of choline oxidation and occurs in minor amounts in food. Under regular laboratory conditions, both remethylation pathways seem to contribute equally in rats (13). The synthesis of methionine through either remethylation pathway is critical for the availability of methionine and the generation of SAM, because at least half of the methionine requirements are provided by remethylation under normal dietary conditions (10). Mthfr-deficient mice have reduced SAM in several tissues, reflecting the importance of folate for maintenance of SAM and transmethylation capacity; SAH levels are increased in Mthfr-deficient mice (14).

The relative importance of folate-mediated methyl neogenesis for homocysteine and methionine homeostasis is dependent on dietary intake of directly transferable, labile methyl groups, and whole-body methyl group demand for transmethylation reactions (10). It has been postulated that mammals utilize preformed methyl groups in preference to newly synthesized methyl groups (15), but the adaptation of one-carbon metabolism to various dietary conditions and endogenous demands is not fully understood. It has been established that there is an absolute dietary requirement for choline in humans and in rodents (reviewed in ref 16), whereas methionine can be replaced by the combination of dietary homocysteine with a methyl donor in the diet (17). The quantitative significance of the choline oxidation pathway *in vivo*, however, remains uncertain. It may be underestimated at present, especially in the human newborn and infant (18).

Betaine supplementation has proven effective in ameliorating the biochemical abnormalities and the clinical course in homocystinuria due to deficiency of cystathionine-β synthase (CBS) (19) or to several remethylation defects (20,21). It lowers the elevated homocysteine levels associated

with these disorders and increases plasma methionine and SAM concentrations. Betaine is believed to directly enhance homocysteine remethylation and, consequently, to increase the availability of methonine for protein synthesis and transmethylation. In addition to increasing methyl group supply and homocysteine turnover, it probably also stimulates homocysteine disposal by activating CBS via SAM (22). However, high-dose betaine treatment does not normalize homocysteine metabolism in homocystinuric patients, as indicated by plasma homocysteine levels that remain elevated 5- to 10-fold (19, 20), leaving these patients exposed to potentially harmful homocysteine concentrations. Betaine administration in moderate hyperhomocysteinemic states has not been extensively studied. Preliminary data suggest a beneficial effect of betaine on plasma homocysteine concentrations in the fasted state and after methionine loading in healthy volunteers (23).

We recently created a murine model for severe and mild MTHFR deficiency, which is a good animal model for human severe and moderate hyperhomocysteinemia, respectively (14). Here, we report our investigation of the effects of betaine supplementation on homocysteine and choline metabolism in wild-type mice and in their littermates with mild or severe MTHFR deficiency. We show that MTHFR deficiency in mice is associated with a higher demand for betaine-dependent remethylation and that BHMT can only partly compensate for this deficiency, even with high-dose betaine treatment. We demonstrate that homocysteine metabolism in mice is dependent on betaine supply and gender, and we provide preliminary evidence for a close relationship between choline/betaine metabolism and homocysteine metabolism in humans with cardiovascular disease.

METHODS

Mice

All mice were generated and housed in our own breeding facility with free access to food and water. Animal experimentation was approved by the Animal Care Committee of the Montreal Children's Hospital and complied with the guidelines of the Canadian Council for Animal Care. Mice with a heterozygous or homozygous disruption of the *Mthfr* gene (14) from F6 or F7 generations of backcrosses to BALB/cAnNCrlBR, together with their wild-type littermates as controls, were used throughout the study. *Mthfr* genotypes were determined by a polymerase chain reaction (PCR)-based method as previously described (14).

The mean starting age was 112.5 (3.1) days for mice on chow, 100.7 (2.7) days in study 1, and 227.3 (7.9) days in study 2, with mean body weights of 24.8 (0.5) g, 22.9 (0.5) g, and 23.5 (0.3) g, respectively. There was no significant difference for these parameters between genotype or treatment groups.

Metabolite levels of mice on regular laboratory chow

Plasma levels of homocysteine, cysteine, betaine, and dimethylglycine were measured in 65 mice (20 females, 45 males) of all three *Mthfr* genotypes fed standard laboratory chow (Purina 5001, Purina Mills). This diet contains choline at 16.1 mmol/kg diet, 0.43% L-methionine, 0.32% L-cystine, and 1.21% L-serine. Total labile methyl group content (sum of methionine, choline, betaine) was 58.2 mmol/kg. Folate content of this diet is high, at least 5.9 mg/kg, which is

threefold higher than the recommended amount for rodents (American Institute of Nutrition [AIN-93M]) (24). Blood was taken by tail clipping under local anesthesia, anticoagulated with EDTA (MicrovetteR 500, Sarstedt, Germany), and immediately placed on ice. Plasma was quickly separated by centrifugation at 10,000g for 5 min and immediately frozen at –70°C until analysis.

Dietary experiments: study 1

Two gender groups of at least six wild-type, six heterozygous, and three homozygous mutant animals were treated either with an amino acid-defined control diet (TD 00310, Harlan Teklad, Madison, WI) or the same diet containing a betaine supplement of 25 mmol/kg diet (TD 00311) for 2 wk. Our control diet had essentially the same composition, including folate content, as the reference rodent diet, AIN-93M (23), but contained choline at 5 instead of 10 mmol/kg diet, 0.30% L-methionine, 0.35% L-cystine, and 0.35% L-serine. Total labile methyl group content (sum of methionine, choline, betaine) was 25.1 mmol/kg as compared with 32.1 (AIN-93M); total sulfur content ws 34.7 mmol/kg (32.1 in the AIN-93M diet). The same design was used to study the effects of two other methyl donors, dimethylsulfonioacetate (DMSA, provided by T. Garrow) and dimethylsulfoniopropionate (DMSP, TCI America) at isomethyl concentrations to betaine. Body weight and food intake were recorded weekly.

After 2 wk, mice were killed in a CO_2 chamber. Blood was collected by heart puncture, anticoagulated with EDTA (MicrovetteR), and immediately put on ice. Plasma was quickly separated by centrifugation at 10,000g for 5 min and immediately frozen at –70°C until analysis. Livers were dissected, and a small piece of liver was excised from the inferior part of the right lobe and fixed in 10% neutral-buffered formalin (Sigma, St. Louis, MO) at 4°C. Brains of nullizygotes were removed and split longitudinally in the midline. Half-brains and liver tissue aliquots were frozen on dry ice and stored at –70°C.

Dietary experiments: study 2

Ten groups, each comprising four adult heterozygous female mice, were placed on control diet TD 00310 and supplemented with increasing amounts of anhydrous betaine in drinking water, ranging from 0 to 640 mmol/l for 2 wk. Water was changed twice weekly. Body weight and food and water consumption were monitored. Betaine intake by drinking water and total intake of labile methyl groups, as a percentage of the theoretical intake by feeding the same amounts of the AIN-93M diet, were calculated. After 2 wk, mice were processed as in study 1. BHMT activities were determined in livers of all groups, and choline metabolites were measured in four subgroups. Heterozygous females on betaine diet from study 1 were included as an additional group in the final evaluation.

Human studies

Plasma samples (122) from a French-Canadian study population with angiographically documented coronary heart disease were analyzed for betaine and DMG concentrations. Population characteristics and other biochemical measurements in plasma aliquots of these patients have been described previously in detail (25, 26).

Metabolites

Total homocysteine and total cysteine concentrations were measured after a reduction of a plasma sample by HPLC as described previously (27). Plasma amino acid concentrations were measured by HPLC, using a previously described method (28). Betaine and DMG concentrations in plasma were analyzed with HPLC as previously reported (29). Choline compounds in tissues were extracted by the method of Bligh and Dyer (30). Choline, glycerophosphocholine, phosphocholine, betaine, and phosphatidylcholine were then measured using liquid chromatography-electrospray ionization-isotope dilution mass spectrometry (LC-ESI-IDMS) (31).

BHMT activity

BHMT activity in crude liver extracts was analyzed as previously described (32).

Liver histology

Liver tissue was fixed in 10% neutral-buffered formalin (Sigma) at 4°C, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin/eosin. Sections were evaluated by microscopic examination at 40× magnification. Steatosis was evaluated by visualization of cytoplasmic droplets. Steatosis was graded as not present, as mild when single hepatocytes contained lipid droplets, as moderate when only microvesicular steatosis occurred, and as severe when macrovesicular lipid deposition was seen and altered the morphology of hepatocytes. Grading was independently confirmed by a second researcher, blinded to treatment.

Statistical analyses

Mean and SE of the mean were used, unless stated otherwise. Because the majority of *Mthfr* –/– mice do not survive beyond the first few weeks of life, sample sizes of this subgroup were sometimes too small to formally test for normal distribution. We therefore used nonparametric tests for statistical analyses involving these mice. Metabolite levels between groups were compared using ANOVA or the Kruskal-Wallis test. If a significant test result was found, single parameters were compared with either a two-sided t test or a two-sided Wilcoxon test. Linear correlation between two parameters was calculated and Spearman's regression coefficient provided. A general linear multiple regression model was used to calculate the multiple regression coefficient R and the partial regression coefficient r. For all analyses, a $P=0.05$ was considered significant.

RESULTS

Mthfr-deficient mice on laboratory chow

We first evaluated plasma metabolite levels of mice with different *Mthfr* genotypes fed standard laboratory chow (Table 1). These values were similar to those we reported earlier (14). Homocysteine correlated negatively with betaine in *Mthfr* +/+ mice ($r=-0.148$, $n=23$), +/– mice ($r=-0.363$, $n=28$), and –/– mice ($r=-0.590$, $n=14$), but statistical significance was achieved only in the last group ($P=0.03$).

Mthfr-deficient mice on control and betaine-supplemented diets

Plasma metabolites

Results were evaluated for mice with both genders combined (see Fig. 1). The proportion of male and female mice was similar in the genotype and treatment groups. Separation by gender decreased the number of available observations but did not change the genotype- or diet-dependent differences. Plasma homocysteine concentrations in mice on the control diet were strongly dependent on *Mthfr* genotypes. *Mthfr +/−* and *Mthfr −/−* mice had a 1.8-fold and 7-fold increase, respectively, over *Mthfr +/+* mice. Plasma methionine concentrations were 50% lower in *Mthfr −/−* mice compared with *Mthfr +/+*. The methionine/homocysteine ratio was significantly reduced from 5.8 (1.1) in wild-type mice to 2.8 (0.5) in *Mthfr +/−* and 0.4 (0.1) in *Mthfr −/−* mice, respectively. Plasma betaine concentrations were not significantly different between genotypes, but DMG levels showed a decrease of 25% and 63% in *Mthfr +/−* and *Mthfr −/−* mice, respectively.

Mice on the betaine-supplemented diet were not different from those on the control diet with respect to food intake and body weight. With the betaine supplement, plasma homocysteine decreased significantly by 56%, 58%, and 50% in *Mthfr +/+, +/−*, and *−/−* mice, respectively. In *Mthfr −/−* mice, methionine increased by 25%, resulting in a significant threefold increase in the methionine/homocysteine ratio. Plasma betaine did not show consistent changes, but DMG increased significantly in *Mthfr −/−* mice. Using two other methyl donors for BHMT, DMSA, and DMSP, we obtained exactly the same degree of homocysteine decrease and the same elevated residual plasma homocysteine (data not shown).

Choline metabolites in tissue and liver BHMT enzyme activity

In liver tissue from mice of both genders on the control diet, betaine, phosphocholine (PCho), and glycerophosphocholine (GPC) were lowest in nullizygotes, intermediate in heterozygotes, and highest in wild-type mice (Fig. 2). Choline (Cho) and phosphatidylcholine (PtdCho; data not shown) concentrations were only weakly negatively correlated to the number of defective *Mthfr* alleles. Most of these differences did not reach statistical significance due to the wide range of values.

Liver metabolite concentrations of mice on the betaine supplement were available for male mice of all three genotypes and only for some female *−/−* mice. We therefore performed statistical analyses only on the respective male groups. With betaine supplementation, liver betaine concentrations increased dramatically. The magnitude of the increase appeared to be genotype-dependent (7-fold, 17-fold, and 34-fold increases in *Mthfr +/+, +/−*, and *−/−* mice, respectively) and abolished the genotype differences found on the control diet (Fig. 2). Liver betaine levels correlated positively with plasma betaine (r=0.59, P<0.05) and plasma DMG (r=0.62, P<0.05) in mice on the betaine diet, and with plasma DMG levels (r=0.49, P<0.05) in mice on the control diet. PCho increased with betaine supplementation, by 2.9-fold, 2.3-fold, and 3.2-fold in *Mthfr +/+, +/−*, and *−/−* mice, respectively. Liver Cho and GPC levels in betaine-supplemented mice did not show significant changes. However, by combining genotypes, we found a significant increase of betaine, PCho, and (data not shown) PtdCho with betaine supplementation.

Brain metabolite concentrations were available only in *Mthfr* −/− mice. In four nullizygotes on betaine supplementation (2 F/2 M), betaine and GPC levels were significantly higher than those in six nullizygotes (2 F/4 M) on the control diet (betaine: 25.6 [1.2] μmol/kg ww vs. 22.0 [1.0] μmol/kg ww; GPC: 1513 [79] μmol/kg ww vs. 1190 [82] μmol/kg ww). Brain choline and PCho also increased with betaine supplementation, but this increase was not statistically significant (choline: 268 [49] μmol/kg ww vs. 229 [19] μmol/kg ww; PCho: 636 [42] μmol/kg ww vs. 522 [21] μmol/kg ww). PtdCho levels did not increase (28431 [816] μmol/kg ww vs. 28264 [269] μmol/kg ww).

Specific BHMT activities in liver were not significantly different between wild-type and *Mthfr* +/− mice on the control diet (189 [17] U/mg protein vs. 216 [22] U/mg protein). *Mthfr* −/− mice had 1.5-fold higher activity compared with wild-type mice (275 [36] U/mg protein); this increase was borderline significant, *P*=0.06.

BHMT activities remained essentially unchanged with betaine supplementation (data not shown). A highly significant negative correlation was found between BHMT activity and the concentration of liver betaine in mice on the control diet (Fig. 3). This correlation was abolished with the betaine supplement.

Liver morphology

We observed severe steatosis in 7 and moderate steatosis in 1 of the *Mthfr* −/− mice on the control diet, whereas none of the 4 *Mthfr* −/− mice with the betaine supplement presented with severe steatosis; 3 had moderate and 1 had mild steatosis (Fig. 4)

Gender-related differences

We found gender differences for some of the plasma metabolites in mice on the control diet (data not shown). Female mice had significantly higher cysteine plasma concentrations than males when all three genotypes were combined (169.3 [6.7] vs. 146.7 [7.1], *P*<0.05). They also had higher methionine concentrations (68.6 [6.3] vs. 58.6 [6.9]), but this difference and the gender differences for homocysteine, betaine, and dimethylglycine did not reach statistical significance. In liver, however, the changes were statistically significant, with higher values for females, compared with males, in their levels of betaine, PCho, and GPC (Table 2). This was evident in the Mthfr +/− and Mthfr +/+ groups; the Mthfr −/− values were not significant because there were only two mice in each gender group. BHMT activity was significantly higher in males, with 1.7-fold, 2.0-fold, and 1.6-fold higher activity in Mthfr +/+, +/−, and −/− mice, respectively. Brain concentrations of choline and PCho in Mthfr −/− mice were higher in females than in males, reaching statistical significance after combining both control and betaine treatment groups (data not shown).

The 15 male and 15 female mice per treatment group responded similarly to the betaine supplement (gender-related data not shown). Plasma homocysteine concentrations decreased to a greater extent in males (64%) compared with females (54%), although this difference did not reach statistical significance. Methionine increased more in *Mthfr* −/− males (ninefold) than in *Mthfr* −/− females (twofold), resulting in methionine/homocysteine ratios in females and males

of 0.77 and 1.41, respectively, compared with ratios of 0.32 and 0.38 in *Mthfr* −/− mice on control diet.

Homocysteine and BHMT with varying betaine supplements

All 10 groups of female *Mthfr* +/− mice on betaine supplements in drinking water and the one additional group of mice from study 1 had similar food (108.6 [0.5] g/kg body weight per day) and water intake (99.1 [1.7] ml/kg body weight per day) and showed no significant changes in body weight or other adverse effects over the 14-day study period. Increasing betaine intake resulted in a significant decline of plasma homocysteine concentrations, which did not further decrease above a betaine intake of 53 mg/kg bw ([Fig. 5](#)). Even with a 120-fold higher betaine intake, homocysteine levels remained at ~40% of the initial level and were still 1.5-fold higher (10.4 [0.8] μmol/l) as compared with *Mthfr* +/+ female mice from study 1 with a dietary betaine supplement (7.2 [0.4] μmol/l). BHMT activity remained unchanged until a betaine intake of 327 mg/kg body weight and then was induced up to 335% of initial activity.

Liver betaine concentrations rose sevenfold with increasing betaine intake from 0 to 480 mg/kg body weight (from 150.4 [15.6] μmol/kg ww to 1086.3 [454.5] μmol/kg ww, $P<0.05$). These findings indicate that the mice were clearly receiving the additional betaine in their diet.

Correlation between homocysteine and betaine in human plasma

One sample out of 122 available plasma samples from patients with cardiovascular disease was excluded due to overt folate deficiency (folate in plasma below detection limit). Betaine and homocysteine concentrations in 121 patients showed a weak, but highly significant, negative correlation with a linear correlation factor $r=–0.254$ ($P<0.005$) ([Fig. 6](#)). DMG showed no correlation with homocysteine. Using multiple regression analysis with homocysteine as the dependent variable and sex, age, creatinine, serum folate, serum cobalamin, MTHFR 677 genotype, and betaine as independent variables, we found a multiple regression coefficient $R=–0.598$ ($n=94$, $P<0.0001$) and a significant negative correlation between betaine and homocysteine with a partial correlation coefficient of $r=-0.222$. Partial correlation coefficients with homocysteine in this model were $r=-0.174$ for folate, $r=-0.344$ for cobalamin, and $r=0.369$ for creatinine.

DISCUSSION

Homocysteine and choline metabolism in Mthfr-deficient mice

Homocysteine levels in mice on regular laboratory chow were similar to those in our earlier report; the other metabolites had not been measured in that study (14). Homocysteine levels were dependent on the *Mthfr* genotype, demonstrating that even *Mthfr* +/− mice, with as much as 60% residual MTHFR activity, were not able to maintain homocysteine homeostasis, despite adequate dietary folate. Plasma methionine levels were much less affected by MTHFR deficiency, as reported for the human disease (8). Cysteine levels in *Mthfr* −/− mice were increased, alluding to an increased flux through the transsulfuration pathway in MTHFR deficiency. Plasma betaine and DMG levels in *Mthfr* −/− mice were decreased, suggesting an inadequate endogenous betaine supply for the alternate remethylation pathway by BHMT. We observed a negative

correlation between plasma homocysteine and plasma betaine in all genotype groups; this correlation reached statistical significance in the nullizygous mice. These findings are consistent with an enhanced dependence on the betaine remethylation pathway in these animals.

To evaluate the influence of methyl group intake, we then used an amino acid-defined control diet with a reduced choline content. Homocysteine concentrations on this folate-repleted amino acid-defined diet were higher than those on laboratory chow but remained strongly associated with *Mthfr* genotype. The administration of these diets enabled us to find a striking relationship between choline metabolism and MTHFR activity. Liver concentrations of betaine, phosphocholine, and glycerophosphocholine were highest in wild-type mice and decreased with an increasing number of disrupted *Mthfr* genes. Plasma DMG showed the same trend. Hence, the disruption of folate-dependent remethylation of homocysteine seems to increase the flow of choline and betaine through the BHMT remethylation pathway and results in a decrease of choline metabolites in liver and, in some cases, in plasma. The decreases in phosphocholine are particularly noteworthy, because phosphocholine is believed to be the intracellular storage form of choline (16). Our findings are consistent with the secondary choline and phosphocholine depletion in liver that has been observed in folate-deficient rats (33).

A low methionine diet, with consequent low SAM levels in tissues, induces choline oxidase (34) and BHMT activity (35). Liver SAM levels are normal in Mthfr +/− and decreased in Mthfr −/− mice (14). We did not observe any induction of BHMT activity in Mthfr +/− mice, but a significantly higher activity in Mthfr −/− mice, possibly due to their lower SAM levels. Specific BHMT activity was negatively correlated with liver betaine on the control diet, suggesting that BHMT activity is an important determinant of betaine concentrations, but it is not induced until betaine intake is dramatically increased.

Low SAM levels decrease the availability of substrate for the enzyme phosphatidylethanolamine N-methyltransferase (PEMT, E.C. 2.1.1.17). PEMT, found mainly in liver, catalyzes SAM-dependent methylation of phosphatidylethanolamine to PtdCho. PEMT is necessary for *de novo* synthesis of PtdCho and choline when the main pathway, the CDP-choline pathway, is compromised, as in dietary choline deficiency (36). Folate-dependent methyl neogenesis will be stressed in choline deficiency (37), because three SAM-dependent steps are then necessary to synthesize one PtdCho molecule that can be catabolized to form one choline molecule. PEMT activity correlates positively with liver PtdCho content (38); is induced by methionine supplementation (39), probably by SAM as well; and is inhibited by SAH (40). MTHFR deficiency, through low SAM and high SAH levels, will affect PEMT activity and disturb *de novo* choline synthesis while enhancing choline metabolism through increased flow along the BHMT remethylation pathway.

Choline deficiency in rodents leads to a decrease of total liver folate (41) and is associated with liver dysfunction and fatty liver, compromised renal function, infertility, growth impairment, bony abnormalities, decreased hematopoiesis, and hypertension. Overt choline deficiency in humans has been observed only in alcoholic liver cirrhosis and during long-term parenteral nutrition and was also associated with fatty liver and liver dysfunction that were reversible upon choline supplementation (reviewed in ref 16). A deficiency of hepatic PEMT activity has also been observed in human alcoholic liver cirrhosis (42).

In human homocystinuria due to severe CBS or MTHFR deficiency, fatty liver occurs frequently for unknown reasons (7, 8). PtdCho, derived from newly synthesized PtdEth via the PEMT pathway, is necessary for the secretion of mainly triacylglycerol-containing very low density lipoprotein (VLDL) particles from hepatocytes (43, 44). Choline deficiency might therefore be associated with neutral lipid deposition in the liver. We found a depletion of choline metabolites in livers of *Mthfr* +/− and *Mthfr* −/− mice and severe fatty infiltration of the liver in *Mthfr* −/− mice. It is possible that MTHFR deficiency and hyperhomocysteinemia lead to fatty infiltration of the liver due to secondary choline deficiency; this deficiency may be a consequence of higher flow of choline through the BHMT pathway and/or limited choline synthesis through the PEMT pathway.

Impact of betaine treatment

All three genotypes responded with a marked decrease of plasma homocysteine following betaine supplementation, with a drop of 50–60% in each genotype group. Our control diet contained only 22% less labile methyl groups than the AIN-93M reference diet (24), and similar diets were considered to be methyl-sufficient in other dietary studies in rodents (35, 45). Because wild-type *Mthfr* mice were also quite sensitive to a change in methyl intake, it appears that even a fully functional folate-dependent remethylation pathway cannot compensate for mildly impaired betaine-dependent remethylation caused by a lower choline intake.

Plasma betaine levels in mice in our study were quite variable and did not reflect betaine intake. This might be due to the dependence of blood levels on individual choline and betaine intakes, the rapid distribution kinetics of betaine (46), or homeostatic control by the kidney (47). However, liver levels of betaine and PCho increased in all three genotypes following betaine supplementation. The increases in liver betaine were genotype-dependent, with the greatest increase occurring in *Mthfr* −/− mice. The PtdCho increase was modest; this large pool was also not affected by *Mthfr* genotype on the control diet and may be maintained at the expense of the other choline metabolites.

Brain betaine and GPC also increased significantly on the betaine diet in *Mthfr* −/− mice (the only genotype group studied in brain), but the increases were more modest than those seen in liver. The increases of other examined metabolites were not significant. Because brain does not express BHMT (15, 48), a direct betaine effect is unlikely. Plasma methionine concentration increased slightly with the betaine supplement in *Mthfr* −/− mice in our study and their increase in the methionine/homocysteine ratio was significant. Plasma methionine increases with betaine treatment in human MTHFR deficiency (8). Plasma choline is actively taken up by the brain and is decreased in choline deficiency due to dietary insufficiency or alcoholic liver cirrhosis (reviewed in ref 16). These findings suggest that the betaine effect on choline metabolism in brain of *Mthfr*-deficient mice could be mediated through hepatic export of either methionine, SAM, or choline.

Betaine supplementation prevented severe fatty infiltration of the liver in nullizygous mice; a beneficial effect of betaine on steatosis has also been demonstrated in nonalcoholic steatohepatitis (49). Betaine may ameliorate these liver problems by enhancing PtdCho

synthesis, although other mechanisms are possible. Irrespective of the mechanism, our results suggest that severe steatosis can be prevented without normalization of homocysteine levels.

Betaine did not induce BHMT activity at the dose of ~300 mg/kg body weight per day used in study 1. We therefore conclude that the (genotype-dependent) absolute differences in homocysteine levels between treatment groups are determined by the change in availability of betaine for BHMT-mediated remethylation, rather than the absolute amount of enzyme, as long as the enzyme is not saturated. The measured liver betaine concentrations are clearly below the high Km of 2.2 mmol/l for betaine (50).

The relative differences in plasma homocysteine between the three genotypes remained fixed with betaine supplementation in study 1. To test the hypothesis that the intake of betaine in this study might not have been high enough to compensate completely for any decrease in folate-dependent remethylation, we monitored plasma homocysteine in heterozygous mice with various betaine intakes, ranging from 0 to an extremely high betaine intake of 6 g/kg body weight per day. Even the highest betaine dose did not lower homocysteine levels below 40% of the initial levels, which is essentially the same percentage that we observed in all three genotypes in study 1. The absolute homocysteine concentrations in these heterozygous mice were still 1.5-fold higher than in wild-type mice on the lower betaine supplement (study 1). The degree of homocysteine lowering by betaine in our study is consistent with the limited human data from homocystinuric patients with severe MTHFR deficiency.

Specific BHMT activity was markedly induced with high betaine supplements, as was previously shown (35, 45). However, betaine-induced high BHMT activity did not influence the homocysteine-lowering effect. The limited homocysteine-lowering effect of betaine in human severe hyperhomocysteinemia has been attributed to product inhibition, because DMG is a strong BHMT inhibitor *in vitro* (51). To test the inhibition hypothesis, we repeated study 1 with two other methyl donors for BHMT, DMSA, and DMSP at an isomethyl dosage level. The respective demethylated products of DMSA and DMSP, methylthioacetate and methylthiopropionate, respectively, have been shown to be much weaker inhibitors of BHMT in vitro as compared with DMG (45). We obtained exactly the same extent of homocysteine decrease and the same elevated residual plasma homocysteine concentrations with these compounds as with betaine (results not shown). Thus, product inhibition of BHMT cannot explain the remaining hyperhomocysteinemia.

BHMT is present only in liver and kidney (48), whereas folate-dependent remethylation is ubiquitous. The intracellular increase in homocysteine generated in other tissues may contribute a substantial portion of circulating homocysteine, which cannot be adequately metabolized by only liver or kidney. Tissue-specific differences in transport and metabolism of various metabolites require consideration. In support of this argument is the relatively minor increase in brain betaine and PCho in nullizygous mice following betaine supplementation, in contrast to the striking increase in betaine in the liver.

Gender differences

We identified a gender difference in homocysteine and choline metabolism, with female mice usually having higher values than males for many of the measured metabolites. Although plasma

measurements were variable, the liver concentrations of betaine, Pcho, and GPC were significantly higher in females. Brain choline and PCho concentrations were higher in female nullizygotes, compared with males of the same genotype. Gender-related differences in enzyme activities and fluxes could explain these observations, because BHMT activity was 60% higher in males than in females of all genotypes. As discussed previously, higher BHMT activity decreases choline metabolites by an increased flux through the BHMT remethylation pathway. The markedly higher BHMT activities for male mice compared with female mice may be attributable to hormonal influences. The presence of steroid hormone binding consensus sites for glucocorticoids and sex hormones in the promoter region of the *Bhmt* gene has recently been demonstrated (45). BHMT activity increased in response to hydrocortisone and testosterone and decreased after injection of thyroxine and estradiol in rats (15). Female rats had only 73% of the MS activity of males in liver (15). In humans, total remethylation activity is higher in males (10). Thus there appears to be more remethylation activity in males compared with females, in several species.

Females have been suggested to have a greater flux of homocysteine through the transsulfuration pathway (10). In agreement with this hypothesis, we found increased plasma cysteine levels in females. Transmethylation is believed to play a quantitatively greater role in males, because of their greater muscle mass and need for creatine synthesis, through guandinoacetate methyltransferase (GAMT, E.C. 2.1.1.2) (10); GAMT is a major contributor to total body homocysteine production through its generation of SAH in the transmethylation reaction. Perhaps the increased remethylation activity in males is required to offset the increased production of homocysteine and the decreased flux through the transsulfuration pathway.

Our findings of a more robust response to a betaine supplement in males, that is, a greater decrease in plasma homocysteine and a particularly strong increase in methionine, are consistent with the hypothesis that males are more sensitive to changes in choline or betaine intake than females because they rely more on BHMT-mediated remethylation.

Interaction of homocysteine and betaine in human plasma

Our murine studies emphasize the close interrelationship between homocysteine, folate, and choline metabolism. In mice on laboratory chow, we found a negative correlation between homocysteine and betaine in plasma that was influenced by the *Mthfr* genotype. Betaine concentrations in livers of mice on the control diet in study 1 were *Mthfr* genotype-dependent. In humans, this interrelationship has not been extensively investigated. In our sample of patients with cardiovascular disease, we also found a surprisingly strong negative correlation between homocysteine and betaine in plasma. In mice, there was a negative correlation between liver betaine and BHMT activity, indicating that betaine concentrations correlate negatively with the utilization of betaine by BHMT.

If the same is true in humans, we would expect lower betaine together with higher homocysteine, at least under conditions where folate-dependent remethylation of homocysteine is compromised, or if flux through BHMT was limited by inadequately low choline intake. One related study in humans reported lower plasma betaine in individuals with folate or cobalamin deficiency (51). In a recent study of patients with chronic renal failure and controls, a significant negative correlation between plasma concentrations of homocysteine and betaine was noted (52). The

significant correlation between plasma homocysteine and betaine in the latter report and in our study population without overt folate deficiency underscores the importance of betaine-dependent remethylation in humans under physiologic conditions.

Choline demands in humans, especially adults, are ill-defined. It is possible that some nutritional habits, for example, avoiding eggs and meat in a strict low-cholesterol diet, or increased choline demands, such as during pregnancy or infancy (16), could lead to a moderate choline deficiency. Choline deficiency has usually been defined by clinical signs such as fatty liver, or elevated liver transaminases. As with the use of methylmalonic acid or homocysteine for the detection of subclinical cobalamin or folate deficiency, respectively, plasma betaine and homocysteine may reflect subclinical choline deficiency in humans.

Our studies in mice demonstrate a strong interaction between homocysteine metabolism and choline/betaine metabolism. Male mice and *Mthfr*-deficient mice may be more sensitive to changes of choline/betaine intake than females or wild-type mice, because they have a greater reliance on BHMT-mediated remethylation. Our preliminary findings in human subjects with coronary artery disease also point to a correlation between homocysteine and betaine metabolism. Individuals with a disruption of folate-dependent remethylation, due to insufficient dietary folate or to the common mutation in MTHFR, may be more sensitive to choline status, with a greater requirement for choline or betaine to offset this disturbance.

ACKNOWLEDGMENTS

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Table 1

Plasma metabolite concentrations [μmol/l] of Mthfr-deficient mice on regular lab chow stratified for genotype[a]

	Wild type (6 F/17 M)	Heterozygous (8 F/20 M)	Nullizygous (6 F/8 M)
Homocysteine	**4.3**	**7.7**[b]	**55.0**[b]
	0.3	0.4	4.9
Cysteine	**151.1**	**149.5**	**170.0**[b]
	3.5	3.3	5.6
Betaine	**65.9**	**67.8**	**58.8**
	4.3	5.2	11.6
Dimethylglycine	**13.3**	**14.0**	**6.3**[b]
	1.5	1.3	1.1

[a]Results are provided as mean and SE in the second line.
[b]$P < 0.05$ compared with wild-type genotype.

Table 2

Liver metabolite concentrations [μmol/kg wet weight] and specific BHMTactivities [U/mg protein] of Mthfr-deficient mice stratified for genotype and gender on control diet. Results are provided as mean (N) and SEM in the second line.

	Wildtype		Heterozygous		Nullizygous	
	Male	Female	Male	Female	Male	Female
Betaine	**84.7** (5)	**246.7** (4)$^\$$	**41.3** (5)*	**150.4** (4)$^\$$	**22.5** (2)	**69.7** (2)
	12.0	*53.2*	*3.7*	*15.6*	*1.9*	*18.9*
Choline	**247.9** (5)	**235.5** (4)	**215.5** (5)	**140.8** (4)	**142.2** (2)	**258.8** (2)
	10.6	*31.1*	*44.8*	*9.3*	21.8	1.0
PCho	**175.1** (5)	**439.7** (4)$^\$$	**103.3** (5)$^{(*)}$	**234.1** (4)$^\$$	**54.1** (2)	**94.9** (2)
	26.9	*77.3*	*18.6*	*37.0*	*31.6*	*13.5*
GPC	**317.3** (5)	**1249.8** (4)$^\$$	**267.5** (5)	**954.5** (4)$^\$$	**188.0** (2)	**281.0** (2)
	46.3	*116.2*	*47.8*	*129.0*	*10.8*	*62.6*
PtdCho	**19034** (5)	**17847** (4)	**17658** (5)	**17196** (4)	**19446** (2)	**17901** (2)
	650	*577*	*870*	*476*	*1488*	*992*
BHMT	**257** (5)	**147** (8)$^\$$	**274** (7)	**135** (5)$^\$$	**336** (2)	**214** (2)
	13	*9*	*9*	*10*	*7*	*21*

*   p < 0.05 compared to wild type genotype of same gender, (*) p = 0.06.
\$ p < 0.05 between male and female group of same genotype
Homozygous mutants were not statistically evaluated due to the small sample size
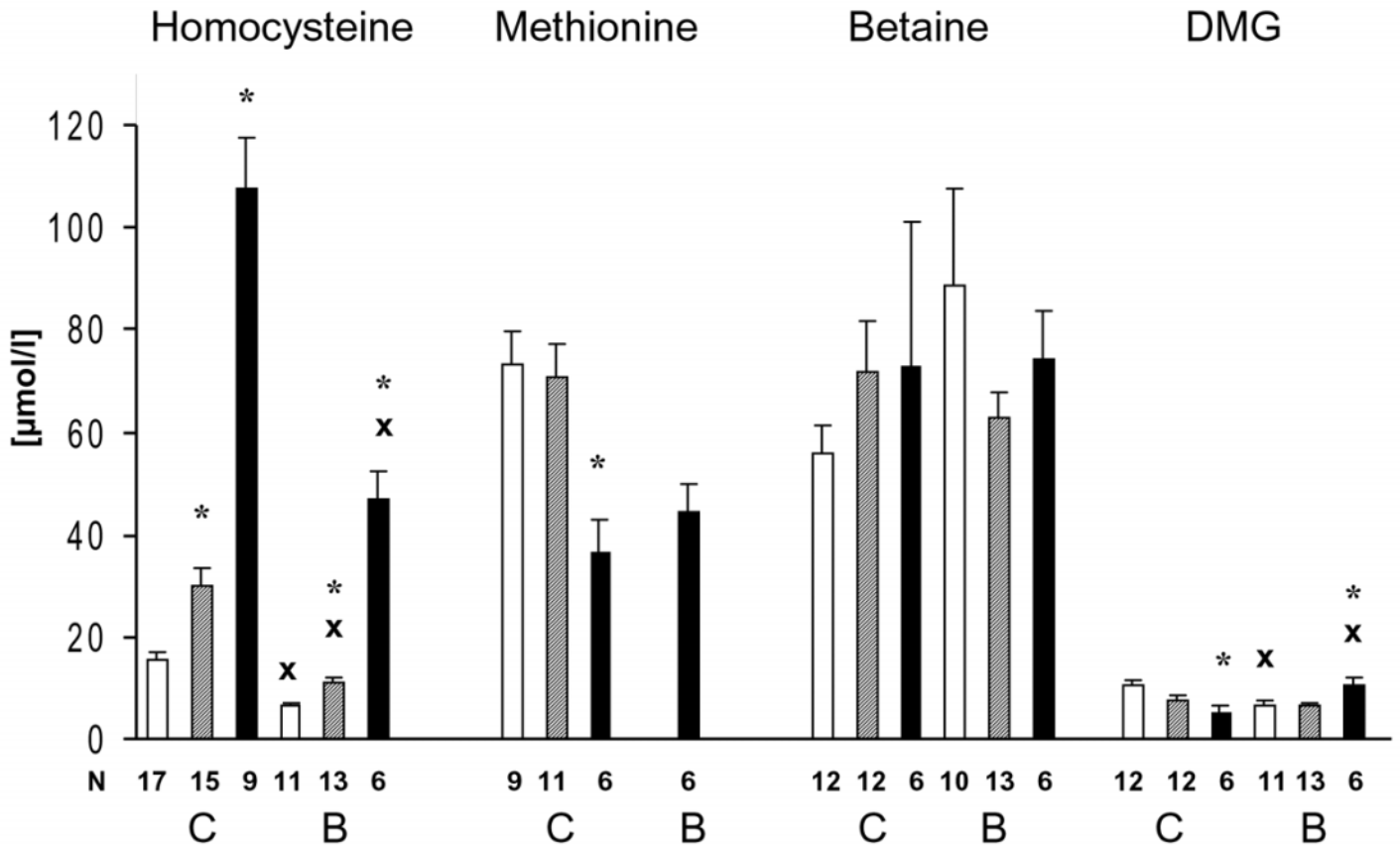
Fig. 1

Figure 1. Plasma metabolites of *Mthfr*-deficient mice of both genders stratified by genotype and diet. C, control diet; B, betaine-supplemented diet. Open bars, wild type; hatched bars, heterozygous; filled bars, nullizygous.Data are presented as mean and SE [µmol/l]. *$P<0.05$ compared with wild-type mice of the same dietary group. X, $P<0.05$ between control and betaine group of the same genotype.
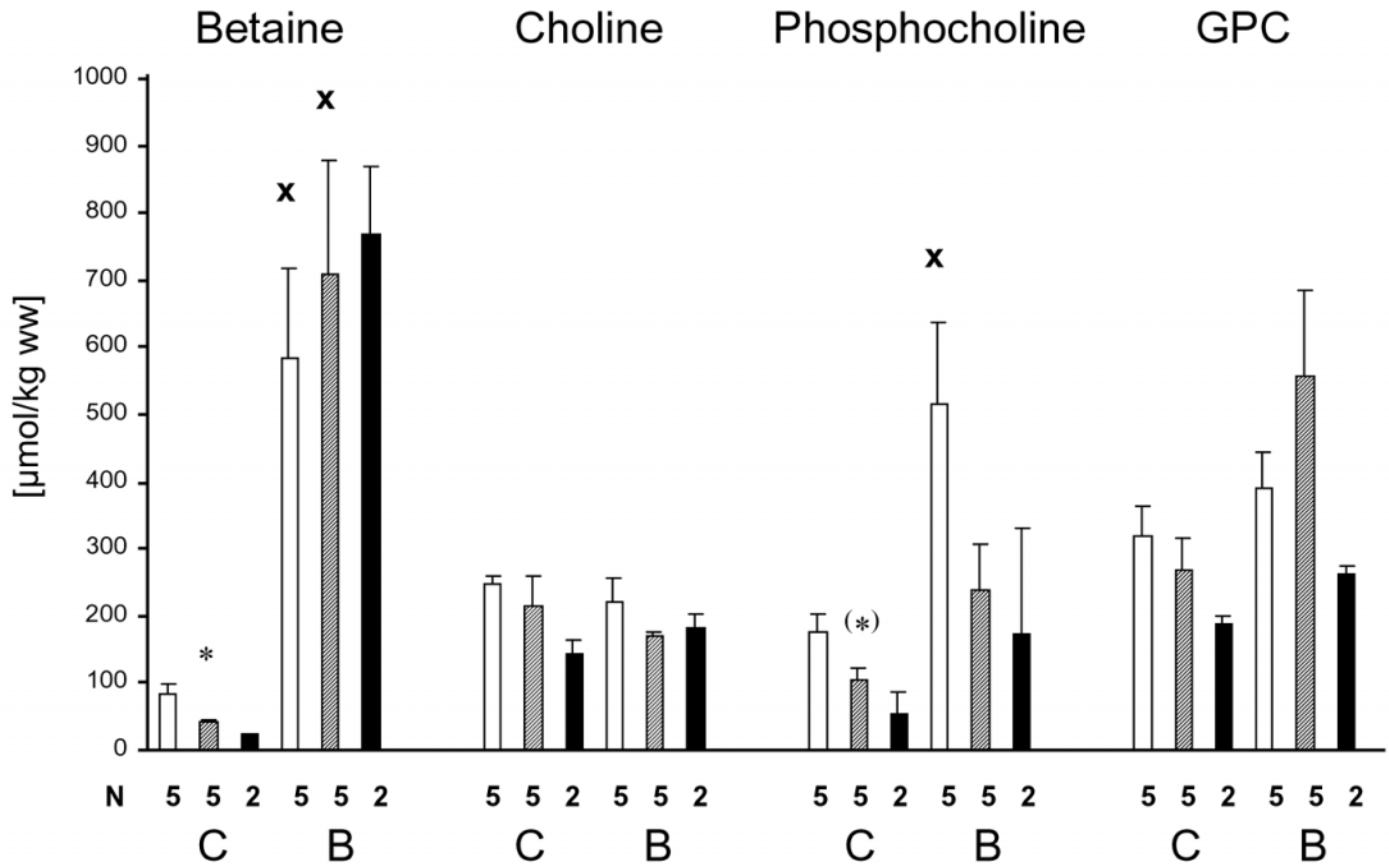
Fig. 2

Figure 2. Liver metabolites of *Mthfr*-deficient male mice stratified by genotype and diet. C, control diet; B, betaine diet. Open bars, wild type; hatched bars, heterozygous; filled bars, nullizygous. Data are presented as mean and SE [μmol/l]. *$P<0.05$ compared with wild-type mice of the same dietary group, (*)$P=0.06$.X, $P<0.05$ between control and betaine group of the same genotype.
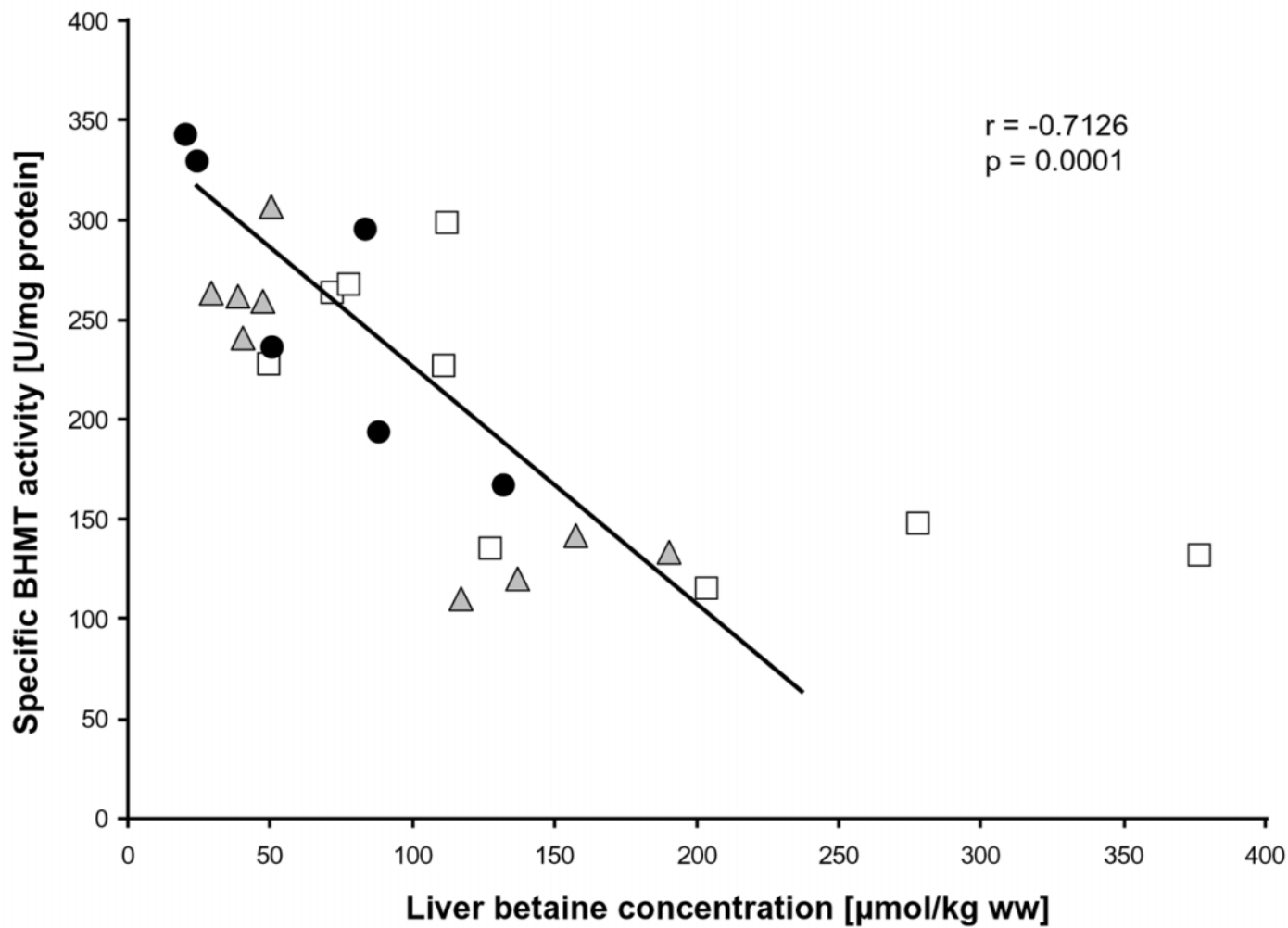
Fig. 3

Figure 3. Linear correlation between specific BHMT activity and liver betaine concentration in 24 mice of all three genotypes on the control diet. Open rectangles, wild type; gray triangles, heterozygous; filled circles, nullizygous.
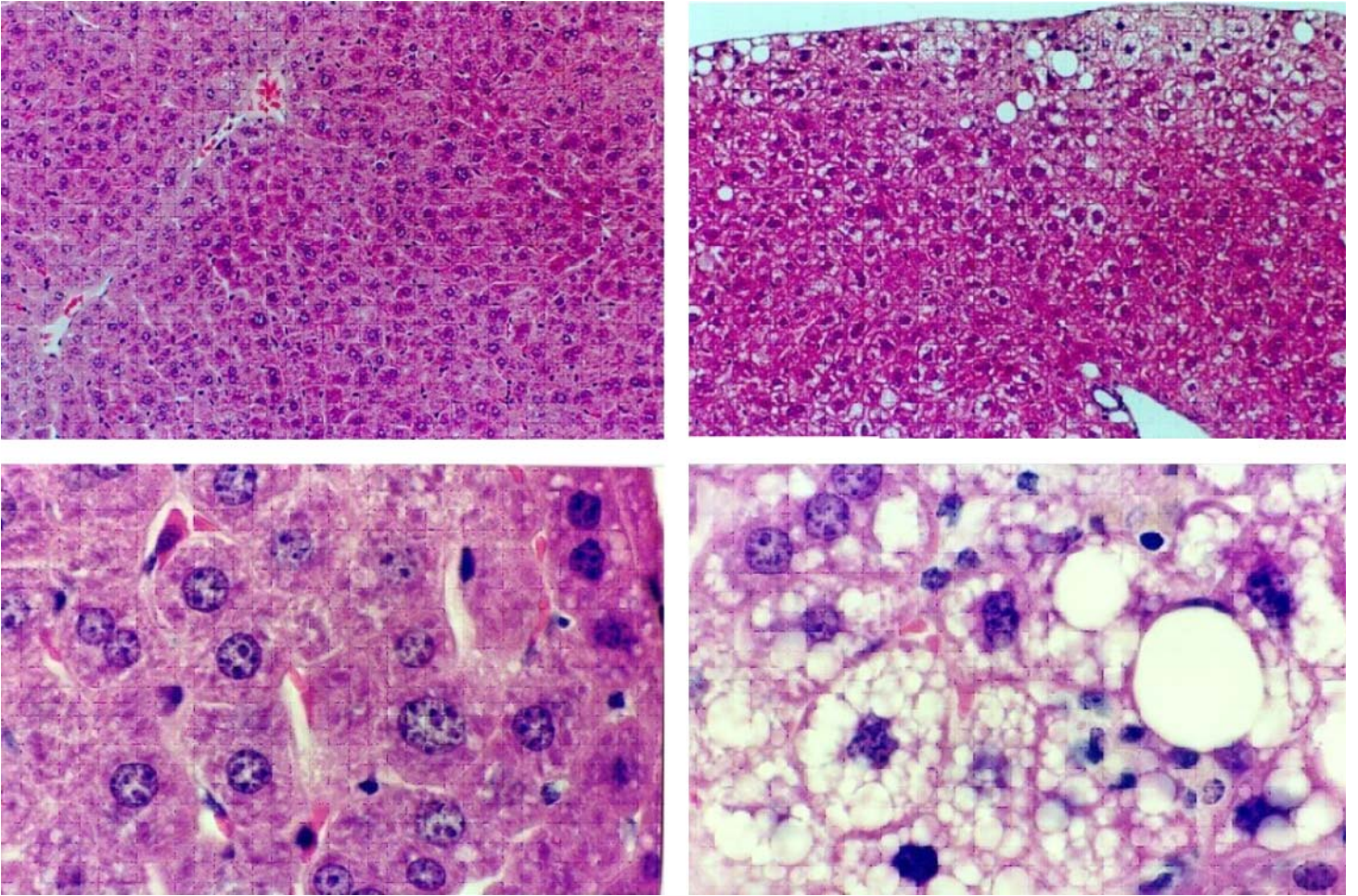
Fig. 4

Figure 4. Liver morphology of Mthfr –/– mice on betaine (left panels) or control (right panels) diet. HE stain, upper panels 20×, lower panels 100× magnification.
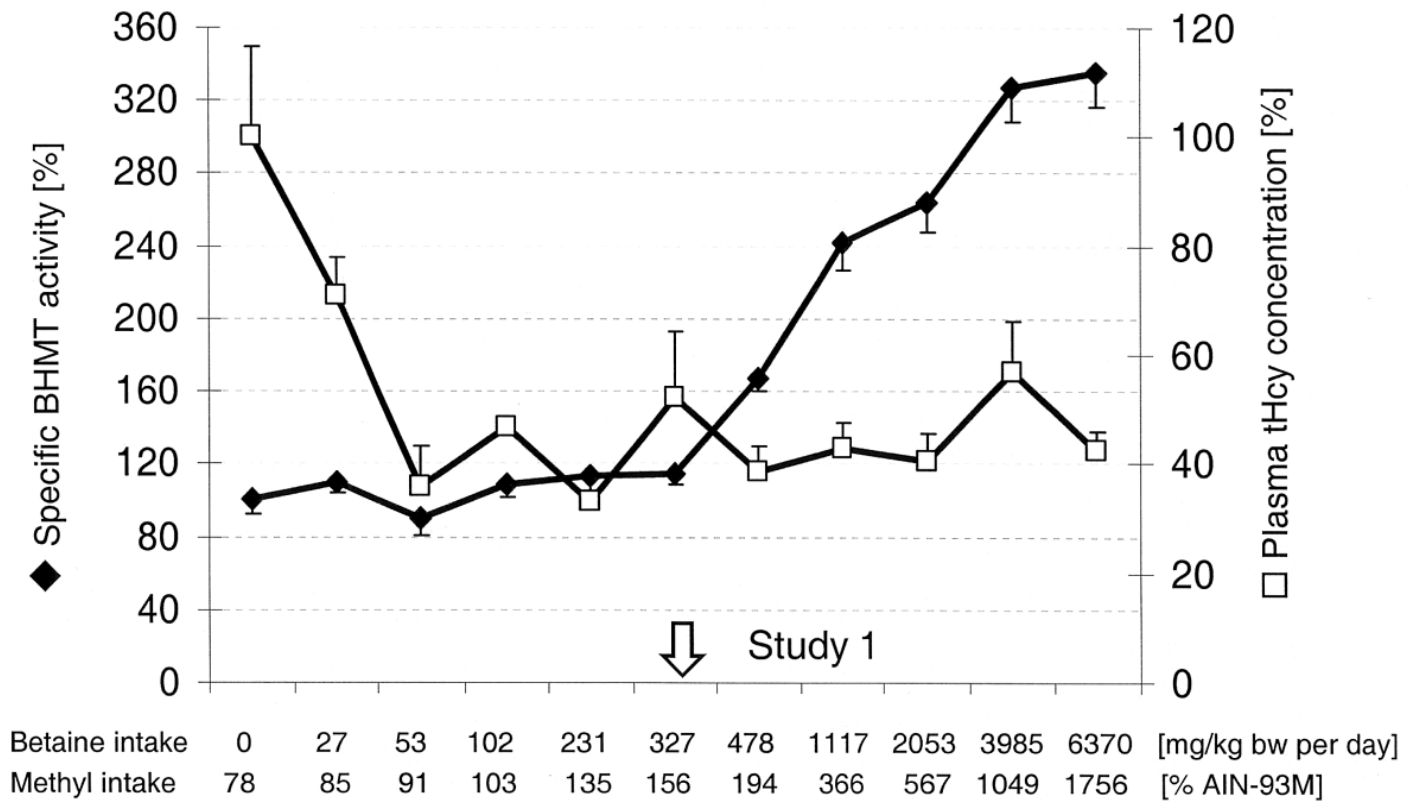
Fig. 5

Figure 5. Effects of increasing betaine intake on plasma homocysteine and on specific activity of liver BHMT in *Mthfr* +/– **mice.** Values have been normalized to the group without the betaine supplement. Data are mean ±SE from four female animals. The arrow indicates the calculated level of betaine intake in study 1. Intake of labile methyl groups was calculated using the actual food intake as described in Materials and Methods and is expressed as the percent of the methyl intake by feeding the same amount of the AIN-93M diet.
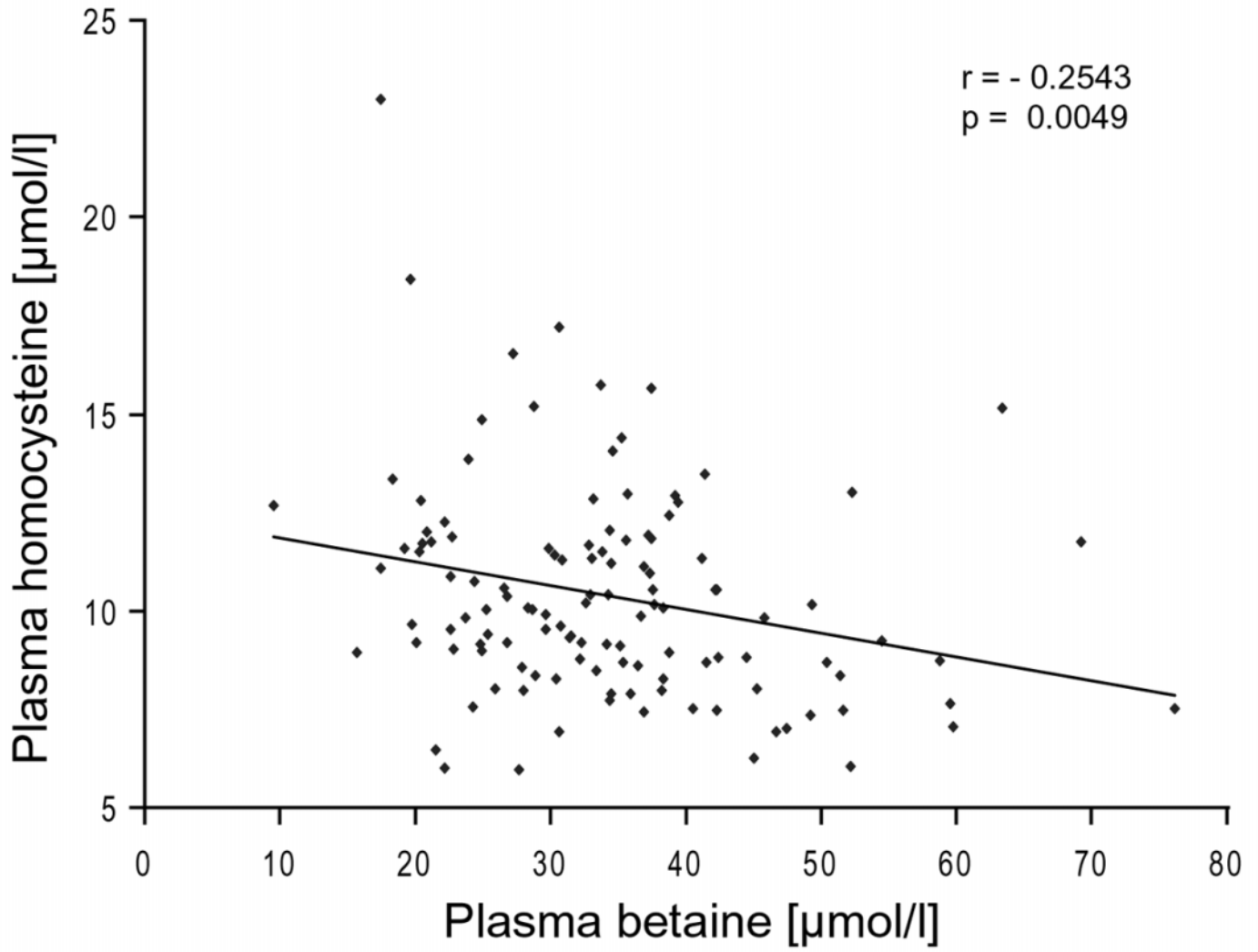
Fig. 6

Figure 6. Linear correlation between plasma betaine and total homocysteine concentrations in 121 patients with angiographically confirmed cardiovascular disease.