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Rolle der betainabhängigen Remethylierung in der Pathogenese und Therapie der Homocystinurie

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1. Zusammenfassung

Hyperhomocysteinämie ist mit zahlreichen multifaktoriellen Erkrankungen assoziiert. Die Kausalität solcher Assoziationen und zugrundeliegende pathogenetische Prinzipien sind noch unzureichend erforscht. Neben nutritiven Mangelzuständen und eingeschränkter Funktion von Nieren oder Leber tragen eine Reihe monogenetischer Defekte zur Hyperhomocysteinämie bei. Die Extremvariante monogenetischer Defekte mit starker Einschränkung der Funktion eines der am Homocysteinstoffwechsel beteiligten Enzyme führt zum Krankheitsbild der Homocystinurie, einem der klassischen „Inborn errors of metabolism“. Die Homocystinurie gilt als behandelbare Erkrankung, allerdings lässt sich durch diätetische Therapie und Vitaminsupplementation in der Regel keine Normalisierung des Stoffwechsels erreichen. Ein Therapieprinzip besteht in der hochdosierten Verabreichung des Cholinmetaboliten und Methyldonors Betain. Betainsupplementation führt zum Absinken der erhöhten Homocysteinkonzentrationen im Plasma. Welchen Stellenwert dieser Therapieansatz für die verschiedenen Formen der Homocystinurie hat, worauf der Therapieeffekt beruht und welche Dosis eingesetzt werden soll, ist trotz langjährigen therapeutischen Einsatzes von Betain nicht hinreichend evaluiert worden.

In der vorliegenden Arbeit werden experimentelle Daten vorgestellt, die den Einfluß der Betainsupplementation auf den Betain- und Homocysteinstoffwechsel dokumentieren. Durch extensive Studien in zwei Tiermodellen der Homocystinurie wurde der Effekt der Betaintherapie auf Überleben, körperliche Entwicklung und biochemische Parameter des Methylstoffwechsels bei gesunden Tieren und bei Tieren mit mässiger und schwerer Hyperhomocysteinämie detailliert belegt. Dosis-Wirkungsbeziehungen wurden im Tiermodell etabliert. Als neuer, wesentlicher Befund der unbehandelten Hyperhomocysteinämie der Maus konnte eine Betaindepletion der Leber identifiziert werden.

Eine Querschnittsstudie an erwachsenen Patienten belegte die negative Korrelation von Betain- und Homocysteinkonzentrationen im Plasma auch bei geringfügig erhöhtem Homocystein, was einen vermehrten Betainverbrauch bei Hyperhomocysteinämie des Menschen nahe legt. In einer Studie an gesunden Probanden wurde die Pharmakokinetik von oral verabreichtem Betain charakterisiert. Durch Vergleich der pharmakodynamischen Daten von Probanden und Patienten mit Homocystinurie konnte ein beschleunigter Transport und Katabolismus von Betain insbesondere zu Beginn der Betainsupplementation nachgewiesen werden, was stark für eine Betaindepletion bei unbehandelter Homocystinurie spricht und die im Tiermodell gefundenen Zusammenhänge bestätigt.

Diese bisher wenig beachtete sekundäre Störung des Cholinstoffwechsels bei Hyperhomocysteinämie könnte einen wesentlichen pathogenen Faktor in der Entstehung der zerebralen und hepatischen Funktionsstörungen und bei der Entstehung atherosklerotischer Läsionen darstellen.

Da die Homocystinurie ein seltenes und heterogenes Krankheitsbild darstellt, wurde die Assoziation verschiedener multifaktoriell bedingter Krankheitsbilder mit der häufigen milden Hyperhomocysteinämie aufgrund eines funktionell wirksamen Polymorphismus des Enzyms Methylentetrahydrofolat-Reduktase untersucht. Die beschriebenen Assoziationen erwiesen sich hierbei in der Regel jedoch als zu schwach um konsistente Hinweise auf pathogenetische Prinzipien zu liefern. Zum weiteren Studium der Pathogenese der Homocystinurie sind daher die transgenen Tiermodelle erforderlich. Die vorgelegte Arbeit unterstreicht die Eignung der beiden verfügbaren Tiermodelle für solche Studien.

2. Abkürzungsverzeichnis

BHMT	Betain:Homocystein Methyltransferase
CBS	Cystathionin-β-Synthase
DMSA	Dimethylsulfonioacetat
DMSP	Dimethylsulfoniopropionat
MS	Methioninsynthase
MTHFR	Methylentetrahydrofolatreduktase
PtdCho	Phosphatidylcholin
PtdEth	Phosphatidylethanolamin
SAH	S-Adenosylhomocystein
SAM	S-Adenosylmethionin

3. Einleitung

Um die Funktion und den Stellenwert der betainabhängigen Remethylierung von Homocystein zu vergegenwärtigen wird zunächst in der Homocysteinstoffwechsel vorgestellt. In der Folge werden natürlicher Verlauf, pathogenetische Prinzipien und Therapiemöglichkeiten der wichtigsten angeborenen Stoffwechselstörungen dargestellt, die zur schweren Hyperhomocysteinämie und Homocystinurie führen. Schließlich werden drei alternative experimentelle Ansätze zur Untersuchung der Fragestellung erläutert: Patientenstudien, Populationsstudien unter Berücksichtigung der häufigen milden Hyperhomocysteinämie und Tierversuche mit den entsprechenden transgenen Tiermodellen der Homocystinurie.

3.1 Pathobiochemie der Hyperhomocysteinämie

Die essentielle Aminosäure Methionin dient nach Aktivierung zu S-Adenosylmethionin (SAM) als universeller Methylgruppendonor der circa 150 verschiedenen Methyltransferasen, die durch Methylgruppentransfer Akzeptoren wie z.B. Nukleinsäuren, Lipide, Phospholipide, Proteine oder Neurotransmitter modifizieren. Durch Methylgruppenverlust wird SAM zu S-Adenosylhomocystein (SAH), das wiederum inhibierend auf zahlreiche Methyltransferasen wirkt. SAH steht in einem reversiblen Gleichgewicht mit seinen durch Hydrolyse entstandenen Produkten Adenosin und Homocystein. Um eine ungestörte Transmethylierung zu gewährleisten müssen diese Produkte sehr effektiv aus der Zelle entfernt werden. Homocystein kann entweder im ersten Schritt des Transsulfurierungsweges mit Serin zu Cystathionin kondensieren oder durch Übertragung einer Methylgruppe von Methyltetrahydrofolat oder Betain zu Methionin remethyliert werden [Finkelstein 1990] (siehe Abbildung 1). Die Methylgruppe des Methyltetrahydrofolats wird aus Einkohlenstoff-Einheiten über verschiedene Stoffwechselwege *de-novo* synthetisiert und aus dem Folsäurestoffwechsel durch das Enzym Methylentetrahydrofolat-Reduktase (MTHFR) bereitgestellt. Das Enzym Methioninsynthase (MS) transferiert diese Methylgruppe unter Vermittlung des Kofaktors Methylcobalamin unter Bildung von Methionin auf Homocystein. Bei geringem Angebot an Methionin werden aus dem Histidinabbau stammende Einkohlenstoff-Einheiten vorwiegend chemisch reduziert und damit der Synthese von Methionin und Purinen und Pyrimidinen zur Verfügung gestellt. Bei hohem Angebot von Methionin, werden endogen gebildete Einkohlenstoffeinheiten vorwiegend oxidiert und

abgebaut. Die beschriebene Regulation des Folatstoffwechsels erfolgt weitgehend über die Konzentration von SAM, welches die Methyltetrayhdrofolatsynthese auf der Ebene der Enzymaktivität der Methylentetrahydrofolatreduktase (MTHFR) inhibiert [Krebs et al. 1976].
Der alternative Methyldonor Betain entsteht durch Oxidation aus Cholin. Cholin und - in geringerem Maß - auch Betain werden direkt durch die Nahrung zugeführt [Zeisel et al. 2003]. Die labile Methylgruppe des Betains (Trimethylglycin) wird durch das in Leber und Niere vorhandene Enzym Betain-Homocystein Methyltransferase (BHMT) unter Bildung von Methionin und Dimethylglycin auf Homocystein übertragen. Weitere präformierte übertragbare Methylgruppen sind lediglich über das in Nahrungsproteinen enthaltene Methionin verfügbar. Ein Mangel an Cholin führt zur verminderten Bereitstellung von Betain als Substrat für das Enzym BHMT und – zumindest im Tiermodell - zu niedrigen SAM-Konzentrationen und einer Verarmung an Folaten. Der Methylgruppenbedarf wird in dieser Situation durch vermehrte folsäureabhängige Remethylierung ausgeglichen. [Niculescu et Zeisel 2002]. Ein Überangebot an exogenen übertragbaren Methylgruppen durch Cholin, Betain oder Methionin hingegen führt zu erhöhten SAM-Konzentration, die, wie oben beschrieben, die methyltetrahydrofolatabhängige Remethylierung hemmen. SAM hemmt darüber hinaus aber auch die Remethylierung von Homocystein durch Betain auf den Ebenen von Transkription und Aktivität des Enzyms BHMT und aktiviert das Enzym Cystathionin-β-Synthase (CBS), welches die Transsulfurierung und damit den Abbau von Homocystein einleitet [Finkelstein 1990].
Bei einem schwerwiegenden Mangel der Aktivität der Enzyme CBS oder MTHFR, durch genetische Defekte der jeweiligen Apoenzyme oder genetisch bzw. diätetisch bedingten Kofaktor-Mangel, bricht die Regulation des Homocysteinstoffwechsels zusammen [Selhub & Miller 1992]. Aus der Akkumulation von Homocystein und SAH resultieren eine Hemmung der Methyltransferasen und weitere toxische Effekte deren Pathomechanismus noch weitestgehend unverstanden ist. Beim CBS-Mangel führt die unzureichende Elimination von Homocystein trotz einer gesteigerten Remethylierungsrate zur Akkumulation von Homocystein, Methionin und SAM. Der MTHFR-Mangel bringt durch Ausfall eines Remethylierungsweges eine verringerte Verfügbarkeit von Methionin und SAM mit sich. Da Cystathionin als Produkt des Transsulfurierungsweges erhöht gefunden wird, scheint die CBS-Aktivität durch die erhöhten Homocysteinkonzentrationen stimuliert zu werden, obwohl das aktivierende SAM nur in niedrigen Konzentrationen vorliegt. Der Mangel an übertragbaren Methylgruppen in Verbindung mit der Hemmung der Transmethylierung durch SAH führt zu einer gegenseitigen Verstärkung der Methylierungsstörung bei MTHFR-

Mangel. MTHFR-Mangel führt außerdem zur vermehrten Verfügbarkeit von Methylentetrahydrofolat und begünstigt damit den oxidativen Abbau von Folaten zu CO_2, was wiederum einen Folatmangel nach sich zieht [nach Krebs et al. 1976]. Diese Faktoren könnten erklären, warum der MTHFR-Mangel ein in der Regel schwereres Krankheitsbild verursacht als der CBS-Mangel. Wichtig ist zu bemerken, dass bei den beiden bekannten und oben beschriebenen Defekten in jeweils einem der drei möglichen Eliminationswege des Homocysteins die beiden anderen verbleibenden Eliminationswege nicht ausreichen um die Homöostase von Homocystein zu erhalten und zum vermehrten Export von Homocystein in die extrazelluläre Umgebung führen.

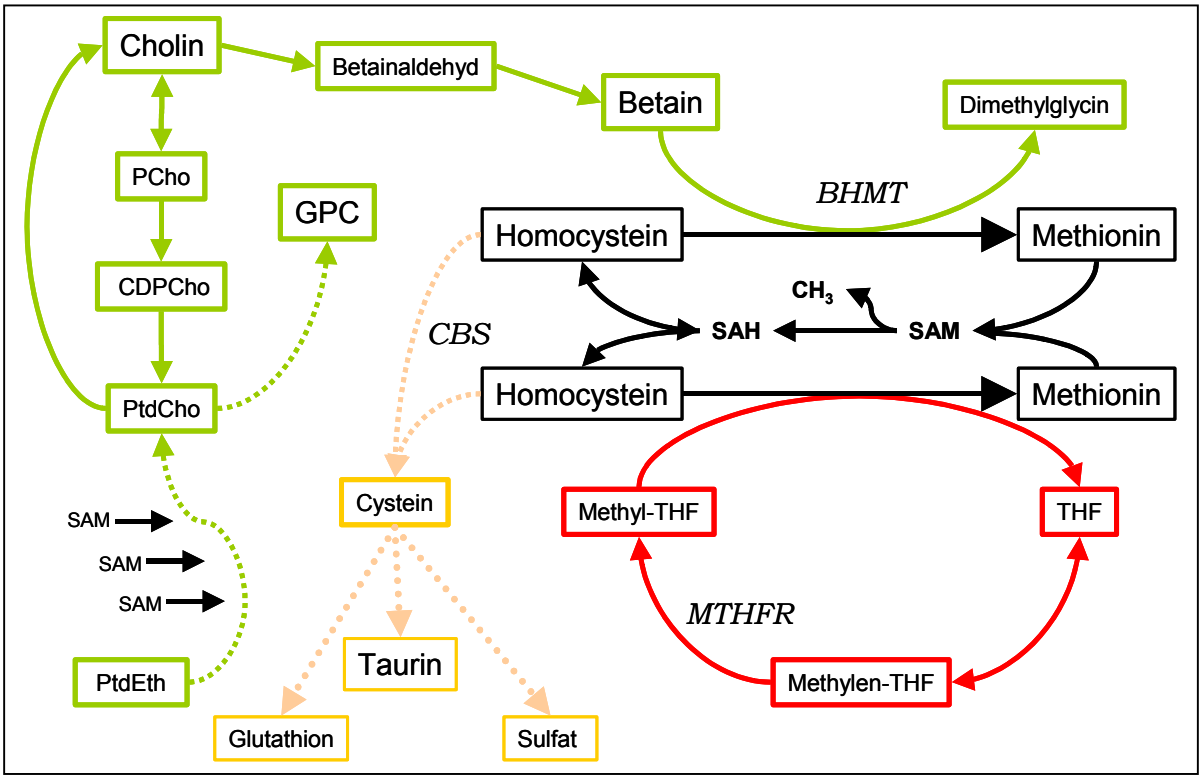

Abb. 1. Cholin- und Methylstoffwechsel in der Leber.
Direkte enzymatisch katalysierte Stoffwechselschritte werden durch solide Pfeile dargestellt, mehrstufige Reaktionen durch unterbrochene Linien. Methylentetrahydrofolatreduktase (MTHFR) ist das Schlüsselenzym zur Bereitstellung von Methyltetrahydrofolat (Methyl-THF) als Substrat für die Remethylierung von Homocystein. Betain trägt über die Betain-Homocystein-Methyltransferase (BHMT) zur alternativen Remethylierung von Homocystein bei. Cystathionin-β-Synthase (CBS) leitet den Katabolismus von Homocystein ein. PtdCho = Phosphatidylcholin, PtdEth = Phosphatidylethanolamin [Schwahn et al. 2003a].

Obwohl Folat-, Methionin- und Cholinstoffwechsel auf der Ebene der Remethylierung von Homocystein eng verflochten sind, wurde die Rolle des Cholinstoffwechsels und der betainabhängigen Remethylierung von Homocystein bei Homocystinurie bisher wenig beachtet. Dies kann zum einen daran liegen, dass die Verfügbarkeit von Cholin aus der Nahrung eingeschränkt ist und bis vor kurzem angenommen wurde, dass die Betainzufuhr über die Nahrung vernachlässigbar gering ist [Zeisel et al. 2003]. Zum anderen wird schwerer Cholinmangel beim Menschen selten beobachtet, ein genetischer Defekt des Cholinkatabolismus oder der betainabhängigen Remethylierung von Homocystein ist bisher nicht bekannt. Darüber hinaus entzieht sich der Cholinstoffwechsel der Untersuchung, da wichtige Reaktionsschritte in der Leber lokalisiert und die entsprechenden Metabolite analytisch nur schwer nachweisbar sind.

3.2 Klinische Symptome der Homocystinurie

Der Begriff Homocystinurie bezeichnet ein seltenes pädiatrisches Krankheitsbild, welches durch typische klinische Zeichen in Verbindung mit pathognomonischen biochemischen Veränderungen definiert ist. Es findet sich eine starke Erhöhung der nicht-proteinogenen Aminosäure Homocystein im Plasma auf über 100µmol/l (Normwerte <15µmol/l), was zur Ausscheidung des Disulfides Homocystin im Urin führt . Klinische Symptome umfassen vorwiegend Zeichen einer chronischen Enzephalopathie mit einer Vielzahl neurologischer oder auch psychiatrischer Auffälligkeiten. Daneben stehen Symptome einer Vaskulopathie mit thrombotischen oder thromboembolischen Gefäßverschlüssen im Vordergrund, ferner findet man Bindegewebsveränderungen mit marfanoidem Körperbau, Linsenluxation, Myopie und Osteoporose. [Carson et al. 1963]. Vor allem autoptisch wurde eine Hepatopathie nachgewiesen [Gaull et al. 1974, Kanwar et al. 1976]. Als häufigste Ursache der Homocystinurie wird ein Defekt des Homocysteinabbaus durch einen schweren Mangel des Enzyms Cystathionin-β-Synthase (CBS) mit einer geschätzten Prävalenz von 1:300.000 gefunden. [Mudd et al. 1995] Nur wenige Zentren weltweit führen ein generelles Neugeborenenscreening zur Erkennung typischer Formen des CBS-Mangels durch; andere Varianten der Homocystinurie konnten bisher aus methodischen Gründen nicht im Screening erfasst werden. Aufgrund dieser Situation ist die wahre Inzidenz der Homocystinurien nicht bekannt. Durch regionales Bevölkerungsscreening mittels molekulargenetischer Marker oder Homocysteinmessungen konnten asymptomatische Menschen mit CBS-Mangel mit einer Prävalenz von über 1:20.000 identifiziert werden. [Gaustadness et al. 1999] Weitere seltene

angeborene Störungen der Transmethylierung von Homocystein zu Methionin durch Defekte der Enzyme Methylentetrahydrofolat-Reduktase (MTHFR), Methioninsynthase (Cbl G), Methioninsynthase-Reduktase (Cbl E) oder im Transport und Stoffwechsel des Kofaktors Cobalamin (Cbl C, D, F) führen ebenfalls zu Varianten der Homocystinurie. [Rosenblatt & Whitehead 1999].

3.3 Natürlicher Verlauf der Erkrankung

Patienten mit Homocystinurie sind in der Regel bei Geburt asymptomatisch. Je nach Schweregrad und Art des zugrundeliegenden Defektes treten Symptome im Lauf der ersten Lebensjahre auf. Meist wird zunächst eine unspezifische psychomotorische Entwicklungsretardierung bemerkt. Weitere der oben erwähnten Symptome, insbesondere Linsenluxation und vaskuläre Komplikationen, treten später in Erscheinung. Allerdings werden nicht alle Betroffenen mit biochemisch voll ausgeprägter Homocystinurie symptomatisch. Die verfügbaren Daten zu Morbidität und Mortalität bei Homocystinurie sind durch die retrospektive Auswertung von Dokumentationen weniger Behandlungszentren an einer Gruppe von über 600 symptomatischen Betroffenen mit CBS-Mangel erhoben worden und repräsentieren damit historische Erfahrungen mit einem selektionierten Kollektiv [Mudd et al. 1985]. Systematische Daten zu milderen Formen des CBS-Mangels oder zu den anderen Varianten der Homocystinurie existieren nicht. Im oben erwähnten Kollektiv von Patienten mit CBS-Mangel wurden bei Erwachsenen folgende Zahlen für typische Komplikationen ermittelt: Linsenluxationen bei > 95%, Osteoporose bei über 50%, ein Risiko für thromboembolische Komplikationen von etwa 4% pro Lebensjahr, der Median des Intelligenzquotienten lag bei 64. Die Faktoren, die den Krankheitsverlauf bei Homocystinurie beeinflussen und zu einem Spektrum von klinischen Ausprägungen zwischen Symptomfreiheit und neonataler Letalität führen, sind nicht hinreichend bekannt.

3.4 Pathogenese der Homocystinurie

Bisher konnte lediglich die Pathogenese einzelner Symptome bei Homocystinurie aufgeklärt werden. Beispielsweise konnte eine Störung der Sekundärstruktur des Fibrillins aufgrund der Spaltung von Disulfidbrücken zwischen proteinständigen Cysteinresten unter Einfluß der

Sulfhydrylgruppen des freien Homocysteins als Ursache des strukturellen Defektes ausgemacht werden, der zur Degeneration der Zonulafasern und zur Linsenluxation führt [Mudd et al. 1995]. Dieser Mechanismus und ein ebenfalls nachgewiesener Defekt in der Lysyloxidase-abhängigen Quervernetzung der Kollagenfasern könnten auch für weitere Bindegewebsveränderungen verantwortlich sein, die den marfanoiden Habitus vieler Patienten mit Homocystinurie verursachen.

Die Thrombophilie und Arteriosklerose bei allen Formen der Homocystinurie wird auf einen schädigenden Einfluß des Homocysteins und seiner Metabolite auf die Funktion von Thrombozyten, Gerinnungsfaktoren und von Endothel und glatter Muskulatur der Blutgefäße zurückgeführt, ohne dass bisher ein hinreichender kausaler Mechanismus gefunden werden konnte. In Tiermodellen der Homocystinurie konnte eine endotheliale Dysfunktion in Abhängigkeit vom Schweregrad der Hyperhomocysteinämie nachgewiesen werden [Dayal et al. 2001, Eberhardt et al. 2000, Devlin et al. 2003]. Homocystein fördert die Entzündungsreaktion der Gefäßwand und beschleunigt die Entstehung von Atherosklerose [Hofmann et al. 2001].

Zur Entstehung der Enzephalopathie bei Homocystinurie tragen Homocystein und dessen Oxidationsprodukte Homocysteinsäure und Homocysteinsulfinsäure bei, für die direkte neurotoxische Effekte belegt werden konnten [Mudd et al. 1995, Goertz et. al. 2004]. Über eine Hemmung der Transmethylierung von Methyl-Akzeptoren wie Phosphatidylethanolamin-Abkömmlingen, Proteinen wie etwa dem basischen Myelinprotein, oder eine Störung des Neurotransmitterstoffwechsels durch Hemmung der Katecholamin-O-Methyltransferase oder Histamin-N-Methyltransferase sind zahlreiche deletäre Wirkungen vorstellbar, jedoch nicht hinreichend in Geweben nachgewiesen worden.

Zur Pathogenese der Leberschädigung bei Homocystinurie ist sehr wenig bekannt. In Analogie zu Modellen der Steatohepatitis aufgrund einer toxischen Hemmung der Remethylierung von Homocystein, durch Störung der Methylierung von Phosphatidylethanolamin, durch experimentell induzierten Cholinmangel mit sekundärer Störung des Lipidexports oder durch Störung des intrazellulären Redoxpotentials kann auf ähnliche Prinzipien bei der Entstehung der Homocystein-assoziierten Leberverfettung geschlossen werden. Zahlreiche Studien belegen den Zusammenhang zwischen einer adäquaten Verfügbarkeit von übertragbaren (labilen) Methylgruppen (insbesondere SAM, Cholin, Betain) und der Empfindlichkeit der Leber auf verschiedene chemische Noxen (Ethanol, Methotrexat, Chloroform, Acetaminophen) oder bakterielle Toxine, mit Nekrose oder Verfettung zu reagieren. Beispielsweise führt die ethanolinduzierte Störung der

folsäureabhängigen Remethylierung von Homocystein zu einer Cholin- und Betainverarmung der Leber [Chern et al. 2000] und zur Steatohepatitis, die wiederum durch Cholin- bzw. Betainsubstitution vermeidbar oder sogar reversibel ist [Barak et al. 1993, Cheng & Kaplowitz 2003]. Auch bei der ätiologisch unklaren nichtalkoholischen Steatohepatitis hat Betain einen signifikanten therapeutischen Effekt [Angulo & Lindor 2001].

Chloroformtoxizität führt zur Hyperhomocysteinämie in Mäusen, die sich durch SAM-Supplementation mildern läßt [Varela-Moreiras et al. 1995]. Zudem verursacht Chloroform eine zentrilobuläre hepatische Steatose, die durch Betaingabe gebessert wird [Junnila et al. 2000]. Da Chloroform über Glutathion gebunden und detoxifiziert wird, wurde hypothetisiert, dass Betain durch Erhöhung des Methylgruppenumsatzes mehr Homocystein als Präkursor für Cystein und damit Glutathion zur Verfügung stellt [Sang et al. 1998]. Die durch Paracetamol induzierte Leberzellnekrose ist ebenfalls mit intrazellulärem Glutathion-Mangel assoziiert. Supplementation mit PtdCho war hier therapeutisch effektiv [Jaeschke et al. 1987]. Insbesondere der PtdCho-Effekt legt einen glutathionunabhängigen protektiven Mechanismus nahe. In der Leber von Mäusen mit CBS-Mangel wurden kürzlich mittels GenChip–Analyse Expressionsänderungen von Genen gefunden, die auf Störungen des Wachstums und der Proliferation von Leberzellen sowie auf ein gestörtes Redoxpotential schließen lassen [Robert et al. 2003].

Dem Cholinmetaboliten Betain kommt neben der oben beschriebenen Funktion als Methyldonor eine Rolle als kompatibler Osmolyt und sogenanntes chemisches Chaperon in Niere und Leber zu. Hydratationsänderungen beeinflussen grundlegende metabolische Funktionen der Leberzelle, wie den Protein- oder Glukosestoffwechsel [Häussinger 1996]. Betain akkumuliert als Reaktion auf hyperosmolare Umgebung in Kupffer-Zellen [Zhang et al. 1996] und Sinusendothelzellen [Wettstein et al. 1998] der Leber, in peripheren Makrophagen [Denkert et al. 1998], sowie in einigen anderen Geweben wie Duodenalepithel, Nierentubuluszellen und Plazenta [Miller et al. 2000]. Phagozytierende Zellen akkumulieren oder verlieren Betain als Reaktion auf osmotisch oder phagozytosebedingte Zellvolumenschwankungen. Exogenes Betain moduliert dort verschiedene Zellfunktionen wie Chemotaxis, Phagozytose, NO-Produktion und Leukotrien- und Zytokinfreisetzung [in Klasing et al. 2002]. Die Steigerung der intrazellulären Betainkonzentration mildert die Induktion von molekularen Chaperonen wie HSP70 und alpha-B-Crystallin bei Hypertonizität und Hitzestress in Nierentubuluszellen. [in Burg 1995]. Die pathogenetische Bedeutung dieser Vorgänge bei durch Hyperhomocysteinämie beeinträchtigtem Betainstoffwechsel sind bisher nicht untersucht worden.

3.5 Therapie der Homozystinurie

Neugeborene mit Homozystinurie zeigen das biochemische Vollbild der Erkrankung, haben jedoch in der Regel keine oder unspezifische Symptome, was nur über eine funktionelle Kompensation durch die Plazenta zu erklären ist. Erkrankte, die als Säuglinge oder im späteren Verlauf ihres Lebens mit einer generalisierten psychomotorischen Entwicklungsverzögerung, einem thromboembolischen Ereignis oder mit einer Linsenluxation symptomatisch werden, weisen in der Regel bereits irreversible Schäden auf bevor eine spezifische Behandlung begonnen werden kann. Dies trifft insbesondere für die an Remethylierungsdefekten leidenden Patienten zu. Da davon auszugehen ist, dass die biochemischen Veränderungen wesentlich zur Pathogenese der Erkrankung beitragen, ist das Ziel der spezifischen Therapie die frühestmögliche Normalisierung der biochemischen Anomalien. Die Behandlung erfolgt diätetisch durch Bilanzierung der Methioninzufuhr und erhöhte Zufuhr des alternativen Methyldonoren Betain. Pharmakologische Dosen der am Methylstoffwechsel beteiligten Kofaktoren sind im Einzelfall wirksam. Oral verabreichtes Pyridoxin führt bei zahlreichen „Pyridoxin-responsiven“ Patienten mit CBS-Mangel über stärkere Bindung von Pyridoxalphosphat an das mutierte Enzym zur Funktionssteigerung und signifikanten Senkung der Morbidität bis hin zur Normalisierung [Mudd et al. 1985]. Parenteral zugeführtes Cobalamin ist bei den Varianten der Homocystinurie aufgrund gestörter Prozessierung von Cobalamin zum Teil wirksam. Weitere Kofaktoren wie Folsäure oder Riboflavin werden zur Vorbeugung von Mangelzuständen, die zur Homocysteinerhöhung führen würden, gegeben. Die diätetische und Betaintherapie der Patienten mit „Pyridoxin-non-responsivem“ CBS-Mangel oder mit Remethylierungsdefekten wie dem MTHFR-Mangel beeinflusst den Verlauf der Erkrankung günstig und senkt den Homocysteinspiegel. Die klinische Wirksamkeit der Therapie wurde für den CBS-Mangel belegt [Mudd et al. 1985, Wilcken et al. 1983, Wilcken et al. 1985, Yap et al. 2000], für die anderen Formen der Homocystinurie existieren jedoch lediglich Berichte mit sehr kleinen Fallzahlen. Systematische Studien zur Wirkung oder Dosierung der Therapiekomponenten fehlen.

3.6 Betaintherapie der Homocystinurie

Erste Versuche der Therapie mit Methyldonoren wie Cholin [Perry et al. 1968] und Betain [Brenton et al. 1966] wurden erfolglos abgebrochen. Eine biochemisch und klinisch erfolgreiche Therapie der Homocystinurie aufgrund CBS-Mangel [Smolin et al. 1981, Wilcken et al. 1983] oder MTHFR-Mangel [Wendel & Bremer 1984] gelang erst nach Verwendung einer höheren täglichen Betaindosis von 100 bis 600mg/kg Körpergewicht in mehreren Einzeldosen. Bei Erwachsenen wurde damit, bei allerdings unklarer Bioverfügbarkeit, eine maximale Methylgruppenzufuhr von über 50mmol pro Tag erzielt, verglichen mit dem täglichen Bedarf von etwa 18mmol Methylgruppen, die in der Transmethylierung benötigt werden und von denen bei Gesunden unter üblicher Ernährung mehr als die Hälfte über die endogene Methylgruppensynthese bereitgestellt wird [Mudd & Poole 1975]. Die Dosis von 100mg/kg KG und Tag bzw. 2 x 3g/Tag für Erwachsene wurde in der Folge beibehalten, ohne dass eine Überprüfung der notwendigen Dosis in kontrollierten Studien erfolgte.

Klinische Auswirkungen der Betainsupplementation bei Homocystinurie waren eine Besserung von Verhaltensstörungen [Wilcken et al. 1983, Wilcken et al. 1985], beim MTHFR-Mangel eine Normalisierung von Myelinisierungsstörungen [Engelbrecht et al. 1997] und Besserung [Holme et al. 1989, Kishi et al. 1994] oder geringere Progredienz neurologischer Symptome im Vergleich zu unbehandelten Patienten [Hyland et al. 1988, Abeling et al. 1999]. Darüber hinaus wurde die Wirksamkeit der Betaintherapie sehr eindrucksvoll am Beispiel eines 10 Jahre alten Jungen mit schwerem MTHFR-Mangel belegt, der nach willkürlichem Absetzen der Betaintherapie psychotische Symptome entwickelte, die nach Wiedereinführen der Therapie reversibel waren [Bönig et al. 2003]. Therapiestudien werden durch die Heterogenität der Patienten und die Seltenheit der Erkrankung erschwert.

Es wird angenommen, dass die Supplementation mit Betain durch erhöhte Substratverfügbarkeit zu einer verstärkten BHMT-vermittelten Remethylierung von Homocystein führt. Nach Beginn der Betaintherapie findet sich ein Abfall der Homocysteinkonzentration im Plasma auf 50-25% des Ausgangswertes und ein Anstieg des Methionins, der beim CBS-Mangel unerwünscht ist und hier in zwei Fällen sogar für die schwerwiegende Komplikation eines Hirnödems verantwortlich gemacht wurde [Yaghmai et al. 2002, Devlin et al. 2004]. Bei den Remethylierungsdefekten führt die Betaingabe jedoch zur Normalisierung der Konzentration von Methionin im Plasma.

Die Kompensation der funktionellen Störung durch die Betaingabe gelingt allerdings nur unvollständig bei Patienten mit „Pyridoxin-non-responsivem“ CBS-Mangel und bei Remethylierungsdefekten. Die Plasmakonzentrationen von Homozystein bleiben unter Therapie weiterhin um das 5 bis 10fache über der Norm erhöht. Warum sich keine Normalisierung erzielen lässt, ist nicht aufgeklärt. Die Therapie mit Betain ist theoretisch nicht unproblematisch. Lediglich der erste Schritt der Demethylierung von Betain zu Dimethylglycin durch das Enzym Betain:Homocystein-Methyltransferase (BHMT) ist kofaktorunabhängig. Die weitere oxidative Abspaltung von Einkohlenstoff-Einheiten von Dimethylglycin über Sarkosin und Glycin benötigt jeweils Tetrahydrofolat als Akzeptor und führt zur Bildung von 5,10-Methylentetrahydrofolat, jener Folatspezies, die beim häufigsten Defekt der Remethylierung, dem MTHFR-Mangel, ohnehin bereits akkumuliert. Damit wird der funktionelle Mangel an Tetrahydrofolat weiter verstärkt [Allen et al. 1993, Krebs et al. 1976]. Dazu kommt, daß das Enzym BHMT durch Dimethylglycin mit einer Inhibitionskonstanten K_i von 10µmol/l *in vitro* bereits in geringen Konzentrationen effektiv gehemmt wird [Allen et al. 1993]. Aus diesen Überlegungen heraus ist zu erwarten, daß ein Sättigungseffekt bei der medikamentösen Therapie mit Betain eintritt.

3. 7 Milder MTHFR-Mangel als Modell zur Aufklärung pathogenetischer Prinzipien?

Die häufigste genetische Störung des Homocysteinstoffwechsels ist der milde MTHFR-Mangel durch einen homozygot vorliegenden Polymorphismus in cDNA-Position 677 mit Basenaustausch von Cytosin zu Thymidin (677C>T). Die 677TT Variante des Enzyms MTHFR ist vermehrt thermolabil und weist eine auf 40% der Norm verringerte katalytische Aktivität auf, wenn suboptimale Konzentrationen von Folaten vorliegen [Frosst et al. 1995]. Etwa 10% der mitteleuropäischen Bevölkerung sind homozygot für diesen Polymorphismus [Gudnasson 1998, Wilcken et al. 2003], der damit einen deutlichen Beitrag zur Prävalenz der Hyperhomocysteinämie leistet. Die Assoziation der milden Hyperhomocysteinämie mit verschiedenen multifaktoriellen Erkrankungen war und ist Gegenstand zahlreicher Studien und die Homocysteinerhöhung gilt inzwischen als unabhängiger Risikofaktor für die Entstehung der atherosklerotischen Gefäßerkrankung [Ueland & Refsum 1989, Refsum et al. 1998, Homocysteine Studies Group 2002]. Weitere mit Hyperhomocysteinämie assoziierte Erkrankungen sind kongenitale Fehlbildungen [Ray & Laskin 1999],

Schwangerschaftskomplikationen [Vollset et al. 2000], psychiatrische Erkrankungen [Nilsson et al. 1996] und Demenz [Smith 2002]. Der Nachweis einer Assoziation des MTHFR-Polymorphismus mit multifaktoriellen Erkrankungen könnte daher Rückschlüsse auf pathogenetische Mechanismen bei diesen Erkrankungen erlauben und die Überprüfung von Betaineffekten sinnvoll werden lassen.

3.8 Tiermodelle der Homocystinurie zum Studium der Erkrankung

Pathogenetische Studien wurden bisher nahezu ausschließlich *in vitro* vorgenommen. Neben der generellen Tatsache, dass *in vitro* – Studien nur eine begrenzte Aussagekraft für die Verhältnisse in einem kompletten Organ oder Organismus haben, stellt sich hier das Problem, dass der Homocysteinstoffwechsel stark kompartimentiert ist und lediglich die Leber die komplette enzymatische Ausstattung des Folsäure- und Methioninzyklus besitzt. Andere Organe sind dagegen mehr oder weniger auf den Export von Homocystein angewiesen, da sie entweder nicht über die Fähigkeit zur Transsulfurierung oder zur betainabhängigen Remethylierung verfügen. Vielversprechend erscheint daher die Verwendung transgener Tiermodelle der Homocystinurie. Es wurden bisher drei transgene Tiermodelle der Homocystinurie beschrieben:

Die *Cbs*-k.o.-Maus [Watanabe et al. 1995] zeichnet sich durch eine hohe postnatale Letalität von 80% in den ersten drei Wochen, schwere Leberverfettung und Wachstumsretardierung aus. Mittleren Plasmakonzentrationen von Homocystein von 6µmol/l bei Wildtyp-Tieren stehen solche von 14µmol/l bei heterozygoten Tieren sowie 204µmol/l bei den homozygoten Tieren unter regulärer Ernährung entgegen. Therapieversuche mit Betain wurden bisher nicht publiziert. Bisher wurden experimentell nahezu ausschließlich heterozygote *Cbs*-k.o.-Tiere verwendet, um den Einfluß der milden Hyperhomocysteinämie auf das Gefäßsystem zu untersuchen [Dayal et al. 2001]

Ein Defekt der Methyltetrahydrofolat-abhängigen Remethylierung durch Unterbrechung des für die Methioninsynthase (MS) kodierenden Gens bei der Maus wurde im Jahr 2001 beschrieben [Swanson et al.]. Der homozygote Verlust des MS Gens ist bereits vor dem 7. intrauterinen Lebenstag embryonal letal. Versuche, das Überleben mit Supplementation von Folsäure, Methionin, Betain, oder Cholin zu verbessern, blieben erfolglos. Plasmakonzentrationen von Homocystein bei heterozygoten Mäusen waren etwa um 50% höher als die der Wildtyp-Wurfgeschwister. Die Funktion der Methioninsynthase scheint für

das frühe embryonale Überleben notwendig zu sein; eine MS-Expression wurde bereits im Zwei-Zell-Stadium nachgewiesen. Die Kompensation des Defekts durch die betainabhängige Remethylierung setzt wahrscheinlich zu spät ein, da das Enzym BHMT erst deutlich später im Verlauf der Entwicklung exprimiert wird. [Swanson et al. 2001]. Weitere Forschungsergebnisse unter Verwendung dieses Modells wurden in der Folge nicht publiziert.
Als drittes und bisher letztes Modell der Homocystinurie wurde die *Mthfr*-k.o.-Maus beschrieben [Chen et al. 2001]. Dieses Modell ist biochemisch am besten charakterisiert. Homozygote *Mthfr* - k.o. Mäuse sind ein sehr gutes Modell für den schweren MTHFR-Mangel des Menschen. Gleiches gilt für die heterozygote *Mthfr* - k.o. – Maus, die als Modell der milden Hyperhomocysteinämie durch den homozygoten MTHFR 677C>T – Polymorphismus des Menschen angesehen werden kann. Diese Aussagen beruhen auf den Daten zur Restenzymaktivität sowie dem Schweregrad der Hyperhomocysteinämie und der begleitenden pathobiochemischen Veränderungen.

4. Zielsetzung

Ziel der vorgelegten Arbeiten war es die Bedeutung des Methyldonors Betain in der Pathogenese und Therapie von Störungen der Homocysteinstoffwechsels zu untersuchen.

Die Fragestellung wurde *in vivo* in zwei experimentellen Modellen bearbeitet. Die Verwendung transgener Tiermodelle der Homocystinurie durch Defekte der Transsulfurierung (*Cbs*-k.o.-Maus) und vor allem der Remethylierung (*Mthfr*-k.o.-Maus) sollten den Vergleich des milde gestörten Homocysteinstoffwechsels durch heterozygote Unterbrechung des *Cbs*- bzw. *Mthfr*-Gens mit der Situation bei schwerem Stoffwechseldefekt der homozygot betroffenen Tiere und mit gesunden Wildtyp-Wurfgeschwistern erlauben. Hier sollten insbesondere biochemische Parameter und klinische Auswirkungen der Betainsupplementation auf den Phänotyp der Homocystinurie charakterisiert werden.

Um die Bedeutung des Betainstoffwechsels beim Menschen zu bewerten, sollte eine mögliche Assoziation der Plasmakonzentrationen von Betain und Homocystein in einem Kollektiv von an Koronarsklerose und milder Hyperhomocysteinämie leidenden Erwachsenen evaluiert werden. In einer pharmakokinetischen Studie der oralen Betaingabe an gesunde Probanden sowie durch pharmakokinetisch-pharmakodynamisches Modelling der Betainwirkung an Probanden und Patienten mit Homocystinurie sollten weiterhin Dosis-Wirkungsbeziehungen von Betainsupplementation und Homocysteinsenkung und Unterschiede der Metabolisierung von Betain bei normalem und gestörtem Homocysteinstoffwechsel beim Menschen untersucht werden.

In einer Auswertung der verfügbaren Studien zu klinischen Auswirkungen des häufigen milden MTHFR-Mangels sollten darüber hinaus die nachgewiesenen klinischen und insbesondere auch pharmakogenetischen Effekte der milden Funktionseinschränkung des Enzyms MTHFR durch den homozygoten MTHFR-677C>T Polymorphismus auf Krankheitsentstehung und Therapie evaluiert werden.

5. Ergebnisse und Diskussion

5.1 Interaktion von Betain und Homocystein in einem Tiermodell der Hyperhomocysteinämie durch Methylentetrahydrofolat-Reduktase (MTHFR) - Mangel

Die Effekte der Betainsupplementierung auf den Homocystein- und Cholinstoffwechsel im Tiermodell standen im Vordergrund einer Serie von Experimenten bei denen Wildtyp Balb/c - Mäuse mit Wurfgeschwistern verglichen wurden, die heterozygot oder homozygot für die induzierte Unterbrechung des *Mthfr*-Gens waren. Damit waren drei experimentelle Gruppen von Tieren mit normaler, auf etwa die Hälfte eingeschränkter und fehlender MTHFR-Aktivität vorhanden. Biochemische Parameter des Homocystein- und Cholinstoffwechsels in verschiedenen Geweben sowie die Aktivität des Betain-metabolisierenden Enzyms BHMT und die Morphologie der Leber wurden unter verschiedenen diätetischen Bedingungen ausgewertet.

Zunächst wurden erwachsene Tiere unter üblicher Laborernährung untersucht, die sich durch einen relativ hohen Gehalt an Folsäure und übertragbaren Methylgruppen in Form von Cholin (als Präkursor von Betain) und Methionin auszeichnet. Hier zeigte sich eine Erhöhung der Homocysteinkonzentrationen im Plasma heterozygoter Tiere mit mildem MTHFR-Mangel (Mthfr +/-) auf 7.7 ± 0.4 µmol/l und bei Tieren mit schwerem Mthfr -Mangel (Mthfr -/-) auf 55.0 ± 4.9 µmol/l und damit auf das knapp doppelte bzw. über 10fache der Werte von Wildtyp - Tieren (4.3 ± 0.3 µmol/l). Die Plasmakonzentrationen von Betain waren nur leicht verändert, es fand sich jedoch eine negative Korrelation zwischen Betain und Homocysteinkonzentrationen, deren Ausmaß abhängig vom Schweregrad der MTHFR-Funktionseinschränkung war (siehe Abbildung 2).

In dieser Versuchanordnung ließ sich bereits ein enger Zusammenhang zwischen Betain- und Homocysteinstoffwechsel nachweisen, der durch die MTHFR-Aktivität moduliert wurde. In einem modifizierten Versuchsansatz wurden alle Tiere mit einer Aminosäuren-definierten Nahrung ernährt, die etwa 50% des üblichen Cholingehaltes aufwies. Es wurden jeweils 30 Tiere (Jeweils 6 Wildtyp, 6 heterozygote, 3 homozygote Mthfr-k.o.-Mäuse für beide Geschlechter) untersucht. Die Reduktion der Zufuhr präformierter übertragbarer Methylgruppen um insgesamt etwa 20% hatte zum Ziel die folsäureabhängige Remethylierung stärker zu beanspruchen um jene Unterschiede zu verstärken, die durch eine eingeschränkte MTHFR-Aktivität bedingt sind.

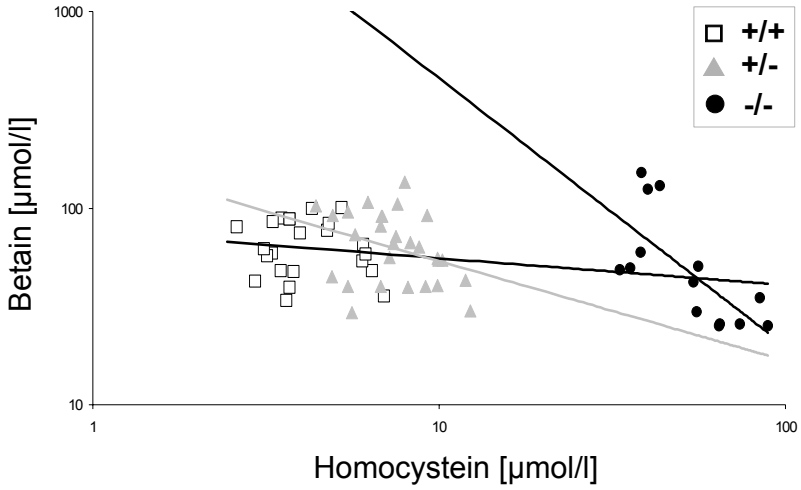

Abb. 2. Korrelation der Plasmakonzentrationen von Homocystein und Betain bei *Mthfr*-Mäusen unter regulärer Ernährung in Abhängigkeit vom Genotyp. +/+ Wildtyp, +/- heterozygot, -/- homozygot für die Unterbrechung des *Mthfr*-Gens.

Mit der cholinreduzierten Nahrung lagen die Konzentrationen von Homocystein im Plasma bei allen Genotypen etwa doppelt so hoch wie unter regulärer Ernährung. Speicherformen des Cholins (Phosphocholin und Glycerophosphocholin) sowie Betain waren genotypabhängig in der Leber verringert und die BHMT-Aktivität war um 50% höher in den Mthfr -/- Tieren. Die Betainkonzentration der Leber korrelierte negativ mit der BHMT-Aktivität. Daneben wurde ein ausgesprochener Geschlechtsdimorphismus im Cholinstoffwechsel offenkundig. Die Akzentuierung der durch die Einschränkung der MTHFR-Aktivität bedingten Hyperhomocysteinämie durch knappe Cholinversorgung zeigte zum einen die Empfindlichkeit der Mäuse gegenüber einer leichten Einschränkung der Versorgung mit präformierten Methylgruppen, zum anderen wies sie erneut auf den starken Einfluß der betainabhängigen Remethylierung auf die Homocysteinkonzentrationen im Plasma hin.

Durch Supplementation mit Betain in einer ermittelten Menge von 327 mg/kg Körpergewicht und Tag konnte eine Reduktion der Homocysteinkonzentrationen im Plasma um 50-58% je nach Genotyp erreicht werden. Die Konzentration von Betain in der Leber stieg sehr stark an und korrelierte positiv mit der deutlich geringer ansteigenden Plasmakonzentration. Die spezifische BHMT-Aktivität blieb allerdings konstant, es fand also keine relevante Induktion der Expression dieses Enzyms unter vermehrtem Substratangebot statt. Die komplette Studie wurde jeweils mit zwei weiteren Methyldonoren, Dimethylsulfoniumpropionat (DMSP) und

Dimethylsulfoniumazetat (DMSA), an jeweils 30 Tieren wiederholt. Die beiden Methyldonoren sind strukturverwandt mit Betain. Sie unterscheiden sich jedoch durch das Vorhandensein eines tertiären Schwefelatoms von Betain, welches ein quaternäres Stickstoffatom besitzt, an denen jeweils eine „labile Methylgruppe“ kovalent gebunden vorliegt. DMSP und DMSA weisen außerdem eine höhere Affinität der Bindung an das metabolisierende Enzym BHMT auf während das jeweilige Demethylierungsprodukt deutlich geringer an BHMT bindet und damit eine sehr geringe Produkthemmung zeigen sollte. Unter Verwendung gleicher Dosen wurde mit den alternativen Methyldonoren jedoch ein ähnlicher Homocystein-senkender Effekt wie mit Betain erzielt, die Betainkonzentration der Leber stieg ebenfalls an, am ehesten im Sinne eines Einspareffekts durch präferentielle Verwendung von DMSP und DMSA. Diese alternativen Methyldonoren erscheinen damit auch zur Anwendung beim Menschen nicht vielversprechender als Betain zu sein. Als weiteres Ergebnis verhinderte das Betainsupplement die bei *Mthfr* -/- Tieren sonst häufig beobachtete Verfettung der Leber (siehe Abb. 3).

In einer weiteren Studie wurden weibliche heterozygote Tiere in 10 Gruppen zu 4 Tieren im Rahmen einer Dosis-Wirkungsstudie mit ansteigenden Dosen Betain von 0 bis 6,4 g/kg KG supplementiert. Homocystein wurde damit auf minimal 40% der Ausgangskonzentration gesenkt, über die Zufuhr von 53 mg/kg KG pro Tag hinaus wurde kein weiterer Effekt mehr erreicht. Die BHMT-Aktivität wurde allerdings erst durch hohe Dosen von Betain auf bis zu 335% der Ausgangswerte induziert, beginnend mit Betaindosen von über 327 mg/kg KG. Betain in der Leber stieg von 150 ± 16 µmol/kg Feuchtgewicht unter Kontrolldiät auf 1086 ± 455 µmol/kg Feuchtgewicht bei einem Betainsupplement von 480 mg/kg Körpergewicht an (siehe Abb. 4).

Die starke Abhängigkeit der Konzentrationen von Homozystein im Plasma und der hepatischen Cholinmetabolite vom *Mthfr*-Genotyp bei Mäusen zeigt, daß eine Einschränkung der MTHFR-Aktivität zum Zusammenbruch der Homocysteinhomöostase führt. Bei *Mtfhr* -/- Mäusen fanden sich erhöhte Cysteinkonzentrationen als Hinweis auf eine vermehrte Umsatzrate der Transsulfurierungsreaktion und darüber hinaus erniedrigte Konzentrationen von Betain in Plasma und Leber als Belege für eine erhöhte Beanspruchung der alternativen Remethylierung durch das Enzym BHMT. Jedoch zeigten die erhöhten Homocysteinkonzentrationen bei homozygoten k.o.- Tieren aber auch bei heterozygoten Tieren an, daß keine komplette Kompensation der ausgefallenen beziehungsweise reduzierten MTHFR – Aktivität erreicht wurde.

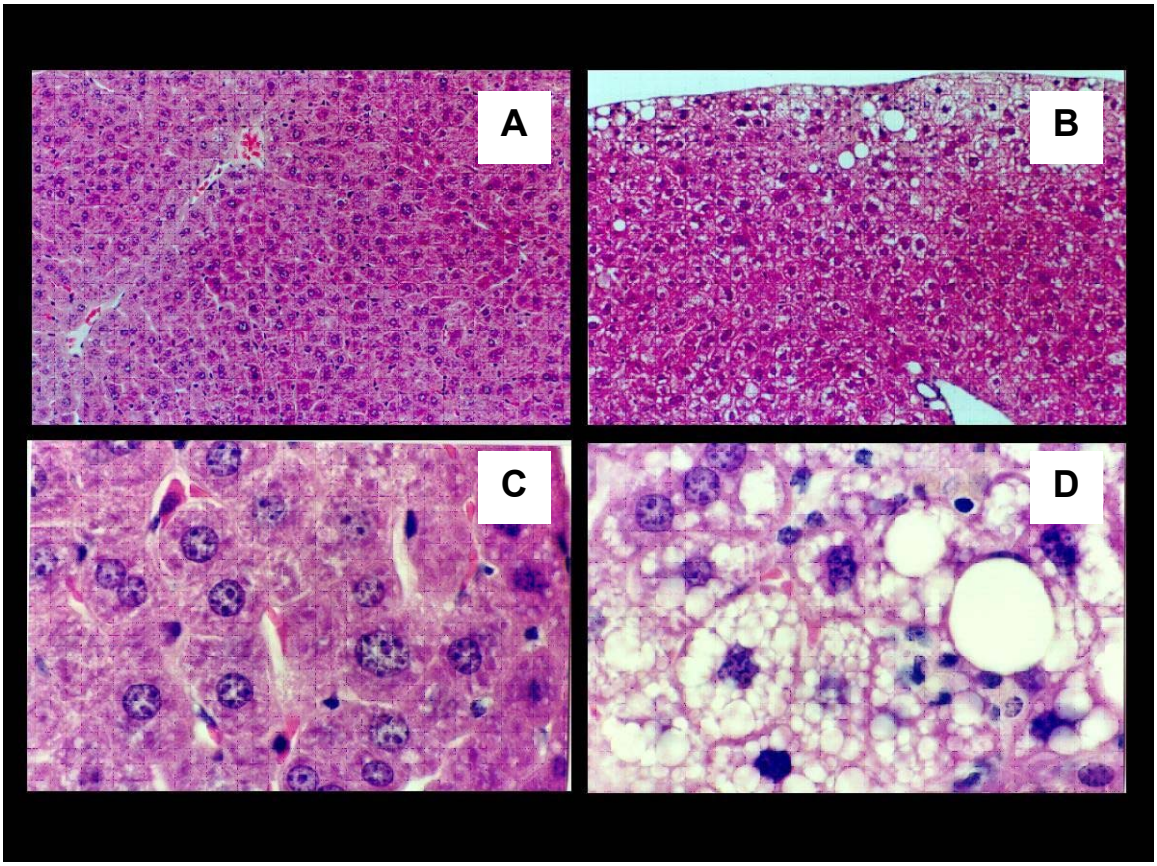

Abb. 3. Morphologie der Leber von Mthfr -/- Mäusen mit (a, c) oder ohne ein Betainsupplement (b, d). HE Färbung, oben x 20, unten x 100 [Schwahn et al. 2003a].

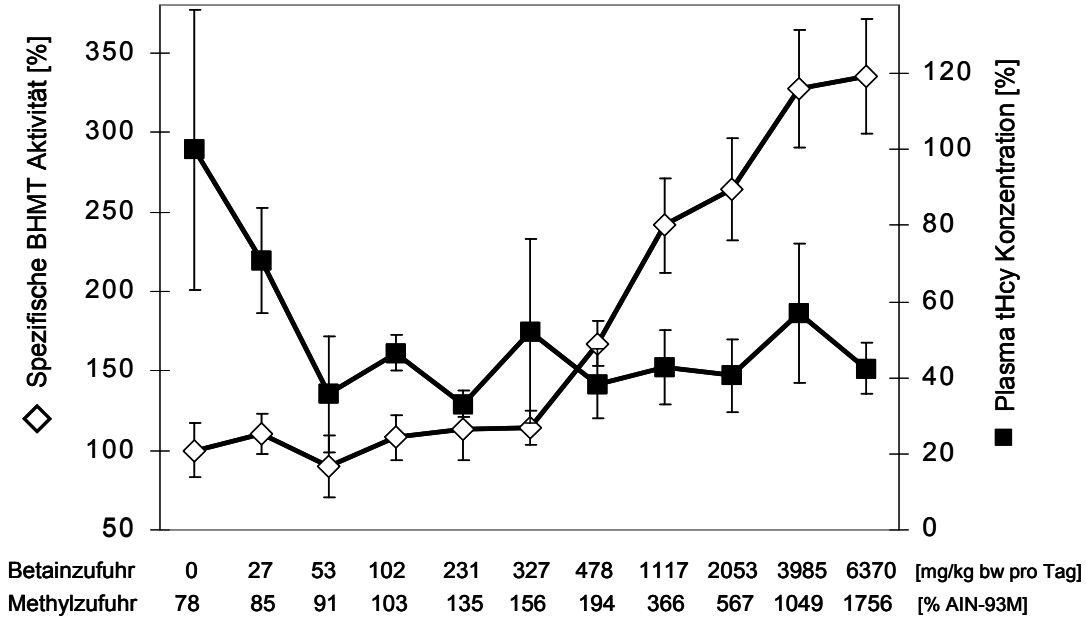

Abb. 4. Homocysteinkonzentrationen im Plasma und hepatische Aktivität des Enzyms Betain-Homocystein Methyltransferase bei heterozygoten *Mthfr*-Mäusen unter ansteigender Dosierung des Methyldonors Betain [Schwahn et al. 2003a].

Eine Konsequenz aus der Dyshomöostase des Homocysteinstoffwechsels und der sekundären Aktivierung des Betainkatabolismus sowie der Transsulfurierung war die Steatose der Leber bei Mthfr -/- Mäusen. Bemerkenswert ist weiterhin, dass die Leberverfettung der Mthfr -/- Mäuse durch das Betainsupplement verhindert wurde, obwohl die Homocysteinkonzentration im Plasma nicht normalisiert wurde. Durch Belastung von humanen Leberzellkulturen mit Homocystein konnte eine gesteigerte Synthese von Triglyzeriden und Cholesterol über Aktivierung einer Stressreaktion des endoplasmatischen Retikulums und Steigerung des Transkriptionsfaktors SREBP-1 erzeugt werden [Werstuck et al. 2001]. Ein protektiver Effekt von Betain wurde in diesem System nicht untersucht. Die Verhinderung der Verfettung trotz persistierender Hyperhomocysteinämie spricht gegen die Hypothese, dass vorwiegend oxidativer Stress aufgrund der erhöhten Homocysteinkonzentrationen für die Verfettung der Leber verantwortlich ist.

Die erstaunlich enge Verbindung zwischen Homocysteinakkumulation und Beanspruchung der betainabhängigen Remethylierung bei Mäusen wurde in einer Stichprobe von 121 menschlichen Plasmaproben aus einem Kollektiv von Patienten mit gesicherter kardiovaskulärer Erkrankung überprüft. Hier zeigte sich eine signifikante negative Korrelation ($r = -0.254$ ($p < 0.005$) zwischen Betain- und Homozysteinkonzentrationen im Plasma (siehe Abbildung 5). Dieses Ergebnis kann dahingehend gedeutet werden, daß Hyperhomocysteinämie als Ausdruck eines beeinträchtigten Homocysteinstoffwechsels auch beim Menschen mit einer vermehrten Inanspruchnahme des Betainkatabolismus verknüpft ist, ein Zusammenhang, der bisher nicht beschrieben wurde. Diese Ergebnisse könnten relevant sein für diejenigen knapp 10% der Bevölkerung die eine Hyperhomocysteinämie aufweisen, darunter die homozygoten Träger des MTHFR 677C>T Polymorphismus mit suboptimaler Folsäureversorgung.

Die Bedeutung einer adäquaten Cholinzufuhr bei Menschen ist nicht geklärt, es gibt keine bekannten angeborenen Defekte in cholinkatabolen Stoffwechselwegen und nur wenige mit schwerem Cholinmangel assoziierte Krankheitszustände. Es erscheint durchaus möglich, dass ein subklinischer Cholinmangel beim Menschen über geringere Bereitstellung von Betain zu pathologischen Reaktionen, wie einer Hyperhomocysteinämie, führen kann.

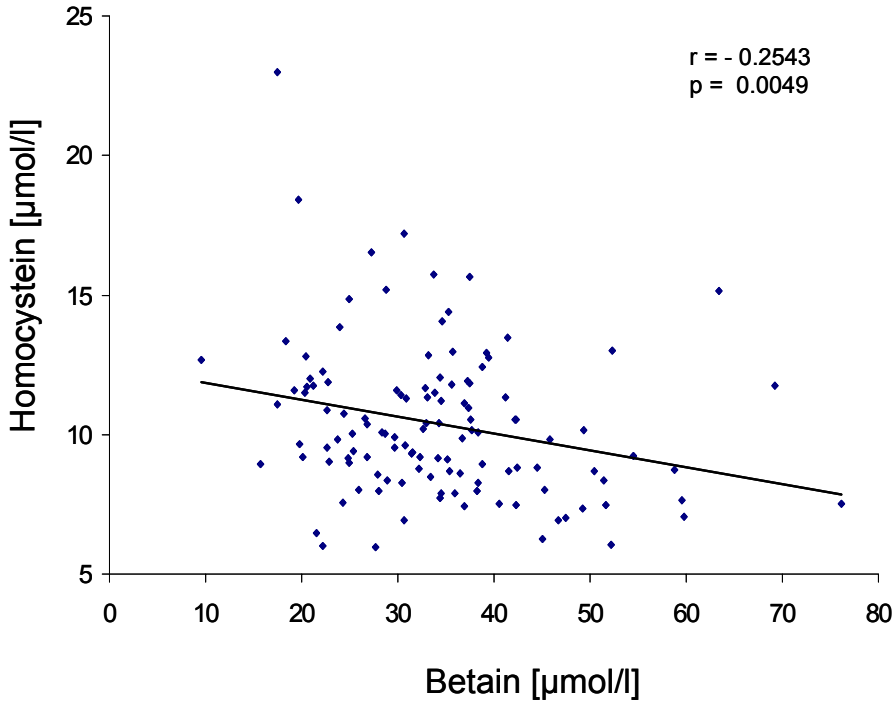

Abb. 5. Korrelation der Plasmakonzentrationen von Homocystein und Betain in einem Kollektiv von 121 an koronarer Herzerkrankung leidenden Patienten [Schwahn et al. 2003a].

5.2 Auswirkungen der Betainsupplementation auf biochemische Parameter in einem Tiermodell der Hyperhomocysteinämie durch Cystathionin-β-Synthase (CBS) - Mangel

Um zu belegen, dass die bei der *Mthfr*-Maus gefundene enge Interaktion zwischen Cholin- und Homocysteinstoffwechsel auch im Rahmen der Homocystinurie aufgrund eines weiteren genetischen Defektes mit anderen pathobiochemischen Auswirkungen vorliegt, wurden Experimente mit einem transgenen Tiermodell des Cystathionin-β-Synthase – Mangels (*Cbs*-Maus) vorgenommen. Hierfür wurden lediglich Tiere mit heterozygotem CBS-Mangel und Kontrollen in Form von Wild-Typ Wurfgeschwistern eingesetzt.
Unter Kontrolldiät wiesen die heterozygoten *Cbs*-Mäuse mit 23,0 ± 2,8 µmol/l eine um etwa 50% höhere Homocysteinkonzentration im Plasma auf als Kontrolltiere mit 15,4 ± 0,4 µmol/l. Plasma Homocystein und hepatische BHMT-Aktivität sowie hepatische Betainkonzentrationen zeigten eine starke negative Korrelation in der Gruppe unbehandelter Tiere. Dies bestätigte die bei *Mthfr*-Mäusen gefundene enge Assoziation zwischen Aktivität der betainabhängigen Remethylierung und Konzentrationen von Homocystein im Plasma. Mit Betainsupplementation von 350 mg/kg KG und Tag sanken die Homocysteinkonzentrationen im Plasma um 37.5 ± 18.2 % in Wildtyp-Tieren und um 48.8 ± 7.0 % in heterozygoten Tieren. Mit dem alternativen Methyldonor DMSA wurde eine Senkung um 55.2 ± 4.5 % in Wildtyp-Mäusen und um 50.4 ± 4.2 % in heterozygoten Tieren erreicht. Auch in dieser Studie blieben die Homocysteinkonzentrationen unter Betainsupplementation im Plasma 1,6fach höher in heterozygoten Tieren als in Wildtyp-Tieren.

In einer zweiten Versuchanordnung wurden jeweils 4 heterozygote Cbs-Mäuse mit unterschiedlichen Betainsupplementen von 0, 1, 10 und 100 mmol/l Trinkwasser (entsprechend 0, 14, 220 und 1550 mg/kg KG und Tag) über zwei Wochen behandelt. Unter der höchsten Betainsupplementation fielen die Homocysteinkonzentrationen im Plasma auf 25.2 ± 2.3 % des Ausgangswertes ab, während die Betainkonzentration der Leber auf das 150fache anstieg und die BHMT-Aktivität eher abfiel. Die Konzentrationen von Homocystein und Methionin im Plasma zeigten eine klare negative bzw. positive Korrelation mit der Betainkonzentration in der Leber in dieser Studie (siehe Abbildung 6).

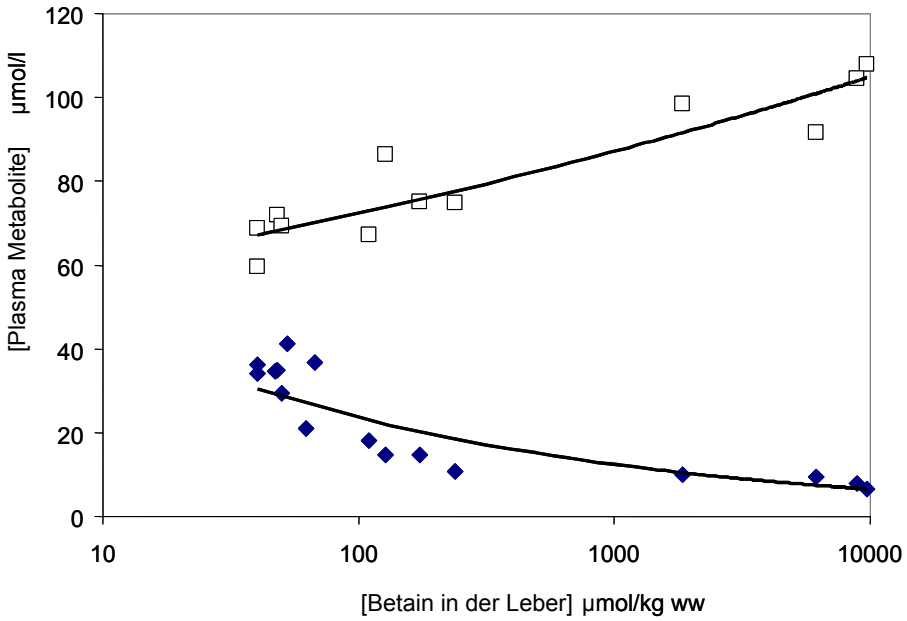

Abb. 6. Korrelation der Konzentrationen von Homocystein (schwarze Rauten) und Methionin (leere Quadrate) im Plasma mit denen von Betain in der Leber. Untersucht wurden Mäuse mit einer heterozygoten Unterbrechung des *Cbs*-Gens unter unterschiedlich hoher Betainsupplemention [Schwahn et al. 2004a].

In dieser zahlenmässig kleinen Studie zeigte sich, daß die Bestimmung einzelner Laborparameter und der Vergleich einzelner Behandlungsgruppen der Mäuse nicht sehr informativ waren. Vielmehr wurde über die Korrelation von Ergebnissen der Gesamtheit aller untersuchten Tiere miteinander ein Muster von pathophysiologischen Zusammenhängen erkennbar, das eine Interpretation möglich machte. Frappierend waren auch hier die starke Korrelation der Betainkonzentration der Leber mit der Homocysteinkonzentration im Plasma und der BHMT-Aktivität. Erneut fanden sich Belege für einen erhöhten Umsatz von Betain bei Hyperhomocysteinämie, hier aufgrund einer eingeschränkten Kapazität der Transsulfurierung. Auch hier war eine Normalisierung des Homocysteinstoffwechsels mit Betainsupplementation und Auffüllen der hepatischen Betainspeicher nicht zu erreichen, was sich durch die stets höheren Homocysteinkonzentrationen bei heterozygoten Tieren im Vergleich zu Wild-Typ - Tieren nachweisen ließ. Auch unter Supplementierung mit DMSA, welches leichter an die katalytische Domäne der BHMT bindet als Betain, wurde ein ähnlicher Effekt erreicht, unter Anstieg der Betainkonzentration in der Leber.

Mit steigender Betaindosierung bis zu 1550 mg/kg KG und Tag wurde eine Senkung der Homocysteinkonzentration im Plasma auf 25% des Ausgangswertes erreicht, wie er auch als maximale Wirkung bei Menschen beschrieben wurde. Jenseits einer Dosis von 220 mg/kg KG

und Tag wurde kein weiterer Effekt mehr erzielt, entsprechend etwa 240 µmol/kg Leber (siehe Abb. 6). Im Vergleich zu den Ergebnissen der Experimente mit der *Mthfr*-Maus sprechen diese Daten dafür, daß entweder Betain effektiver bei CBS-Mangel metabolisiert wird als bei Remethylierungsdefekten, oder daß die Betainspeicher vor Supplementation hier stärker entleert waren als bei der *Mtfhr*-Maus. Zwar sind beide Mäuse auf einem unterschiedlichen genetischen Hintergrund gezüchtet worden. Homocysteinkonzentrationen im Plasma und hepatische BHMT-Aktivität waren jedoch nicht signifikant unterschiedlich zwischen den beiden verwendeten Mausestämmen (BALB/c und C57BL/6). Allerdings waren Cholin, Phosphocholin und Phosphatidylcholinkonzentrationen in der Leber in Wild-Typ *Cbs*-Mäusen niedriger und Betain leicht erniedrigt gegenüber denen bei Wild-Typ *Mthfr*-Mäusen.

Serin im Plasma stieg unter Betainsupplemention in heterozygoten *Cbs*-Mäusen an, in Übereinstimmung mit menschlichen Daten bei CBS-Mangel [Dudman et al. 1987]. Serin dient als Ein-Kohlenstoffüberträger im Rahmen der *de-novo*-Methylgruppensynthese für die folsäureabhängige Remethylierung. Offensichtlich kommt es auch hier zu einer Depletion durch vermehrte Inanspruchnahme, die durch Betainsupplementation ausgeglichen wird.

Durch diese Experimente konnte gezeigt werden, daß Betain gut absorbiert und auch in der Leber angereichert wird. Weitere Cholinmetabolite blieben jedoch unverändert und die BHMT-Aktivität war unter der höchsten Dosis sogar verringert. Die erreichten Betainkonzentrationen in der Leber lagen mit 6,6 mmol/kg Feuchtgewicht jenseits der Sättigung der BHMT, für die eine K_m von 2,2 mmol/l beschrieben wurde. Eine Induktion der BHMT-Aktivität durch vermehrte Genexpression wird erleichtert bei geringen Methioninkonzentrationen [Park et al. 1999]. Neben einer Inhibition der Enzymaktivität durch SAM konnte gezeigt werden, dass SAM die Transkription des *Bhmt*-Gens in Hepatozyten inhibiert. In der Leber von *Cbs*-Mäusen sind erhöhte Konzentrationen von Methionin und SAM unter Betainsupplementation zu erwarten, die möglicherweise zur Repression der *Bhmt*-Expression geführt haben.

Erhöhte Konzentrationen von Homocystein im Plasma können aufgrund dieser Ergebnisse bei Mäusen als Indikator für knappe Betainversorgung angesehen werden, vor allem wenn der Homocysteinstoffwechsel beeinträchtigt ist. Es ist durchaus möglich, dass diese Zusammenhänge auch bei Menschen wirksam werden, die durch Vitaminmangel oder als heterozygote Träger pathogener Mutationen im *CBS*- oder *MTHFR*- Gen oder als homozygote Träger des häufigen *MTHFR*-Polymorphismus 677C>T einen eingeschränkte Kompensationsfähigkeit ihres Homocysteinstoffwechsels aufweisen.

5.3 Betainsupplementation verringert Letalität, Morbidität und Entwicklungsrückstand in einem Tiermodell der Homocystinurie durch Methylentetrahydrofolat-Reduktase (MTHFR) - Mangel

Homocystinurie aufgrund eines schweren MTHFR - Mangels führt unbehandelt häufig innerhalb der ersten Lebensmonate zum Tode. Es besteht eine mässig gute Korrelation zwischen der in Fibroblasten gemessenen verbliebenen Enzymaktivität und dem Schweregrad der Erkrankung. Es gibt zur Zeit kein Neugeborenenscreening auf Homocysteinerhöhungen. Daher werden die meisten Patienten mit MTHFR-Mangel erst verzögert nach Auftreten oft schwerwiegender Symptome diagnostiziert und behandelt.
Es ist nicht überraschend, dass auch die *Mthfr*-k.o.-Mäuse eine hohe postnatale Letalität aufweisen. In einer Serie von Tierexperimenten wurde der natürliche Verlauf der Erkrankung dokumentiert und die Auswirkungen der Betaintherapie auf Überleben und Krankheitszeichen sowie den Homocysteinstoffwechsel untersucht.

Die heterozygoten Mütter der zu untersuchenden Würfe wurden bereits präkonzeptionell und während der gesamten Tragezeit mit 2% Betain im Trinkwasser supplementiert. Die Supplementation wurde mit Entwöhnen der Würfe im Alter von spätestens drei Wochen beendet. Diese Supplementation wurde ohne erkennbare unerwünschte Wirkungen vertragen und führte zu einer Betaineinnahme der Mütter von etwa 3 g/kg Körpergewicht und Tag. In verschiedenen Körpergeweben von erwachsenen Mäusen wurde der Anstieg von Betain unter dieser Supplementationsdosis gemessen. Die Gewebekonzentrationen waren deutlich geringer bei heterozygoten Tieren als in Wildtyp- Tieren. Es wurden Betainkonzentrationen bis zu 9,4 mmol/kg Feuchtgewicht in der Leber bei Wildtyp – Tieren erreicht. Die Messung von Betain und Dimethylglycin in Plasma von 12 Tage alten und zu diesem Zeitpunkt ausschließlich gesäugten Nachkommen von Mäusen mit Betainsupplement zeigten eine erhöhte Betainverfügbarkeit an.
Insgesamt wurden 314 Tiere aus 50 Würfen unbehandelter Mütter untersucht und mit 249 Tieren aus 38 Würfen von mit Betain supplementierten Müttern verglichen. Hierzu wurden die Geschlechts- und Genotypverteilung bestimmt und zwischen den Behandlungsgruppen verglichen. Die Tiere wurden postnatal täglich gewogen und biochemische Parameter des Homozystein- und Betainstoffwechsels sowie die Morphologie der Hirnentwicklung in verschiedenen Lebensaltern dokumentiert. Die Ergebnisse wurden zwischen beiden Behandlungsgruppen und zwischen den jeweiligen Wurfgeschwistern mit normalem

Homozysteinstoffwechsel (+/+), heterozygoten (+/-) und homozygoten (-/-) Trägern des MTHFR- Mangels verglichen.

Die Betainsupplementation führte zu einem dramatischen Rückgang der Sterblichkeit von 83% auf 26% der *Mthfr* -/- Tiere (siehe Abbildung 7) und verbesserte die Gewichtsentwicklung ausgehend vom ersten postnatalen Tag an (siehe Abbildung 8a und b).

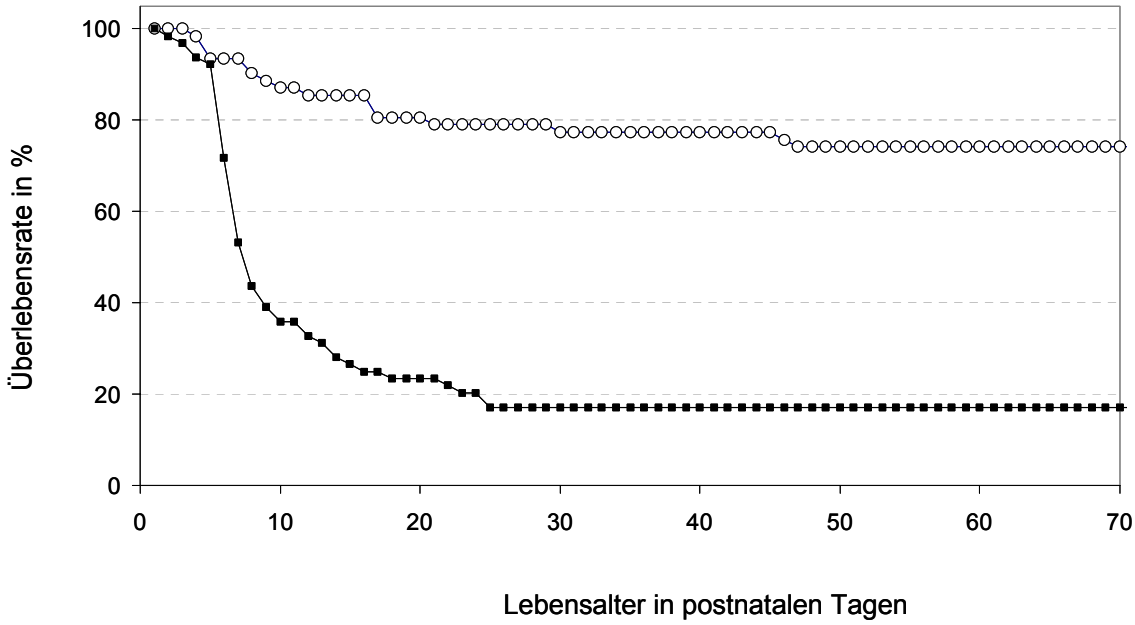

Abb. 7. Überlebensrate von Mthfr-k.o.-Mäusen ohne (schwarze Quadrate) und mit (offene Kreise) Betainsupplementation der Mütter während Tragzeit und Säugeperiode [Schwahn et al. 2004b].

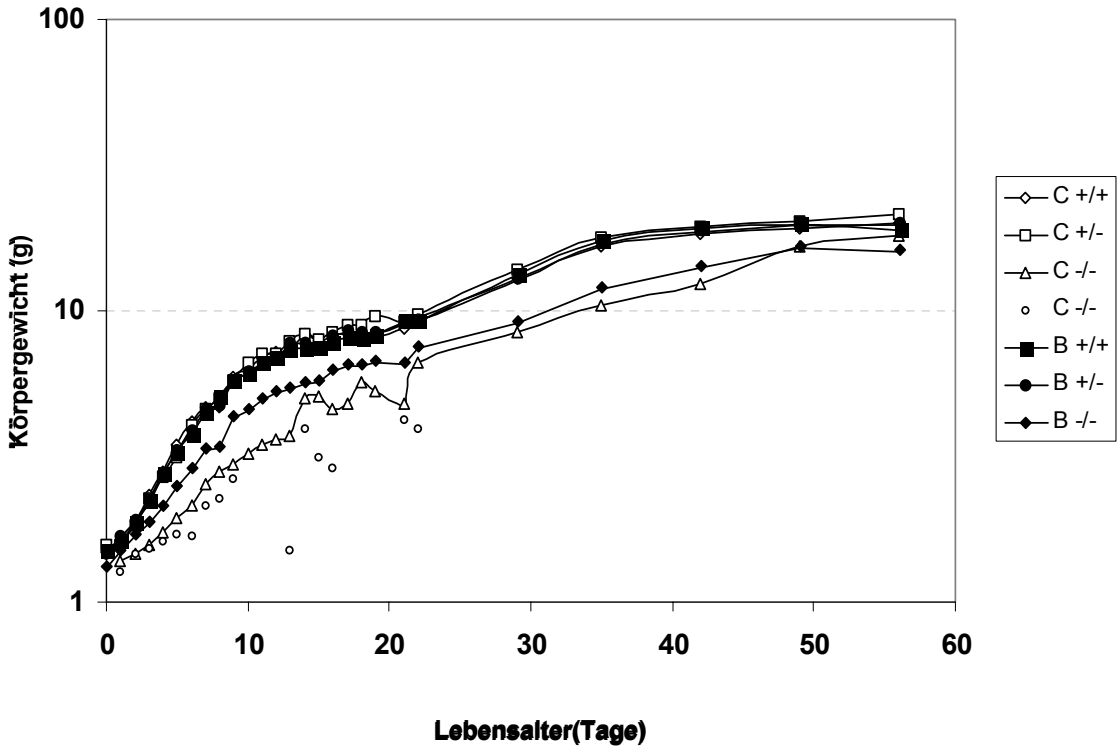

Abb. 8a. Postnatale Gewichtsentwicklung von *Mthfr*-Mäusen mit (B) und ohne (C) Betainsupplemention der Mütter. +/+ = Wild typ, +/- = heterozygot, -/- = homozygot für die Unterbrechung des *Mthfr*-Gens [Schwahn et al. 2004b].

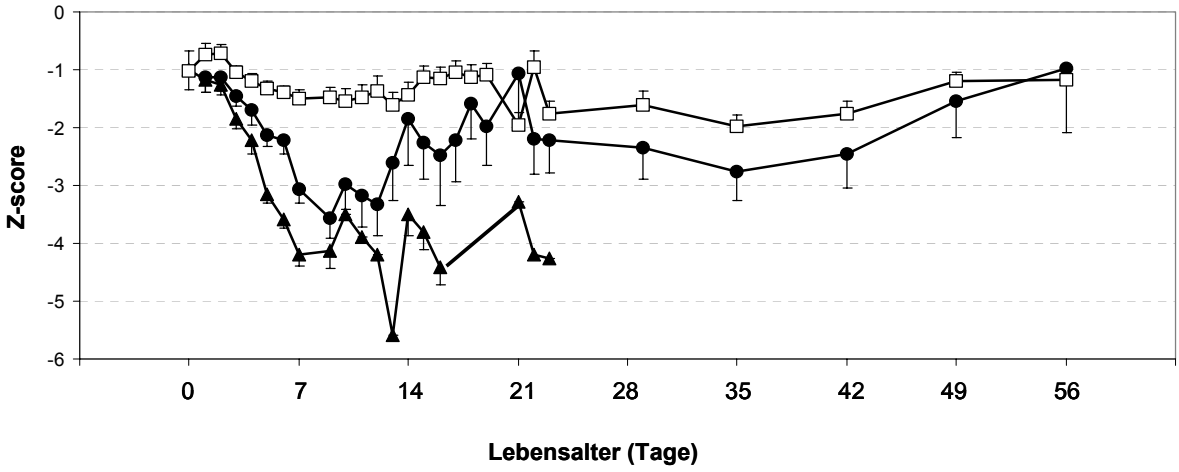

Abb. 8b. SD-Scores der Gewichtsentwicklung von 55 unbehandelten und 62 betainsupplementierten *Mthfr* -/- Mäusen relativ zu der ihrer +/+ und +/- Geschwistertiere über 56 Tage. Offene Dreiecke symbolisieren überlebende Tiere, offene Kreise im Verlauf an der Erkrankung verstorbene Tiere [Schwahn et al. 2004b].

Bei unbehandelten *Mthfr* -/- Tieren konnte neben einer generellen Entwicklungsverzögerung eine bevorzugte Störung der Entwicklung des Kleinhirns festgestellt werden. Foliation und Differenzierung der Kleinhirnrinde waren stark verzögert und zeigten einen rostro-kaudalen Gradienten in der Ausprägung des Schweregrads. Darüber hinaus fand sich eine Differenzierungsstörung der neuronalen Schichten im Gyrus dentatus des Hippocampus. Mit dem Betainsupplement waren alle diese Auffälligkeiten in ihrer Ausprägung deutlich abgemildert.

Biochemische Veränderungen wurden in Leber und Gehirn von jeweils 8 bzw. 6 Tieren der drei Genotypen und beider Behandlungsgruppen am 6. postnatalen Lebenstag gemessen. Genotyp - bedingte Unterschiede in der Kontrollgruppe zeigten sich vor allem in der Akkumulation von Homocystein und SAH und in der Erhöhung von Cystein und Cystathionin bei Tieren mit eingeschränkter MTHFR-Aktivität als Ausdruck einer gesteigerten Transsulfurierung.

Mit Betain supplementierte Tiere wiesen eine geringere Anhäufung von Homocystein und einen geringeren Umsatz der Transsulfurierung von Homocystein zu Cystein auf. Im Hirngewebe 6 Tage alter Nachkommen von Betain-supplementierten Müttern zeigte sich außerdem eine Zunahme der globalen DNA-Methylierung im Vergleich zur unbehandelten Kontrollgruppe. Diese funktionelle Auswirkung der Betainsupplementation könnte ein Mechanismus sein, der im Sinne eines generellen epigenetischen Effekts durch Normalisierung der DNA-Methylierung, und damit der Regulation der Genexpression, Überleben und Entwicklung der *Mthfr* -/- Mäuse verbessert hat.

Die ausgeprägten Effekte der Betainsupplementierung mit Vermeidung schwerer biochemischer und morphologischer Veränderungen sowie der eindrucksvolle Rückgang der Letalität beweisen die Bedeutung des Betainstoffwechsels für die postnatale Entwicklung und die Rolle der Betain- bzw. Cholindepletion in der Pathogenese der Homocystinurie bei Mäusen.

Auf die Situation beim Menschen übertragen, sprechen diese Daten für eine möglichst frühe Diagnosestellung und Betainbehandlung bei MTHFR – Mangel. Ein Ziel, welches nur durch ein generelles und frühzeitiges Screening auf Hyperhomocysteinämie erreicht werden kann.

5.4 Die veränderte Pharmakokinetik von Betain bei Patienten mit verschiedenen Formen der Homocystinurie im Vergleich zu Gesunden lässt auf eine Betainverarmung und erhöhten Umsatz schließen

Systematische Studien zur Betainwirkung bei Menschen sind selten, insbesondere Studien bei Patienten mit Homozystinurie. In einer pharmakokinetischen Studie zur oralen Applikation von Betain bei gesunden männlichen Probanden wurden zunächst basale pharmakologische Daten zur Behandlung mit Betain ermitteltet. Diese Daten dienten der Planung von pharmakokinetischen Studien bei Patienten mit verschiedenen Formen der Homozystinurie. Daten aus beiden Studien lagen dann einer Modellierung der Betainwirkung in einem pharmakokinetisch-pharmakodynamischen Modell zugrunde.

12 gesunde männliche Probanden erhielten eine Einzeldosis von 50 mg/kg KG Betain. Serielle Blutentnahmen und Urinsammlungen mit Bestimmung von Betain und dessen Metaboliten Dimethylglyzin (DMG) erlaubten die Darstellung der Plasmakonzentrationen über die Zeit, die Berechnung des Betainumsatzes und der renalen Ausscheidung. Nach einer Auswaschphase von 1 – 2 Wochen schloß sich eine Periode von 5 Tagen mit regelmässiger Einnahme von jeweils 50mg/kg Betain alle 12h an um die Kinetik von Betain unter kontinuierlicher Einnahme zu erfassen. In einer anschließenden Abklingphase wurde die Elimination von Betain über 72h in Plasma und über 24 h in Sammelurin verfolgt.
Betain wurde rasch absorbiert ($t_{1/2}abs$ 0.28h, SD 0.17), die maximale Plasmakonzentration C_{max} von 0.94mmol/l (SD 0.19) wurde nach t_{max} von 0.90h (SD 0.33) erreicht. Die Verteilungshalbwertszeit $t_{1/2}a$ wurde mit 0.59h (SD 0.22) bestimmt. Die Halbwertszeit der Elimination $t_{1/2}b$ *betrug* 14.38h (SD 7.17) (siehe Abbildung 9).

Unter kontinuierlicher Betaineinnahme waren eine deutlich langsamere Verteilung ($t_{1/2}a$ (1.77h, SD 0.75) und Elimination ($t_{1/2}b$ 41.17h, SD 13.50) zu beobachten. Die Konfidenzintervalle der Unterschiede betrugen 0.73-1.64h für die Verteilungshalbwertszeit $t_{1/2}a$ und 19.90-33.70h für die Eliminationshalbwertszeit $t_{1/2}b$. Die Absorptionsgeschwindigkeit war nicht unterschiedlich. Die Plasmakonzentrationen für DMG stiegen signifikant an und akkumulierten in einem ähnlichen Ausmaß wie Betain im Plasma.

Die renale Ausscheidung war gering und betrug nur etwa 4% der aufgenommen Dosis. Zusammenfassend konnte nachgewiesen werden, dass Betain rasch aufgenommen und verteilt wird und dass es zum weit überwiegenden Teil metabolisch eliminiert wird. Unter kurzdauernder kontinuierlicher Gabe stellt sich innerhalb weniger Tage ein neues Fließgleichgewicht ein, welches durch eine verlangsamte Verteilung und Metabolisierung gekennzeichnet ist (siehe Abbildung 10).

Einerseits zeigte der Anstieg des Betainmetaboliten DMG in Plasma und Urin einen vermehrten Katabolismus von Betain an, andererseits konnte dieser vermehrte Katabolismus die Akkumulation von Betain nicht verhindern.

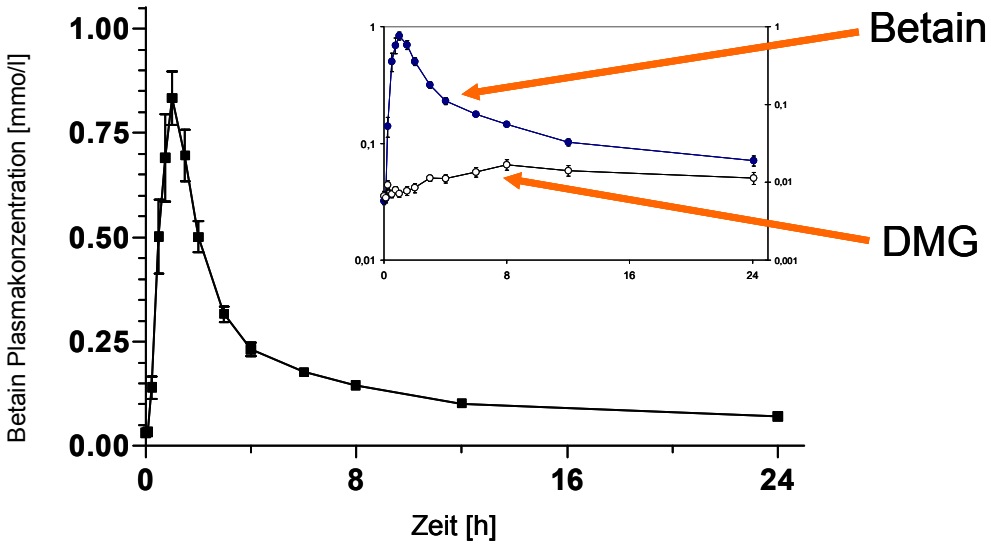

Abb. 9. Plasmakonzentrationen von Betain nach Einmalapplikation von 50mg/kg KG bei zwölf gesunden Probanden (Mittelwert und 1 SEM). Der Ausschnitt zeigt die semilogarithmische Darstellung der Plasmakonzentrationen von Betain und Dimethylglycin im gleichen Versuch. Der Anstieg des Betainmetaboliten DMG weist die Metabolisierung von Betain nach [Schwahn et al. 2003b].

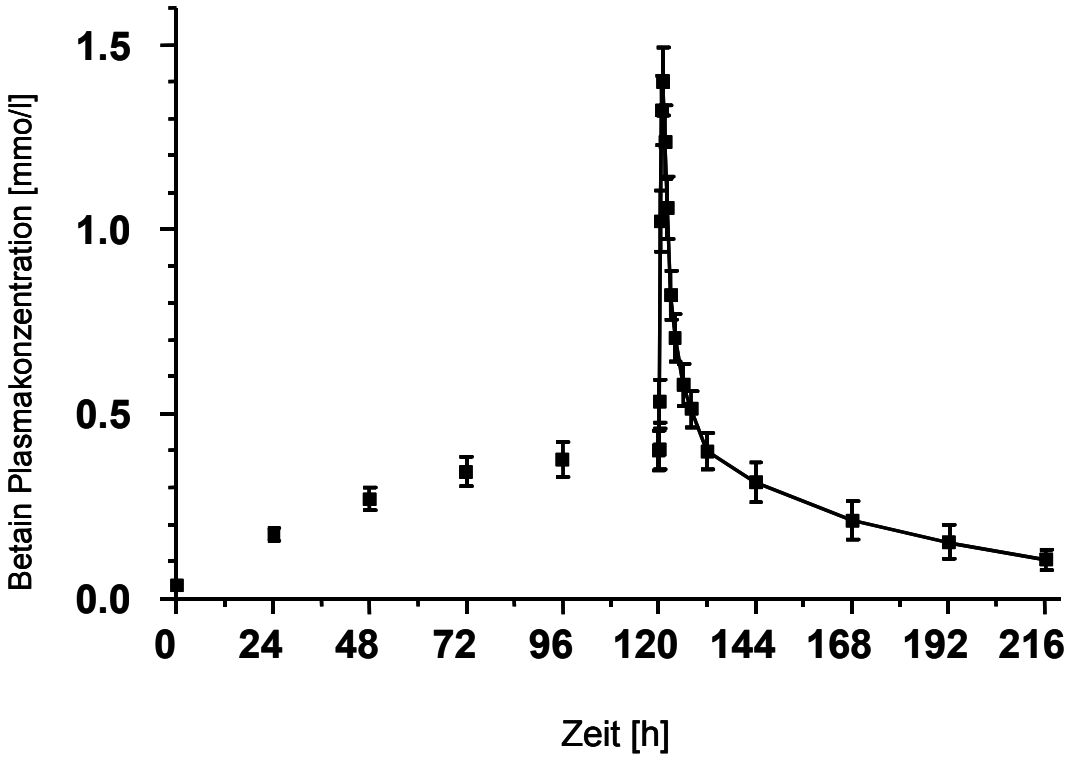

Abb. 10. Plasmakonzentrationen von Betain nach wiederholter Applikation von 2 x 50 mg/kg KG pro Tag über 5 Tage bei zwölf gesunden Probanden. Datenmodellierung (Mittelwert und SEM) [Schwahn et al. 2003b].

In dieser Studie wurden ebenfalls drei Patienten mit Homocystinurie untersucht, zwei Patienten mit schwerem MTHFR-Mangels und eine Patientin mit CBS-Mangel. Alle drei Patienten wiesen nach einer Einnahmepause von 8 Tagen erniedrigte Ausgangskonzentrationen von Betain im Plasma auf. Verteilungsvolumen und Gesamtclearance von Betain waren deutlich erhöht gegenüber der gesunder Probanden. Die Beobachtungen an dieser geringen Zahl von Patienten lassen den Schluß zu, dass es bei Homocystinurie zu einer Verarmung an Betain im Organismus, vor allem in der Leber, kommt. Die Betaindepletion führt zu einer rascheren Verteilung und Metabolisierung exogen zugeführten Betains als bei gesunden Probanden. Allerdings spricht die Abnahme der Verteilungs- und Eliminationsraten bei gesunden Probanden unter Dauersupplementation mit Betain im Sinne einer Sättigung ebenfalls für eine Untersättigung des Organismus bezogen auf die maximale Betainmetabolisierungskapazität.

Zu den Auswirkungen der Betainsupplementation auf gesunde Probanden existieren nur wenige Publikationen:
Mit Hilfe von mit stabilen Isotopen markiertem Methionin wurden Stoffwechselumsatzmessungen bei Probanden mit oder ohne ein Supplement von je 4g Betain über 5 Tage vorgenommen. Am Ende der Behandlungsperiode wurde in der Betaingruppe eine etwas vermehrte Remethylierung und deutlich gesteigerte Transmethylierung sowie Transsulfurierung von Homocystein berechnet, die Steigerung der Transsulfurierung betrug hier etwa 50% der durch die vermehrte Transmethylierung verursachte Produktion von Homocystein. [Storch et al. 1991].
Die Dosis von 6g Betain über 6 Wochen senkte die Homocysteinplasmakonzentrationen von gesunden Probanden geringfügig und milderte den Homocysteinanstieg nach standardisierter Methioninbelastung ab [Schwab et al. 2002, Steenge et al. 2003, Wilcken et al. 1985].

Trotz des therapeutischen Einsatzes von Betain seit über 20 Jahren, existierten bis vor kurzem keine entsprechenden pharmakologischen Daten. Eine methodisch angreifbare Studie an drei gesunden Probanden und einem Patienten mit MTHFR-Mangel lieferte erstmals vorläufige Ergebnisse und wies auf die raschen Änderungen der Plasmakonzentration von Betain unter oraler Supplementation hin [Sakura et al. 1998].
Eine erst kürzlich vorgenommene Studie zu Pharmakokinetik und Wirkung oraler Betainsupplementation an 6 Patienten mit CBS-Mangel erbrachte vergleichbare Ergebnisse zur Kinetik und lieferte darüber hinaus durch Modellierung der Daten in einem pharmakodynamischen Modell Hinweise auf einen Sättigungseffekt der Betaintherapie jenseits einer Dosis von 150 mg/kg Körpergewicht [Matthews et al. 2002].

In Fortsetzung der Arbeiten zur Kinetik von Betain bei Probanden und Patienten mit Homocystinurie wurden weitere 7 Patienten mit schwerem MTHFR-Mangel und 6 Patienten mit schwerem CBS-Mangel untersucht und die gemessenen Daten zu Betain, Dimethylglycin und Homocystein unter Verwendung eines hierfür entwickelten pharmakokinetisch-pharmakodynamischen Modells ausgewertet. Vorläufige Ergebnisse zeigen auch hier erniedrigte Ausgangskonzentrationen von Betain im Plasma sowie einen beschleunigten Umsatz von Betain in der Patientengruppe zu Beginn der Betainsupplementation bei unbehandelten Patienten. Unter fortgesetzter Behandlung kommt es zur Akkumulation von Betain zur Sättigung des Homocystein-senkenden Effektes ab einer Dosis von etwa

100 mg/kg KG und Tag (siehe auch Abbildung 10). Hieraus lässt sich folgern, dass die starke Wirkung der Betainsupplementation zu Beginn der Therapie auf einem Auffüllen entleerter Betainspeicher beruht. Mit Erreichen von Konzentrationen im Sättigungsbereich des betainkatabolisierenden Enzyms BHMT lässt sich eine geringere Wirkung finden. Hieraus lässt sich ein therapeutischer Betainbedarf von maximal 100 mg/kg und Tag zur Aufrechterhaltung des Effektes ableiten [Balkenhol et al. 2003].

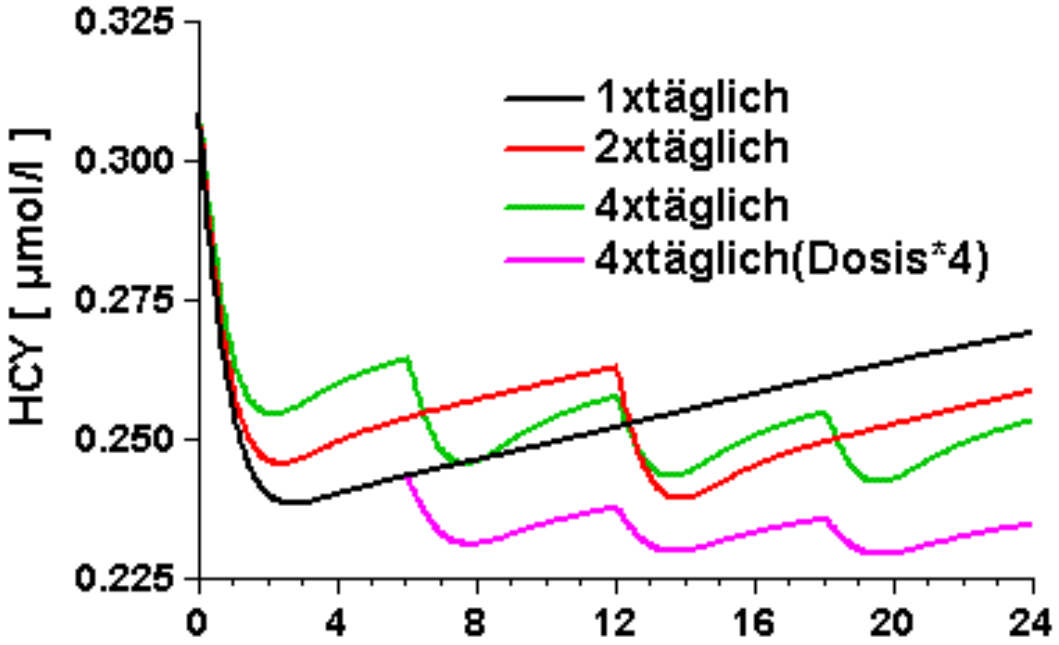

Abb. 11. Pharmakodynamisches Modell der Senkung der Plasmakonzentrationen von Homocystein bei einer Patientin mit CBS-Mangel durch Wirkung von Betain. Modelliert wurden die Homocysteinkonzentrationen nach Applikation der gleichen Dosis als Einmalgabe oder Mehrfachgabe bzw. nach Vervierfachung der Dosis (unterste Kurve).

5.5 Klinische Relevanz der MTHFR - Polymorphismen beim Menschen

Als epidemiologisch bedeutsames Modell für einen gestörten Homocysteinstoffwechsel gilt die milde Variante des MTHFR-Mangels aufgrund eines homozygot vorliegenden nt677C>T Polymorphismus. In Analogie zu den klinischen Symptomen des schweren MTHFR-Mangels, insbesondere in Anbetracht der vaskulären Komplikationen, wird seit über 10 Jahren der Hypothese nachgegangen, dass der milde MTHFR-Mangel mit gesundheitlichen Risiken assoziiert ist. Aufgrund der ungleich höheren Prävalenz des milden Defekts gegenüber der Homocystinurie sollte eine pathogenetische Bedeutung der Funktionseinschränkung des Enzyms MTHFR in epidemiologischen Studien leicht aufzudecken sein. Stimuliert durch die zahlreichen Studien zur Assoziation der milden Hyperhomocysteinämie mit dem Risiko für mehrere multifaktorielle Erkrankungen, sind inzwischen mehrere hundert Publikationen erschienen, die die funktionelle Relevanz des Polymorphismus und statistische Assoziationen mit Erkrankungen zum Gegenstand hatten. Ziel der vorgelegten und unabhängig begutachteten Übersichtsarbeit war es, Grundlagen von Funktionsstörungen des Enzyms MTHFR und das aktuelle Wissen zur klinischen Bedeutung der wichtigsten Polymorphismen zusammenzufassen und zu bewerten.

5.5.1 Polymorphismen

Zwei Varianten des *MTHFR*-Gens kommen mit hoher Frequenz beim Menschen vor: Ein Basenaustausch von Cytosin zu Thymidin in Position 677 mit der Folge eines Aminosäurenaustauschs von Alanin zu Valin (A222V) und ein Basenaustausch von Adenosin zu Cytosin in Position 1298 der cDNA, der zum Austausch von Glutamat zu Alanin führt. Der 677C>T-Polymorphismus wurde bereits intensiv untersucht. Deutlich weniger Daten liegen zum 1298A>C Polymorphismus vor. Daneben existieren mindestens drei weitere Polymorphismen, die keine funktionelle Relevanz zu besitzen scheinen.

Der 677C>T-Polymorphismus tritt im katalytischen Zentrum des Enzyms MTHFR auf [Frosst et al. 1995] und ist eine wichtige Einflußgröße auf die Homocysteinkonzentrationen im Plasma, wenn die Folsäureversorgung knapp ist [Jacques et al. 1996, Gudnason et al. 1998]. In dieser Situation ist die Enzymaktivität auf etwa 45% der Norm erniedrigt [Weisberg et al. 2001]. Der 1298A>C Polymorphismus hat lediglich bei Vorliegen in Kombination mit dem 677C>T Polymorphismus eine funktionelle Relevanz. Immerhin findet man in

Mitteleuropa nur bei etwa 56,5% aller Menschen einen *MTHFR*-Genotyp, der keine funktionelle Einschränkung der MTHFR-Aktivität mit sich bringen kann [Meisel et al. 2001].

5.5.2 Funktionelle Konsequenzen des Polymorphismus

Die Plasmakonzentration von Homocystein bei nüchternen Menschen wird durch zahlreiche nutritive und genetische Faktoren bestimmt. Die wichtigste genetisch bedingte Ursache der milden Hyperhomocysteinämie, definiert als Homocysteinkonzentrationen von 16 - 30 µmol/l [Malinow 1994], ist das homozygote Vorliegen des 677C>T Polymorphismus im *MTHFR*-Gen [Jacques et al. 1996]. Insbesondere die Träger des Polymorphismus reagieren auf die Supplementation mit Folsäure mit einer Normalisierung der Homocysteinkonzentration im Blut, was auf eine Stabilisierung der Tertiärstruktur des Enzyms zurückgeführt werden kann [Guenther et al. 1999]. Weitere funktionelle Auswirkungen des Polymorphismus sind eine Erniedrigung der Methyltetrahydrofolatkonzentrationen in Plasma und Erythrozyten und ein relatives Überwiegen von oxidierten Folaten durch die vermehrte Verfügbarkeit von Methylentetrahydrofolat [Bagley & Selhub 1998]. Das erhöhte Homocystein und die erniedrigten Methyltetrahydrofolatkonzentrationen sind vermutlich verantwortlich für die geringere globale DNA-Methylierung, die bei Trägern des Polymorphismus nachgewiesen wurde [Stern et al. 2000].

5.5.3 Assoziationen mit klinischen Symptomen und Medikamentenwirkung

Der milde MTHFR-Mangel durch den homozygoten C zu T Polymorphismus in cDNA Position 677 alleine hat keine gesicherten Auswirkungen auf das Risiko für kardiovaskuläre oder thrombotische Ereignisse, erhöht jedoch statistisch das Risiko für Neuralrohrdefekte von Kindern betroffener Mütter und für Schwangerschaftskomplikationen. Dagegen ist sein Vorliegen mit einem geringeren Risiko für die Entstehung von Kolonkarzinomen und akuter Leukämie assoziiert und wirkt sich im Sinne pharmakogenetischer Effekte auf die Therapie mit Antifolaten und anderen Medikamenten aus, deren Stoffwechsel methylierungsabhängig ist [Schwahn & Rozen 2001, Schwahn & Rozen 2004]. Eine Reihe von Medikamenten wurde bereits auf solche pharmakogenetischen Effekte des *MTHFR*-Polymorphismus hin untersucht (siehe Tabelle 1).

Tab. 1. Übersicht über die Medikamente, die auf mögliche pharmakogenetische Effekte des *MTHFR* 677C>T Polymorphismus hin untersucht wurden [nach Schwahn & Rozen 2004].

Folat	Levodopa	Methotrexat
Cobalamin	Typische Neuroleptika	Raltitrexed
Betain	Valproat	Fluoropyrimidine
Riboflavin	Carbamazepin	Fenofibrat
	Phenytoin	Bezafibrat
	Östrogen	Cholestyramin

Offenbar sind die Effekte des Polymorphismus' und der aus dem milden Remethylierungsdefekt resultierenden Hyperhomocysteinämie in der Regel so schwach, dass sie nur in grossen epidemiologischen Studien detektiert werden können. Zudem wurde in vielen Studien nicht berücksichtigt, dass die funktionellen Auswirkungen des Polymorphimus abhängig von der Folsäureversorgung sind. Ohne Korrektur der entsprechenden Daten in der Multivarianzanalyse können so Effekte verschleiert werden. Aufgrund der vorliegenden Daten erscheinen die Träger des *MTHFR* 677TT Genotyps nicht als Modell geeignet um pathogenetische Prinzipien oder Therapieoptionen der schweren Hyperhomocysteinämie *in vivo* zu untersuchen.

Dagegen scheint die heterozygote *Mthfr*-Maus aufgrund der Übereinstimmung ihrer biochemischen Daten mit denen der homozygoten Träger des 677C>T Polymorphismus grundsätzlich als Modell für das Studium des milden MTHFR-Mangels beim Menschen geeignet. Die homozygoten *Mthfr*-k.o.-Tiere spiegeln die Situation des schweren MTHFR-Mangels beim Menschen wider. Somit erscheint die *Mthfr*-Maus geeignet zum Studium pathogenetischer Zusammenhänge der milden und schweren Hyperhomocysteinämie (siehe Tabelle 2).

Tab. 2. Biochemische Folgen der eingeschränkten MTHFR-Aktivität bei Mensch und Maus. Prozentangaben beziehen sich jeweils auf den Wildtyp des Enzyms [nach Schwahn & Rozen 2001].

	Milder **MTHFR - Mangel**		**Schwerer** **MTHFR - Mangel**	
	Mensch	**Maus**	**Mensch**	**Maus**
Genetischer Defekt	677 T/T	*Mthfr* +/-	zwei path. Mutationen	*Mthfr* -/-
MTHFR Aktivität	39 %	60 %	< 10 %	0 %
Plasma Homocystein	120-190 %	168 %	~ 1000%	~ 1000 %
5-Methyl-THF [% der Folate]	71 %	58 %	"niedrig"	4 %
DNA Hypomethylierung	161 %	208 %	-	224 %

6. Schlussfolgerungen und Ausblick

Die vorgelegten Arbeiten belegen die enge gegenseitige Verflechtung von Folsäure-, Cholin- und Homocysteinstoffwechsel. Die ausreichende Bereitstellung von Methylgruppen als aktiviertes Methionin setzt die Funktionsfähigkeit aller drei Stoffwechselwege voraus. Die geringe Funktionseinschränkung eines der beteiligten Systeme zieht keine unmittelbaren Krankheitserscheinungen nach sich, führt jedoch bereits zur deutlich nachweisbaren (Über)beanspruchung der kompensatorischen Stoffwechselwege, wie es schematisch am Beispiel des MTHFR-Mangels in Abbildung 12 gezeigt wird. Beim kompletten Ausfall eines Systems kommt es zur schweren Dyshomöostase, die weitere, sekundäre Stoffwechselstörungen nach sich ziehen kann. Dieses Prinzip wurde am Beispiel der sekundären Störung des Cholinstoffwechsels bei schwerer Hyperhomocysteinämie belegt.

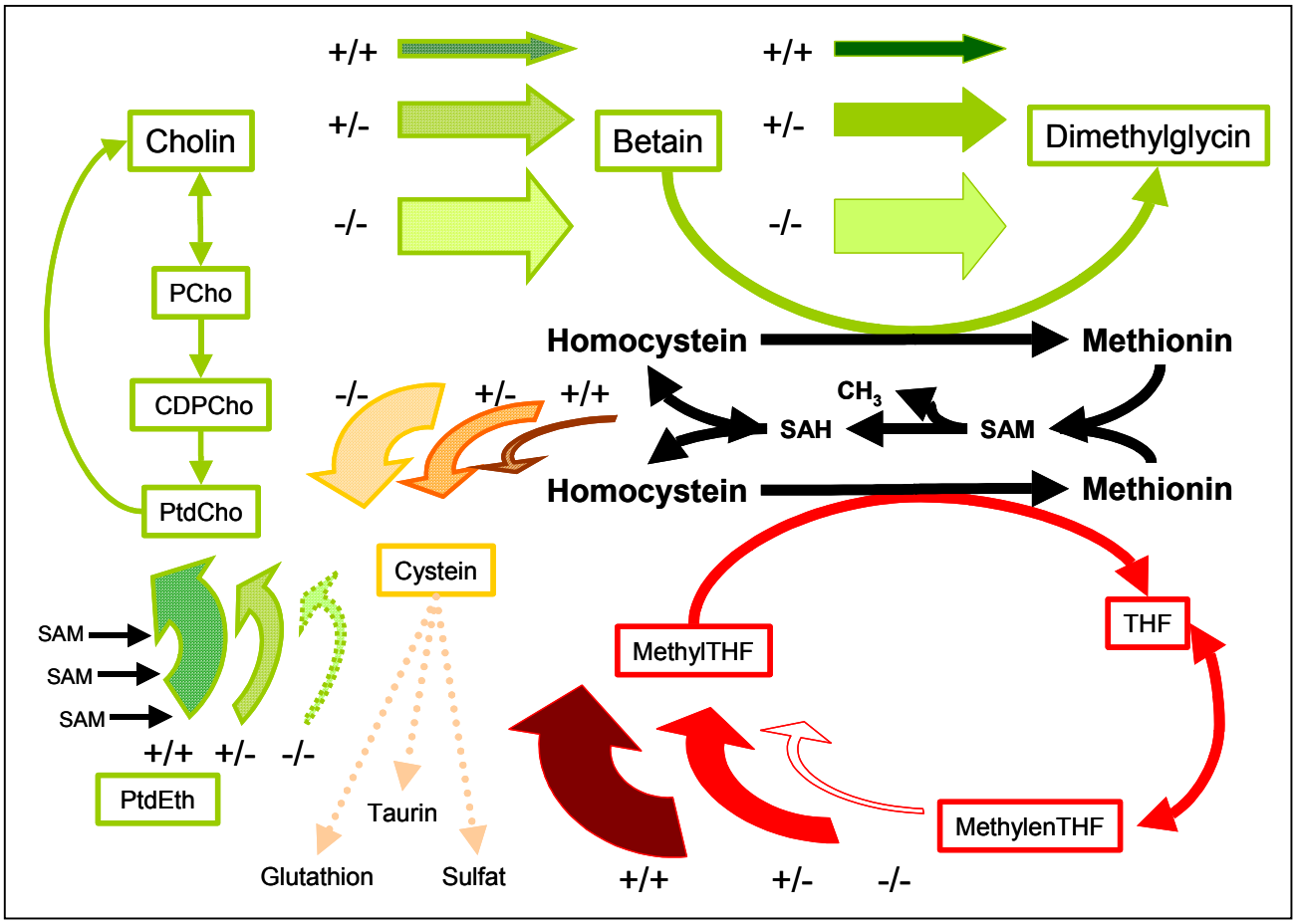

Abb. 12. Schematische Darstellung der Auswirkungen einer eingeschränkten MTHFR-Aktivität auf den Cholinstoffwechsel. +/+ Wildtyp, +/- heterozygoter MTHFR-Mangel, -/- homozygoter MTHFR-Mangel [modifiziert nach Schwahn et al. 2003a].

Der Stellenwert der Betaintherapie bei Homocystinurie liegt nach den Ergebnissen der zusammengefassten Studien in erster Linie im Ausgleich des entstandenen Betain- bzw. Cholinmangels und nicht, wie oft hypothetisiert, in einer Steigerung der normalen Betainmetabolisierung durch erhöhtes Substratangebot. Dies erklärt auch den rasch eintretenden Sättigungseffekt nach Beginn der Betaintherapie und die fehlende Effektivität einer Dosissteigerung unter Betaindauertherapie.

Es ist derzeit noch nicht geklärt, wie die Normalisierung des Cholinstoffwechsels durch Betainsupplementation zu den teils dramatischen Effekten auf Letalität und somatische Entwicklung im Tiermodell sowie auf die geistige Entwicklung und das vaskuläre Risiko bei der menschlichen Erkrankung führt. Die Aufklärung von Pathomechanismen ist weiteren Studien unter Verwendung der transgenen Tiermodelle vorbehalten. Hier kann die Untersuchung von Genexpressionänderungen unter Einfluß der Betainsupplementation weiterhelfen. Die Rolle von Betain als organischer Osmolyt in Leber, Niere und Gehirn sowie eine Störung der Signaltransduktion bei Cholinmangel und die Adaptation der zerebralen Neurotransmittersysteme an die Hyperhomocysteinämie sind weitere viel versprechende Ansätze, denen in zukünftigen Experimenten nachgegangen werden soll.

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9. Anhang

Sonderdrucke der voranstehend zusammengefassten Publikationen:

Schwahn B, Rozen R (2001)
Polymorphisms in the methylenetetrahydrofolate reductase gene: clinical consequences.
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Polymorphisms in the Methylenetetrahydrofolate Reductase Gene

Clinical Consequences

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Contents

Abstract

5,10-Methylenetetrahydrofolate reductase (MTHFR) plays a key role in folate metabolism by channeling one-carbon units between nucleotide synthesis and methylation reactions. Severe enzyme deficiency leads to hyperhomocysteinemia and homocystinuria, with altered folate distribution and a phenotype that is characterized by damage to the nervous and vascular systems. Two frequent polymorphisms in the human *MTHFR* gene confer moderate functional impairment of MTHFR activity for homozygous mutant individuals. The C to T change at nucleotide position 677, whose functional consequences are dependent on folate status, has been extensively studied for its clinical consequences. A second polymorphism, an A to C change at nucleotide position 1298, is not as well characterized.

Still equivocal are associations between *MTHFR* polymorphisms and vascular arteriosclerotic or thrombotic disease. Neural tube defects and pregnancy complications appear to be linked to impaired MTHFR function. Colonic cancer and acute leukemia, however, appear to be less frequent in individuals homozygous for the 677T polymorphism. *MTHFR* polymorphisms influence the homocysteine-lowering effect of folates and could modify the pharmacodynamics of antifolates and many other drugs whose metabolism,

biochemical effects, or target structures require methylation reactions. However, only preliminary evidence exists for gene-drug interactions.

This review summarizes the biochemical basis and clinical evidence for interactions between MTHFR polymorphisms and several disease entities, as well as potential interactions with drug therapies. Future investigations of MTHFR in disease should consider the influence of other variants of functionally-related genes as well as the medication regimen of the patients. Animal models for genetic deficiencies in folate metabolism will likely play a greater role in our understanding of folate-dependent disorders.

1. 5,10-Methylenetetrahydrofolate Reductase (MTHFR): Overview of Enzyme, Gene, and Mutations

1.1 MTHFR Enzyme and Gene

5,10-methylenetetrahydrofolate reductase (MTHFR, EC 1.5.1.20[1]) is a ubiquitous cytosolic enzyme that catalyzes the reduction of 5,10-methylenetetrahydrofolate (CH2-THF) to 5-methyltetrahydrofolate (CH3-THF) [fig. 1]. CH2-THF and its derivative, formyltetrahydrofolate, are essential for the synthesis of pyrimidine and purine nucleotides, whereas CH3-THF, the most abundant circulatory form of folate, provides methyl groups for the cobalamin (vitamin B12)-dependent methylation of homocysteine to methionine. Methionine is the only source of homocysteine; homocysteine can be remethylated back to methionine as part of the methionine cycle. Methionine is then activated to S-adenosylmethionine (AdoMet) which is the universal methyl donor in numerous transmethylation reactions, including methylation of nucleic acids, proteins, lipids and phospholipids, neurotransmitters, creatine, carnitine, and polyamines.[3] MTHFR provides the link between folate and homocysteine metabolism. It is also a critical enzyme in diverting one-carbon moieties from nucleic acid synthesis to methionine synthesis and methylation reactions.

Mammalian MTHFR is a dimer,[4] with tissue-specific subunit sizes of 77 or 70 kiloDalton (kDa), based on Western blotting.[5] The most abundant polypeptide of 77 kDa has two domains: an N terminal catalytical domain of 40 kDa, which contains the binding sites for flavin adenine dinucleotide (FAD), nicotine adenine dinucleotide phosphate (NADPH), and CH2-THF, and the C terminal regulatory domain of 37 kDa, which has a binding site for AdoMet, an important allosteric inhibitor of MTHFR. The corresponding enzyme in *Escherichia coli* is a 33 kDa homotetramer, which shares 30% amino acid identity with the human catalytic domain.[6] This bacterial enzyme does not contain the regulatory domain that is present in the human enzyme. However, the recent structure determination of the bacterial enzyme has been very important for modeling of human mutations that occur in the highly homologous catalytic domain.[7]

A partial cDNA for the human enzyme was first cloned in 1994 and the gene mapped to chromosomal region 1p36.3.[6] This cDNA sequence was used to designate the nucleotide positions of mutations in subsequent studies. Expression of a longer 2.2 kb human cDNA in *E. coli* or *Saccharomyces cerevisiae* results in a catalytically-active protein.[5,8,9] This longer cDNA corresponds to a genomic region of over 20kb, with 11 exons.[10] The long 5′ and 3′ untranslated regions and the different tissue-specific transcript sizes suggest the presence of tissue-specific isoforms, as already observed on Western blotting, and extensive transcriptional or translational regulation.[11] The mouse enzyme and the 2 yeast enzymes share approximately 90 and 35% amino acid identity with the human enzyme, respectively.[9,10]

1.2 Mutations in Severe MTHFR Deficiency

Defects in the *MTHFR* gene cause the most common inherited disorder in folate metabolism. Severe MTHFR deficiency, with residual enzyme activities between 0 and 20% of controls in cultured fibroblasts, is one of the enzymatic defects that contributes to homocystinuria. Twenty-four different mutations have been reported in 22 homocystinuric patients, with most of them located in the 5′ region encoding the catalytic domain.[6,12-15] These mutations are rare and have not been observed in controls. Most of them are missense mutations, but nonsense and splice site mutations as well as one deletion have also been described. Some of these mutations have been expressed *in vitro* and confirmed to impact on enzyme function.[16]

1.3 Mutations (Polymorphisms) in Mild MTHFR Deficiency

Two MTHFR variants are highly polymorphic: a cytosine to thymidine substitution at nucleotide position 677 and an adenosine to cytosine substitution at nucleotide position 1298, causing amino acid changes from alanine to valine or glutamic acid to alanine, respectively. The first variant has been studied extensively, whereas the second has been identified relatively recently. In addition, 3 other single nucleotide polymorphisms

1 International Union of Biochemistry, Enzyme Commission (EC) nomenclature.

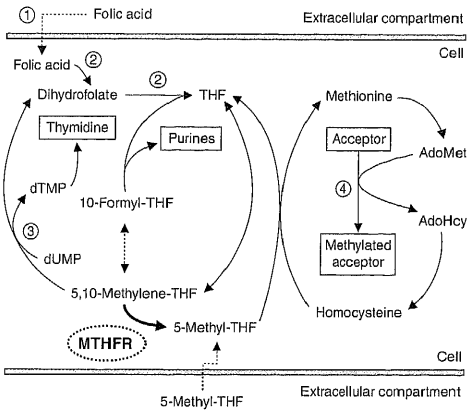

Fig. 1. Simplified pathways of folate metabolism with potential sites for drug interactions. Direct sites of interference by methotrexate are: 1) folate uptake and 2) dihydrofolate reductase.[1] 3) 5-fluorouracil inhibits thymidylate synthase.[2] 4) Other pharmacogenetic interactions with MTHFR may impact the methylation of acceptors, such as drugs, drug metabolites or drug targets.

(SNPs) have been observed in our laboratory, but there is no evidence to suggest that they have functional consequences. A thymidine to cytosine change at nucleotide position 1068[12] and another thymidine to cytosine change at nucleotide position 1317,[17] each with allele frequencies below 10%, do not change their respective codons. A frequent thymidine to cytosine change at nucleotide position 1178 + 31 (allele frequency 29%) occurs in intron 6.[13]

1.3.1 Polymorphism at Nucleotide Position 677

Enzymatic assays of lymphocytes from patients with hyperhomocysteinemia and cardiovascular disease had identified a thermolabile form of MTHFR, which was observed more often in patients than in control individuals. This thermolabile enzyme had reduced enzymatic activity and was associated with higher total plasma homocysteine levels.[18] It was later shown that this variant was due to a polymorphic missense mutation, 677C→T, that converted an alanine to a valine residue (Ala^{222}→Val) in the catalytic domain.[5] The homozygous 677T genotype is an important determinant of plasma homocysteine levels, particularly when plasma folate is low.[19,20]

This variant is quite common in North American, European, and many Asian populations, with homozygosity frequencies of up to 30%.[21] The highest incidences have been reported in Southern Mediterranean populations and Hispanic populations in North America. The mutation is relatively infrequent in sub-Saharan African and American Blacks (1% homozygosity or less).[22-24]

1.3.2 Polymorphism at Nucleotide Position 1298

A second common mutation in the *MTHFR* gene (1298A→C) results in a glutamic acid to alanine change (Glu^{429}→Ala) in the C terminal regulatory domain. This variant is present at a similar frequency (approximately 9 to 10% homozygosity) to that of the 677C→T variant in Caucasian populations.[17,25,26] This mutation does not appear to disrupt enzymatic function sufficiently to alter homocysteine remethylation. Comparison of the activities of the recombinant enzymes expressed *in vitro* has shown that the activity associated with the 1298C mutant enzyme is approximately 68% of control activity, and is intermediate between that of the wild-type enzyme and the enzyme with the 677T mutation (45% of control).[27] These values are similar to the respective enzyme activities reported in lymphocytes of homozygous mutant individuals.[5,17,25] The homozygous 1298C genotype does not confer thermolability and, on its own, is not associated with significant hyperhomocysteinemia.[17,25,26,28] However, individuals who are heterozygous for both the 677 and the 1298 polymorphism, may be at risk for hyperhomocysteinemia and reduced plasma folate levels.[25,27]

Double heterozygotes almost invariably have the two variants in the trans configuration. It is likely that the two mutations occurred independently and recombination has not taken place frequently enough to place them on the same allele.[26,27] One recent study in 1000 healthy middle-European individuals determined the haplotype frequencies for the SNPs at nucleotide position 677, 1298, and 1317. When individuals homozygous for either 677T, 1298C, or heterozygous for both variants were excluded, only 56.5% of this sample carried a genotype that conferred no functional impairment of MTHFR.[26]

2. Biochemical and Clinical Features of MTHFR Deficiency

Although homocystinuria due to severe MTHFR deficiency is a rare inborn error of metabolism, the features seen in these patients serve as a model for marked impairment of enzyme function and can provide some insight into the biochemical and clinical consequences of the milder deficiency.

2.1. Severe MTHFR Deficiency

Prominent biochemical abnormalities in these patients are severe hyperhomocysteinemia (more than 10-fold greater than normal), hypomethioninemia, decreased levels of circulating folates

(corresponding to low CH3-THF), and decreased levels of AdoMet,[29-31] CH3-THF, and neurotransmitters[32] in the cerebrospinal fluid (CSF), as well as decreased CH3-THF levels in the brain.[33] Total folates in liver and kidney have been reported to be normal, with low levels of CH3-THF and elevated levels of oxidized folate species.[34]

The phenotype of severe MTHFR deficiency has two characteristic features: first, various neurologic and psychiatric symptoms caused by neuropathy and encephalopathy with neuronal loss and demyelination; and second, symptoms of vasculopathy and coagulopathy caused by recurrent, primarily venous, thromboses or thromboembolic events. Vascular morphologic changes include intimal hyperplasia, variable thickening of vessel walls with fibrosis, and disruption of elastic lamellae in the aorta wall.[35]

2.2 Mild MTHFR Deficiency

Fasting plasma total homocysteine levels are influenced by many genetic and non-genetic factors, and reference ranges have to be established for every population. However, moderate hyperhomocysteinemia has been defined as a plasma level between 16 and 30μmol/L and intermediate hyperhomocysteinemia between 31 and 100μmol/L.[36] Homozygosity for the 677T allele is the most important genetic determinant for moderate hyperhomocysteinemia, but the effect of the variant is dependent on folate levels.[19] Individuals with this genotype are particularly responsive to homocysteine lowering by folate, often resulting in normalization of homocysteine levels;[37,38] this normalization does not pertain to patients with renal failure.[39,40] Folate-dependent stabilization of the mutant thermolabile enzyme, as demonstrated for human and bacterial MTHFR, may contribute to this folate responsiveness.[7] The same study also demonstrated stabilization of MTHFR by FAD, the cofactor of the enzyme.[7] This finding has led to the investigation of riboflavin, the vitamin precursor of FAD, as a possible determinant of plasma homocysteine. One recent study supports the concept of riboflavin as a determinant of homocysteine, with the relationship confined to individuals carrying at least one mutated *MTHFR* allele.[41] Another influence on MTHFR-dependent hyperhomocysteinemia may be plasma cobalamin. The negative slope of the relationship between plasma homocysteine and plasma cobalamin has been reported to be more pronounced in individuals with two 677T alleles.[42] This relationship is presumably due to the fact that methyl transfer from CH3-THF to homocysteine, catalyzed by methionine synthase, requires cobalamin as a cofactor.

Other metabolic consequences for homozygotes of the 677T allele are decreased CH3-THF levels in plasma,[43] red blood cells, and whole blood, as well as a relative increase of formylated folates through the increased availability of CH2-THF.[44,45] The elevated plasma homocysteine and/or the decreased CH3-THF levels may contribute to the increased DNA hypomethylation seen in homozygous 677T individuals,[46] since MTHFR contributes methyl groups for the synthesis of S-adenosylmethionine and methylation reactions. An increase in homocysteine might also result in decreased methylation through increased production of S-adenosylhomocysteine (AdoHcy), by reversal of the AdoHcy-hydrolase reaction; AdoHcy is an inhibitor of AdoMet-dependent methyltransferases.

3. Association of *MTHFR* Polymorphisms with Disease

The 677 variant has been extensively studied in a wide variety of disorders. The disorders discussed in this review do not represent the entire list, but are limited largely to those with multiple reports or those of particular interest. The available data for the 1298 variant will be discussed together with the 677 variant.

3.1 Vascular Disease

Moderate elevations in plasma total homocysteine level are more frequent in individuals with atherosclerotic or atherothrombotic disease and venous thromboembolism than in those unaffected by these disorders. There is a positive correlation between severity of disease and homocysteine level (reviewed in Ueland et al.[47]).

Many studies have shown a positive association of the homozygous *MTHFR* 677T genotype with vascular disease. A higher incidence of homozygosity for the 677T allele has been found in patients with premature coronary artery disease,[48-53] cerebral infarction,[54] and venous thrombosis.[55-58] The incidence of the homozygous 677T genotype was increased in children of parents with cardiovascular disease.[59]

Patients with renal failure have elevated plasma homocysteine and a high incidence of cardiovascular disease.[60,61] Although the pathophysiology is not well understood, the homocysteine elevation is influenced by the *MTHFR* variant[62] as well as by poor nutritional status and other undetermined factors.[63] As in other populations, folate-responsiveness in patients with renal disease is most pronounced in homozygotes for 677T, but folate supplementation often does not normalize homocysteine levels.[39,40] In patients undergoing hemodialysis, serum homocysteine levels were positively correlated with serum cysteine levelss but negatively associated with the number of 677T alleles present, although the mutations correlated positively with serum homocysteine concentrations.[64] One cross-sectional study found a negative correlation between the incidence of 677T homozygosity and the age of patients entering end-stage renal disease.[65]

Although the incidence of the homozygous 677T genotype does not seem to be associated with renal disease or renal transplant survival,[66] the polymorphism appears to have functional consequences in renal failure. Its clinical impact, however, has not been clarified.

Several studies have observed a lack of association between homozygosity for 677T and the risk of vascular disease.[67-70] Meta-analyses of studies including more than 5800 or 2600 patients, respectively, showed no significant relationship between the *MTHFR* genotype and vascular disease,[71] or venous thromboembolism,[72] whereas an earlier meta-analysis of almost 2500 patients reported a modest increase in risk for coronary artery disease in individuals with the homozygous 677T genotype.[68] However, few of these studies stratified their genotype groups according to folate levels or measured homocysteine levels (reviewed by Fletcher and Kessling[73]). If the association of the homozygous 677T genotype with vascular disease is mediated by increased plasma total homocysteine levels, the effect would be seen only in individuals with low folate status, and large sample sizes and well-matched control groups would be required to observe this association.[47] Considering the variation in frequency of this mutation in different populations, study groups of cases and controls also need to be well matched with respect to ethnicity. The positive associations seen in several Japanese studies[50,51,54] and the negative associations seen in some American studies[67,69] could reflect the mixed background of the American population.

Moreover, an interaction of the 677 polymorphism with other environmental and genetic, proatherogenic and prothrombotic risk factors has been shown in some[52,74-76] but not all, studies.[77,78] In one study of 2400 Caucasians, it was shown to be a significant risk factor in individuals with a high risk profile.[53] Another study has suggested that it may play a role in patients with earlier onset of disease, as expected for a genetic risk factor.[79]

In addition to the controversy regarding the role of MTHFR in altering the risk of vascular disease, there is still some debate over whether plasma homocysteine actually promotes vascular disease or its complications, or is a phenomenon secondary to the vasculopathy itself.[72] Ongoing prospective intervention trials to reduce plasma homocysteine with folate supplementation should help to resolve these issues (as discussed by Eikelboom and coworkers[80]).

3.2 Neoplastic Disease

Epidemiologic, animal, and human intervention studies have suggested that folate deficiency increases, and supplementation may reduce, the incidence of neoplastic disease. Since MTHFR diverts folates to different metabolic pathways, changes in MTHFR activity and folate supply have direct consequences for many of the factors that have been implicated in tumorigenesis including nucleotide balance, RNA and DNA synthesis, DNA repair, and DNA methylation (see Kim[81] or Potter[82] for reviews). The homozygous 677T genotype has been shown to be associated with a variable risk of certain neoplasias, depending on folate status. In several studies,[83-85] the incidence of homozygosity for the 677T allele was significantly lower in patients with colon cancer. The lower attributable risk for colon cancer in individuals homozygous for 677T is modified by dietary habits and lifestyle; folate deficiency,[83,85] high alcohol intake,[83,84] or low methionine intake[84,85] may abolish the risk reduction.

In contrast, a decreased incidence of the homozygous 677T genotype was not observed in patients with precancerous intestinal lesions, such as colorectal hyperplastic polyps[86] or adenomas.[87,88] One study found an increased incidence of homozygosity for 677T only in patients with colorectal carcinomas whose intakes of folate, pyridoxine (vitamin B6), cobalamin (vitamin B12), and methionine were low,[89] again stressing the importance of folate status for the assessment of tumor risk.

A higher incidence of the homozygous 677T genotype has been reported in patients with bilateral breast cancer and those with both breast cancer and ovarian carcinoma, as opposed to those with unilateral breast cancer,[90] and in patients with cervical intraepithelial neoplasia, or endometrial cancer.[91,92] One study reported a clearly lower incidence of homozygosity for the 677T and 1298C alleles in adult acute lymphocytic leukemia, compared with healthy individuals and patients with adult onset acute myeloid leukemia.[93] In a recent study of childhood acute leukemia, both variants were associated with a reduced risk of hyperdiploid leukemias.[94] Most of these studies did not consider the folate status of the study population and are therefore difficult to interpret. The association with homozygosity for 1298C is also surprising, considering its relatively small impact on enzyme activity and lack of influence on homocysteine levels.

There has been limited experimentation regarding the mechanistic link between MTHFR deficiency and tumorigenesis. Genomic hypomethylation, which has been linked to suppression of intestinal neoplasia in mice,[95] requires consideration. Individuals with the homozygous 677T genotype have increased DNA hypomethylation in leukocytes[46] and *MTHFR*-deficient mice have increased hypomethylation in several tissues.[96] Increased expression of the tumor suppressor gene *p16INK4* was found in lung cancer specimens homozygous for the 677T allele, compared with other genotypes;[97] this could be due to effects on DNA methylation.

3.3 Neural Tube Defects

Supplementation of women with folic acid during the periconceptional period reduces the ocurrence and recurrence of neural tube defects (NTD) in their children.[98,99] Mothers affected by an NTD pregnancy often have altered folate metabolism and hyperhomocysteinemia.[45,100,101] Several studies,[45,102] but not all,[21,103,104] have reported a higher incidence of the homozygous 677T genotype in children with NTDs and in their mothers. The attributable risk may be increased further in individuals with low-folate status.[105] Combined heterozygosity for the 677 and 1298 polymorphisms was shown to occur more frequently in affected children than in controls, although this was not statistically significant.[25] A recent meta-analysis calculated a pooled odds ratio of 1.8 for having an NTD [95% confidence interval (CI) 1.4-2.8] for individuals homozygous for 677T, and an odds ratio of 2.0 (95% CI 1.5-2.80) for having a child with NTD for homozygous 677T mothers.[21] Although this variant is the first reported genetic risk factor for NTD, only a small fraction of all NTD cases (approximately 10%) are attributable to *MTHFR* genetic variation,[21] and low blood folates in NTD pregnancies are only partly explained by the 677T variant.[106]

More direct biologic evidence for a causal relationship between MTHFR function and NTD occurrence has been obtained in animal experiments. Downregulation of the *MTHFR* gene using antisense oligonucleotides in cultured mouse embryos increased the incidence of NTD in a concentration–dependent manner.[107,108] The incidence could be decreased by co-injecting CH3-THF into the amniotic sac.[108] Additional genetic and environmental risk factors for folate-preventable NTD remain to be identified.

3.4 Other Congenital Anomalies

A reduced occurrence of different congenital anomalies by periconceptional use of folic acid[109] or multivitamin[110] supplementation has been reported. Several studies investigated a possible association between the 677T polymorphism of MTHFR and non-syndromic cleft lip/cleft palate[111-113] or trisomy 21.[114,115] While the results for cleft lip or cleft palate have not been consistent, a higher incidence of the 677T allele in mothers of children with trisomy 21 was found in 2 studies. A higher incidence of the mutant genotype has been reported in mothers of children with fetal anticonvulsant syndrome.[116] However, additional studies are needed to assess the role of MTHFR in this syndrome and in other congenital malformations.

3.5 Pregnancy Complications/Fertility

Hyperhomocysteinemia is frequently found in women with a history of recurrent early pregnancy loss[117] or placental abruption.[118] Several studies have examined the association of complications in pregnancy with the incidence of the homozygous 677T genotype, with differing results. One review reported calculated pooled odd's ratios (OR) and 95% confidence intervals (CI) for three typical pregnancy complications: recurrent pregnancy loss (OR 3.3, CI 1.2 to 9.2), placental abruption (OR 2.3, CI 1.1 to 4.9), and pre-eclampsia (OR 2.6, CI 1.4 to 2.9).[119] Pre-eclampsia is defined as the development of edema, arterial hypertension and proteinuria during pregnancy. Two other recent meta-analyses calculated pooled ORs for recurrent pregnancy loss of 1.4 (CI 1.0 to 2.0)[117] and of 2.0 (CI 1.4 to 2.9)[120] for pre-eclampsia. The latter report did not observe an association between the homozygous 1298C genotype and pre-eclampsia. The risk conferred by the *MTHFR* genotype may be influenced by the presence of other polymorphisms, such as Factor V Leiden and the 20210 polymorphism of the prothrombin gene;[121-125] this interaction may explain some of the discrepancies in the conclusions from the various studies.

In one study, an increase in the incidence of the mutant 677 genotype in newborns was observed after folic acid supplementation of pregnant women was introduced into one population. The authors concluded that the mutant genotype may have been associated with fetal loss when folate status was low in pregnant women.[126] Another study found a decreased proportion of homozygosity for 677T in female newborns compared with males; this could indicate reduced fetal viability associated with this genotype in females.[127] These reports justify studies with larger sample sizes to validate the benefits of periconceptional folate supplementation.

Folate supplementation had beneficial effects on sperm count and motility in a study of infertile males.[128] One recent study reported that infertile men had a significantly higher incidence of the homozygous 677T genotype than healthy controls.[129]

3.6 Neurologic/Psychiatric Disease

Deficiencies of folate and cobalamin are associated with neurologic and psychiatric disease.[130] The association of diseases of the nervous system with abnormalities in one-carbon metabolism may relate to the fact that homocysteine and homocysteic acid, an oxidized metabolite of homocysteine, have excitatory and excitotoxic neurotransmitter properties[131,132] or to the decreased availability of AdoMet for AdoMet-dependent methylation reactions, e.g. those involved in phospholipid and catecholamine metabolism.[133] Several studies have explored the

role of the 677 polymorphism in some of the more frequent disorders.

Hyperhomocysteinemia has been reported in patients with auratic migraine.[134] A recent study reported an increased incidence of homozygosity for the 677T allele in patients with migraine.[135] Disturbances of folate and cobalamin metabolism may be involved in cognitive dysfunction (reviewed by Selhub et al.[136]). Decreased CSF[137] or brain[138] levels of AdoMet and elevated plasma homocysteine[139,140] have been found in patients with Alzheimer's disease. One report on the *MTHFR* 677 variant in patients with cognitive impairment suggested that it was not a risk factor in persons over 85 years of age.[141] A recent study reported a higher incidence of the homozygous 677T genotype among hyperhomocysteinemic patients with vascular dementia compared with hyperhomocysteinemic patients with cerebral infarction but without dementia. Homozygous 677T patients without hyperhomocysteinemia had no increased incidence of vascular dementia.[142]

One study reported a high incidence of hyperhomocysteinemia (52%) together with low serum and red blood cell folate, CSF folate, CSF AdoMet and CSF monoamine metabolite levels in 46 patients with severe depression.[143] In a Japanese population, an increased incidence of homozygosity for the 677T allele in patients with depression was observed.[144]

Hyperhomocysteinemia is a frequent finding in patients with schizophrenia,[145] and abnormal methyl metabolism has been demonstrated in this disorder.[146] A small study of 11 patients with schizophrenia, selected for hyperhomocysteinemia, showed a disproportionately high incidence of homozygosity for the 677T allele.[147] This finding was confirmed by another study of unselected patients with schizophrenia in a Japanese population,[144] and by a recent study of a subgroup of patients with schizophrenia with a good prognosis due to responsiveness to antipsychotic treatment.[148] In the latter study, the more severe cases of schizophrenia without drug responsiveness had no association with 677T homozygosity.[148] Since the schizophrenia phenotype is variable, reflecting genetic heterogeneity, maternal epigenetic effects during early brain development, and environmental factors during later life,[149] additional large-scale studies with careful selection of patients and controls are needed to clarify the role of *MTHFR* polymorphisms in the pathogenesis of this disorder.

4. Pharmacogenomic Aspects of *MTHFR* Polymorphisms

There is a great deal of heterogeneity in individual responses to drug therapy. In addition to such nongenetic factors as age, lifestyle, and nutrition, genetic factors are becoming increasingly recognized as modulators of drug effects. Polymorphisms in genes affecting drug transport, metabolism, or drug targets may directly influence drug response. Other genetic variants that determine subclinical individual disease phenotypes may indirectly alter response to drug therapy.[150] *MTHFR* polymorphisms could potentially modify the therapeutic effects of any drug that interferes with folate transport and metabolism,[1,2] but they could also impact on response to those drugs whose metabolism, biochemical effects, or targets are influenced by methylation reactions (fig. 1). Thus far, only a small number of drugs have been evaluated with respect to the impact of *MTHFR* polymorphisms.

4.1 Antifolate Drugs

Methotrexate and 5-fluorouracil are well-characterized antifolates. Methotrexate is a dihydrofolate analog which inhibits the enzyme dihydrofolate reductase, causing intracellular depletion of reduced folates[151] and increased homocysteine levels.[152,153] The pyrimidine analog 5-fluorouracil affects thymidine synthesis by CH2-THF-dependent inhibition of thymidylate synthase.[154] It was not associated with significant hyperhomocysteinemia in a study in which short term effects of high dose 5-fluorouracil were addressed.[155] High dose methotrexate or 5-fluorouracil are used in cancer treatment because of their antiproliferative properties. Preliminary data suggest an increased toxicity of the chemotherapeutic combination of cyclophosphamide, methotrexate, and 5-fluorouracil in individuals with the homozygous 677T genotype.[156]

Low dose methotrexate is effective in immunomodulation of rheumatic disease, together with other anti-inflammatory drugs, such as sulfasalazine. Sulfasalazine has been shown to interfere with intestinal folate absorption.[157] In rheumatoid arthritis patients receiving methotrexate therapy, plasma homocysteine levels were found to be elevated; when methotrexate was given together with sulfasalazine, a further increase occurred.[158] Sulfasalazine alone, however, did not cause persistent homocysteine elevation. Comparing pretreatment with post-treatment levels, significant changes in homocysteine occurred only in patients heterozygous or homozygous for the 677C allele, but not in those homozygous for 677T.[158]

4.2 Anticonvulsant Drugs

The common anticonvulsants valproic acid (sodium valproate), carbamazepine, phenytoin, and phenobarbital (phenobarbitone) interfere with folate metabolism[159-163] and have been shown to be associated with elevated plasma homocysteine levels.[164,165] A decreased activity of MTHFR after phenytoin treatment in mice has been reported.[166] Children born to mothers

taking anticonvulsant drugs during pregnancy have an increased incidence of congenital malformations, including neural tube defects.[167] Patients with epilepsy who were homozygous for 677T and receiving phenytoin or carbamazepine had significantly higher plasma homocysteine and lower plasma folate levels, compared with healthy individuals with the same genotype.[168] However, the few patients with the homozygous 677T genotype receiving folate supplementation had normal plasma homocysteine levels in this study. In a study of pediatric epilepsy, the anticonvulsants carbamazepine and valproic acid were associated with higher homocysteine levels. The *MTHFR* genotype influenced plasma folate and homocysteine levels of the children treated with carbamazepine.[169]

4.3 Antiparkinsonian Drugs

Levodopa, the most common drug in the treatment of Parkinson's disease, is a potent methyl acceptor, and long term treatment has been found to be associated with lower brain[170] or CSF[171] levels of AdoMet. The observation of elevated plasma homocysteine levels[172-174] and decreased CSF levels of AdoMet[175] in patients with Parkinson's disease triggered a *MTHFR* genotype study in patients treated with a combination of levodopa, carbidopa, and other antiparkinsonian drugs.[176] All 3 *MTHFR* 677 genotype groups had higher plasma homocysteine levels compared with healthy controls of the same genotypes, but only the homozygotes for 677T had significantly elevated homocysteine levels.

4.4 Hormone Replacement Therapy

Premenopausal women generally have lower fasting plasma homocysteine than men. These values rise after menopause to the level of those of men of similar age,[177,178] but can be reduced by estrogen replacement therapy.[179,180] One report of women with adequate folate status suggested that the homocysteine-lowering effect of hormone replacement therapy could be reduced in women who are homozygous for the 677T allele.[181]

4.5 Lipid-Lowering Drugs

Two classes of lipid-lowering drugs have been shown to interfere with homocysteine metabolism or disposition by unknown mechanisms. In one study, children with hypercholesterolemia who were heterozygous or homozygous for the *MTHFR* 677T allele had an increase in plasma homocysteine levels upon treatment with cholestyramine.[59] Treatment with fenofibrate or bezafibrate, drugs used to lower triglyceride levels and to increase HDL levels in certain dyslipidemias, increased fasting and postprandial plasma homocysteine levels.[182,183] *MTHFR* genotypes did not modify fenofibrate-induced homocysteine changes in one study of men with cardiovascular disease and hypertriglyceridemia, but the sample size of this study was quite small.[183]

Table I. Biochemical consequences of impaired methylenetetrahydrofolate reductase (MTHFR) activity in humans with homozygosity for the 677T allele, and in mice with a heterozygous disruption of the *MTHFR* gene, relative to the wild-type genotype

	Human (%)	Mouse (%)[a]
Residual MTHFR activity	39[b]	60[c]
Plasma total homocysteine	120-190[d]	163
5-MethylTHF as percent of total folates	71[e]	58[c]
DNA hypomethylation	161[f]	203[c]

a All mouse data are derived from Chen et al.[94]
b Measured in peripheral leukocytes.[184]
c Data from mouse liver tissue.
d Data from different populations as reviewed by Gudnason et al.[18]
e Measured in red blood cells.[42]
f Measured in peripheral leukocytes.[44]

5. An Animal Model for Mild and Severe MTHFR Deficiency

We recently generated a mouse model for MTHFR deficiency.[96] As outlined in table I, mice with a heterozygous knockout of the *MTHFR* gene share many of the biochemical features of individuals who are homozygous for the 677T allele. Enzyme activity is in the range of 40-60% of control values, and homocysteine levels are elevated by 1.5- to 2-fold. The decrease in the percentage of CH3-THF is seen in the lymphocytes and liver of humans and mice, respectively. Methylation capacity, as indicated by global DNA methylation, is also compromised in both species. Heterozygous and homozygous *MTHFR* knockout mice have lipid deposition in the proximal aorta.[96] These findings are consistent with a role for MTHFR in vascular disease. Additional studies of *MTHFR*-deficient mice should be useful in studying pathogenetic mechanisms, in evaluating the interaction of hyperhomocysteinemia with other risk factors for vascular disease, and in exploring drug response related to *MTHFR* genotypes.

6. Conclusions

The most commonly used biochemical marker for functional consequences of *MTHFR* polymorphisms is plasma homocysteine. This parameter, however, is known to be influenced by environmental factors, by underlying disease, and probably by many other genetic variants of proteins involved in the transport and metabolism of homocysteine and folate. Moreover, plasma homocysteine might not properly reflect homeostasis in the intra-

cellular compartment of different organs or tissues. It is also possible that impaired metabolic flux through MTHFR might have consequences for folate redistribution at a level of residual MTHFR activity in which hyperhomocysteinemia does not occur. It might be useful, therefore, to distinguish between the association of diseases with hyperhomocysteinemia or with the mutant genotype alone. Other biochemical parameters that have been shown to directly influence MTHFR activity, such as folate or possibly riboflavin, should always be taken into account. At least one study has observed a high incidence of neural tube defects in an indigenous population with constitutional low folate intake and significantly decreased plasma folate, yet homocysteine concentrations were low, suggesting disturbed folate metabolism without hyperhomocysteinemia.[185]

Clinical association studies have suggested a role for MTHFR and homocysteine in a wide variety of disorders, although not all studies have reached the same conclusion. Many of the caveats in the interpretation of the epidemiologic data have already been discussed. It is possible that additional association studies will lead to more satisfying conclusions if sample sizes are markedly increased. However, additional basic biological investigations are required. We have chosen to use *MTHFR*-deficient mice to address some of these issues.

The conflicting results for MTHFR variants and clinical associations may also relate to the medication of the study group. As mentioned above, in a study of patients with schizophrenia, the positive association between the *MTHFR* 677T allele and disease was observed only in the patients who were responsive to antipsychotic medication. Considering the wide variety of cellular processes that require folates, including proliferation, DNA methylation (with effects on gene expression), amino acid synthesis, neurotransmitter metabolism, and phospholipid/membrane biosynthesis, it is likely that *MTHFR* genetic variation will modulate response to many other pharmaceutical compounds.

Although folate supplementation is an effective means of lowering homocysteine levels in individuals who are homozygous for the 677T allele, the requirements for folate might be even higher in homozygotes receiving anticonvulsants or antiparkinsonian drugs, for example. Consequently, the need to identify homozygotes for the 677T allele may arise in certain treatment groups to individualize drug therapy. Since there is still some controversy regarding the relationship between hyperhomocysteinemia or *MTHFR* genotype and disease, such therapy stratification must be implemented in a clinical research setting to allow proper evaluation of outcome.

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Homocysteine–betaine interactions in a murine model of 5,10-methylenetetrahydrofolate reductase deficiency[1]

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SPECIFIC AIMS

The original aim of our study was to examine the interaction between homocysteine and choline/betaine metabolism and to determine whether betaine was effective in treating moderate hyperhomocystinemia, a risk factor for cardiovascular disease.

PRINCIPAL FINDINGS

1. Plasma levels of homocysteine and liver levels of betaine and phosphocholine, the intracellular storage form of choline, are strongly dependent on *Mthfr* genotype in mice

As a model for moderate and severe hyperhomocystinemia, we used our recently generated mice with a heterozygous (+/−) and homozygous (−/−) disruption of the gene for 5,10-methylenetetrahydrofolate reductase *(Mthfr)*. This enzyme provides newly synthesized transferable methyl groups in the form of 5-methyltetrahydrofolate for remethylation of homocysteine to methionine by methionine synthase. An alternate methyl donor for homocysteine remethylation is betaine, derived from choline oxidation; betaine:homocysteine methyltransferase (BHMT) transfers the methyl group from betaine to homocysteine, resulting in demethylated betaine (dimethylglycine, DMG) and methionine. As an initial step in examining the interaction between homocysteine and betaine metabolism, we measured relevant plasma metabolites in mice on regular lab chow. Homocysteine levels were strongly positively correlated with the number of disrupted *Mthfr* alleles. Other significant changes were elevated cysteine and decreased DMG in *Mthfr* −/− mice; the decrease in betaine was not statistically significant. To evaluate the effect of methyl group intake, we used an amino acid-defined control (not supplemented with betaine) diet that was slightly reduced (by 20%) in transferable methyl groups, due to a decrease in choline. Homocysteine levels were still genotype dependent, but uniformly higher in all three genotype groups than the values from mice on lab chow. In liver, betaine, phosphocholine (PCho), and glycerophosphocholine (GPC) were lowest in *Mthfr* −/−, intermediate in +/−, and highest in +/+ mice. Specific activities of BHMT were not significantly different between *Mthfr* +/+ and *Mthfr* +/− mice, whereas *Mthfr* −/− mice had 1.5-fold higher activity. A highly significant negative correlation was found between BHMT activity and betaine concentration in liver. We identified gender differences for some of the choline metabolites in plasma, liver, and brain and for BHMT activity. Female mice tended to have higher metabolite levels than males; males had higher BHMT activity.

2. Betaine supplementation for 2 wk lowers plasma homocysteine in mice with normal *Mthfr* as well as in *Mthfr*-deficient mice

With a betaine supplement of 25 mmol/kg of the aforementioned diet, plasma homocysteine decreased significantly by 56%, 58%, and 50% in *Mthfr* +/+, +/−, and −/− mice, respectively. In *Mthfr* −/− mice, methionine increased by 25%. Liver betaine levels increased dramatically with the supplement; this increase appeared to be genotype dependent (7-fold, 17-fold, and 34-fold increases in *Mthfr*+/+, +/−, and −/− mice, respectively). 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. PCho increased with betaine supplementation

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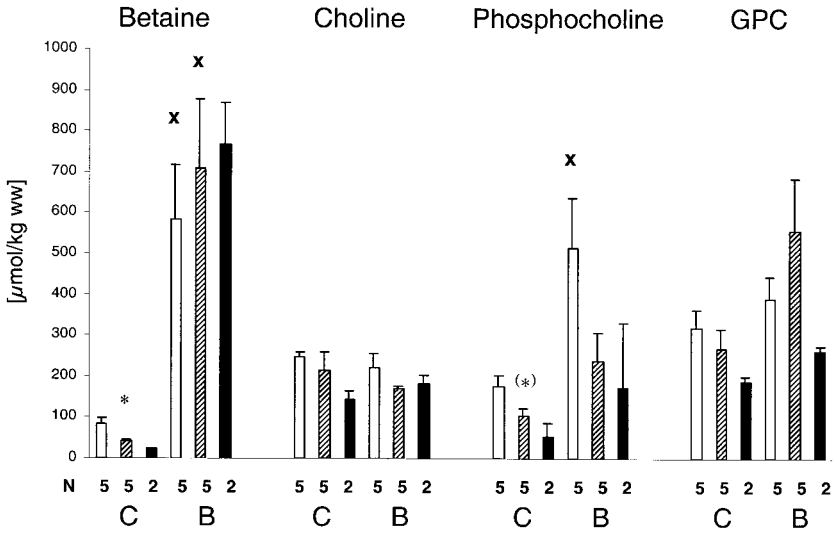

Figure 1. Liver metabolites of *Mthfr*-deficient male mice stratified by genotype and diet. C, control diet; B, betaine diet. Open bars, wild-type; hatched bars, heterozygous; filled bars, nullizygous. Data are presented as mean and SE [μmol/l]. $*P < 0.05$ vs. wild-type mice of the same dietary group, $(*)P = 0.06$. **X** = $P < 0.05$ between control and betaine group of the same genotype.

2.9-fold, 2.3-fold, and 3.2-fold in *Mthfr* +/+, +/−, and −/− mice, respectively. Brain betaine, GPC, choline, and PCho levels were higher in nullizygotes on betaine supplementation than those on the control diet, although changes in the latter two metabolites were not statistically significant (**Fig. 1**). BHMT activities remained essentially unchanged with betaine supplementation. The same degree of homocysteine lowering was observed with two other methyl donors used by BHMT, dimethylsulfonioacetate and dimethylsulfoniopropionate.

We observed severe steatosis of the liver in seven and moderate steatosis in one of the *Mthfr* −/− mice on the control diet, whereas none of the four *Mthfr* −/− mice with the betaine supplement presented with severe steatosis; three had moderate and one had mild steatosis.

The 15 male and 15 female mice per treatment group responded similarly to the betaine supplement, but homocysteine plasma concentrations decreased more in males (64%) than females (54%). Methionine increased more in *Mthfr* −/− males (9-fold) than in *Mthfr* −/− females (2-fold).

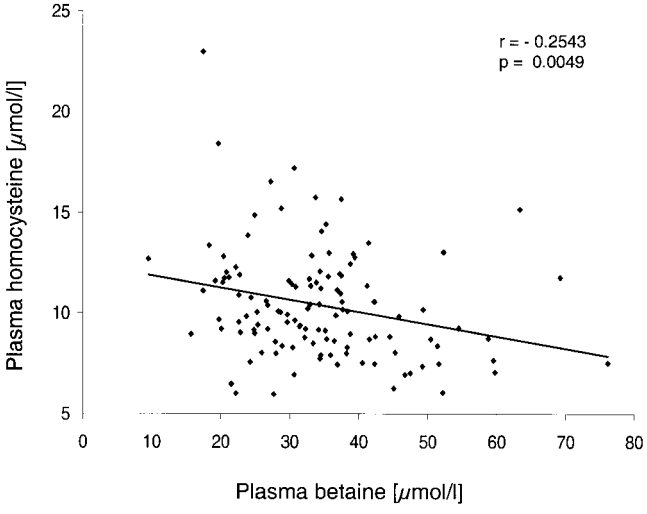

Figure 2. Linear correlation between plasma betaine and total homocysteine concentrations in 121 patients with angiographically confirmed cardiovascular disease.

3. There is a threshold dose for betaine intake beyond which additional reductions in plasma homocysteine cannot be achieved; this phenomenon is not related to induction of BHMT activity

In a dose-response study of *Mthfr* +/− mice, we increased betaine in the drinking water from 0 to 6.37g/kg body weight per day. Betaine supplementation resulted in a significant decline of plasma homocysteine which did not further decrease above an intake of 53 mg/kg body weight. Even with a 120-fold increase over this intake of betaine, homocysteine levels remained at ~40% of the initial level and were still 1.5-fold elevated over +/+ mice. BHMT activity remained unchanged until a betaine intake of 327 mg/kg body weight, then was induced to 335% of initial activity. Liver betaine rose sevenfold with increasing betaine intake from 0 to 480 mg/kg body weight.

4. Plasma homocysteine levels in mice and humans with cardiovascular disease are significantly negatively correlated with plasma betaine, suggesting that betaine may be useful in homocysteine lowering in humans with moderate hyperhomocystinemia

Homocysteine correlated negatively with plasma betaine in *Mthfr* +/+ mice ($r=-0.148$, $n=23$), +/− mice ($r=-0.363$, $n=28$), and −/− mice ($r=-0.590$, $n=14$) on lab chow, but significance was achieved only in the last group. Betaine and homocysteine concentrations in 121 human plasma samples showed a weak, but highly significant, negative correlation with a linear correlation factor $r = -0.254$ ($P<0.005$) (**Fig. 2**).

CONCLUSIONS AND SIGNIFICANCE

The dependence of plasma homocysteine and liver choline metabolite levels on *Mthfr* genotype demon-

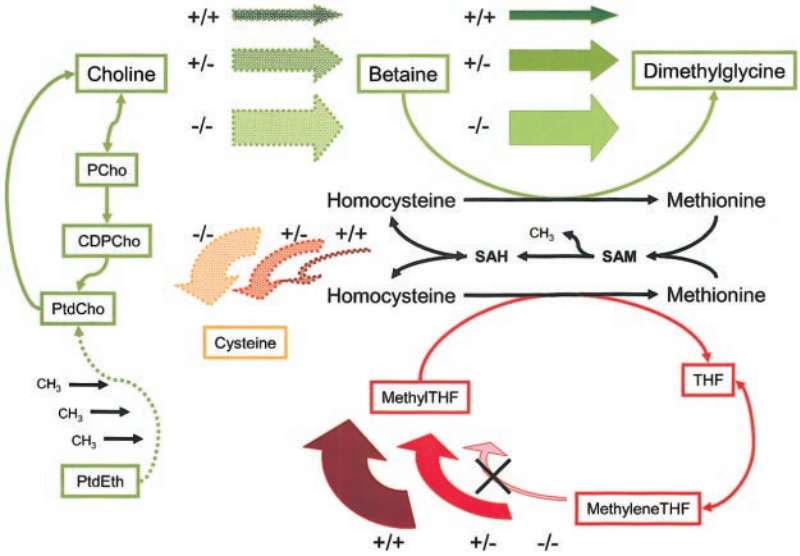

Figure 3. Schematic diagram. Enhanced metabolism of choline and betaine when folate-dependent remethylation of homocysteine is disrupted. +/+, Wild-type; +/−, heterozygous; −/−, nullizygous for disruption of the *Mthfr* gene. Solid arrows represent single enzymatic reactions; dotted arrows represent multiple enzymatic reactions.

strates that a decrease in MTHFR activity results in an inability to maintain homocysteine homeostasis with consequent disturbances of choline/betaine metabolism. The increased homocysteine is observed in the presence of increased flux through the catabolic (transsulfuration) pathway for homocysteine, as indicated by higher cysteine levels in *Mthfr* −/− mice, and in the presence of enhanced flux through the alternate remethylation pathway, as suggested by decreased levels of betaine and DMG. Hyperhomocystinemia seems to promote conversion of choline to betaine to enhance homocysteine outflow through BHMT, thereby leading to depletion of betaine and other choline metabolites in liver. The substantial decrease in choline metabolites in *Mthfr* −/− mice was associated with severe steatosis in liver. Our findings in these mice have relevance for human populations, in which mild MTHFR deficiency is common due to a polymorphism at bp 677 that results in decreased enzyme activity and moderate hyperhomocystinemia if folate status is low.

Since wild-type *Mthfr* mice were as sensitive as *Mthfr* +/− or *Mthfr* −/− mice 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 lower choline intake. This is a new finding and suggests that mild choline deficiency might be another important cause of moderate hyperhomocystinemia, in addition to deficiency of folate and other vitamins. Betaine supplementation prevented severe steatosis of the liver in *Mthfr* −/− mice, even though homocysteine levels were not normalized, providing indirect evidence for a causal relationship between choline deficiency and steatosis. The gender-related differences in metabolite concentrations may be explained by different rates of trans- and remethylation, caused by different metabolic needs and enzyme activities in males and females. Male gender and MTHFR deficiency may be associated with greater sensitivity toward choline deficiency and betaine supplementation, at least in mice.

There is a threshold dose for betaine beyond which additional homocysteine lowering cannot be observed irrespective of an induction of BHMT, the catabolic pathway of betaine. This threshold effect is not due to product inhibition of BHMT by DMG, since alternate methyl donors showed the same degree of homocysteine lowering as betaine. Supplementation of betaine resulted in a maximal homocysteine lowering effect at ~40% of the value without betaine and did not result in normalization of homocysteine in *Mthfr* +/− mice to the values observed in wild-type mice. This finding has also been described in humans with homocystinuria, and might be caused by tissue-specific differences in production and elimination of homocysteine.

Our murine studies emphasize the close interrelationship between homocysteine, folate, and choline metabolism (**Fig. 3**). In mice on lab chow, we found a negative correlation between homocysteine and betaine in plasma, influenced by the *Mthfr* genotype, and between liver betaine and BHMT activity, indicating that betaine levels correlate negatively with the flux of betaine through the BHMT pathway. In humans, this interrelationship has not been extensively investigated. In our sample of patients with cardiovascular disease, we found a surprisingly strong negative correlation between homocysteine and betaine in plasma.

Choline requirements in humans, especially adults, are ill-defined. It is possible that some nutritional habits, e.g., avoiding eggs and meat in a strict low-cholesterol diet or increased choline demands, such as during pregnancy or infancy, could lead to a moderate choline deficiency. As with the use of methylmalonic acid or homocysteine to detect subclinical cobalamin or folate deficiency, respectively, plasma betaine and homocysteine may reflect subclinical choline deficiency in humans. Our findings suggest that betaine may be useful for lowering plasma homocysteine in humans with hyperhomocystinemia and that maintenance of adequate dietary choline may be particularly important when folate-dependent homocysteine remethylation is disturbed. FJ

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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→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 10,000*g* 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 10,000*g* 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.

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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** 0.3	**7.7**[b] 0.4	**55.0**[b] 4.9
Cysteine	**151.1** 3.5	**149.5** 3.3	**170.0**[b] 5.6
Betaine	**65.9** 4.3	**67.8** 5.2	**58.8** 11.6
Dimethylglycine	**13.3** 1.5	**14.0** 1.3	**6.3**[b] 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) *12.0*	**246.7** (4)$^{\$}$ *53.2*	**41.3** (5)* *3.7*	**150.4** (4)$^{\$}$ *15.6*	**22.5** (2) *1.9*	**69.7** (2) *18.9*
Choline	**247.9** (5) *10.6*	**235.5** (4) *31.1*	**215.5** (5) *44.8*	**140.8** (4) *9.3*	**142.2** (2) 21.8	**258.8** (2) 1.0
PCho	**175.1** (5) *26.9*	**439.7** (4)$^{\$}$ *77.3*	**103.3** (5)$^{(*)}$ *18.6*	**234.1** (4)$^{\$}$ *37.0*	**54.1** (2) *31.6*	**94.9** (2) *13.5*
GPC	**317.3** (5) *46.3*	**1249.8** (4)$^{\$}$ *116.2*	**267.5** (5) *47.8*	**954.5** (4)$^{\$}$ *129.0*	**188.0** (2) *10.8*	**281.0** (2) *62.6*
PtdCho	**19034** (5) *650*	**17847** (4) *577*	**17658** (5) *870*	**17196** (4) *476*	**19446** (2) *1488*	**17901** (2) *992*
BHMT	**257** (5) *13*	**147** (8)$^{\$}$ *9*	**274** (7) *9*	**135** (5)$^{\$}$ *10*	**336** (2) *7*	**214** (2) *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

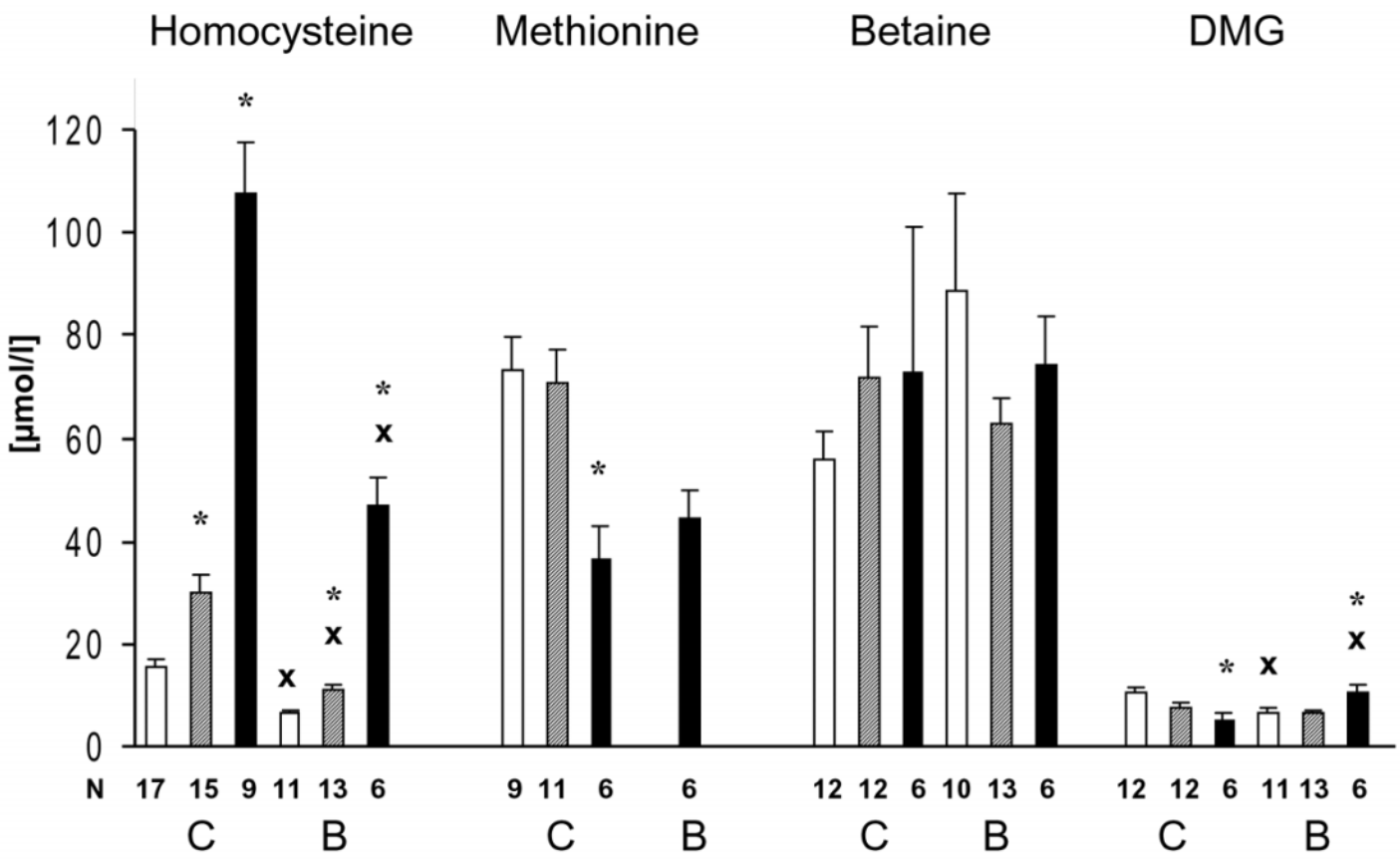

Figure 1. Plasma metabolites of *Mthfr*-deficient mice of both genders stratified by genotype and diet. C, control diet; B, betaine-supplemented diet. Open bars, wild type; hatched bars, heterozygous; filled bars, nullizygous.Data are presented as mean and SE [µmol/l]. **P*<0.05 compared with wild-type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

Fig. 2

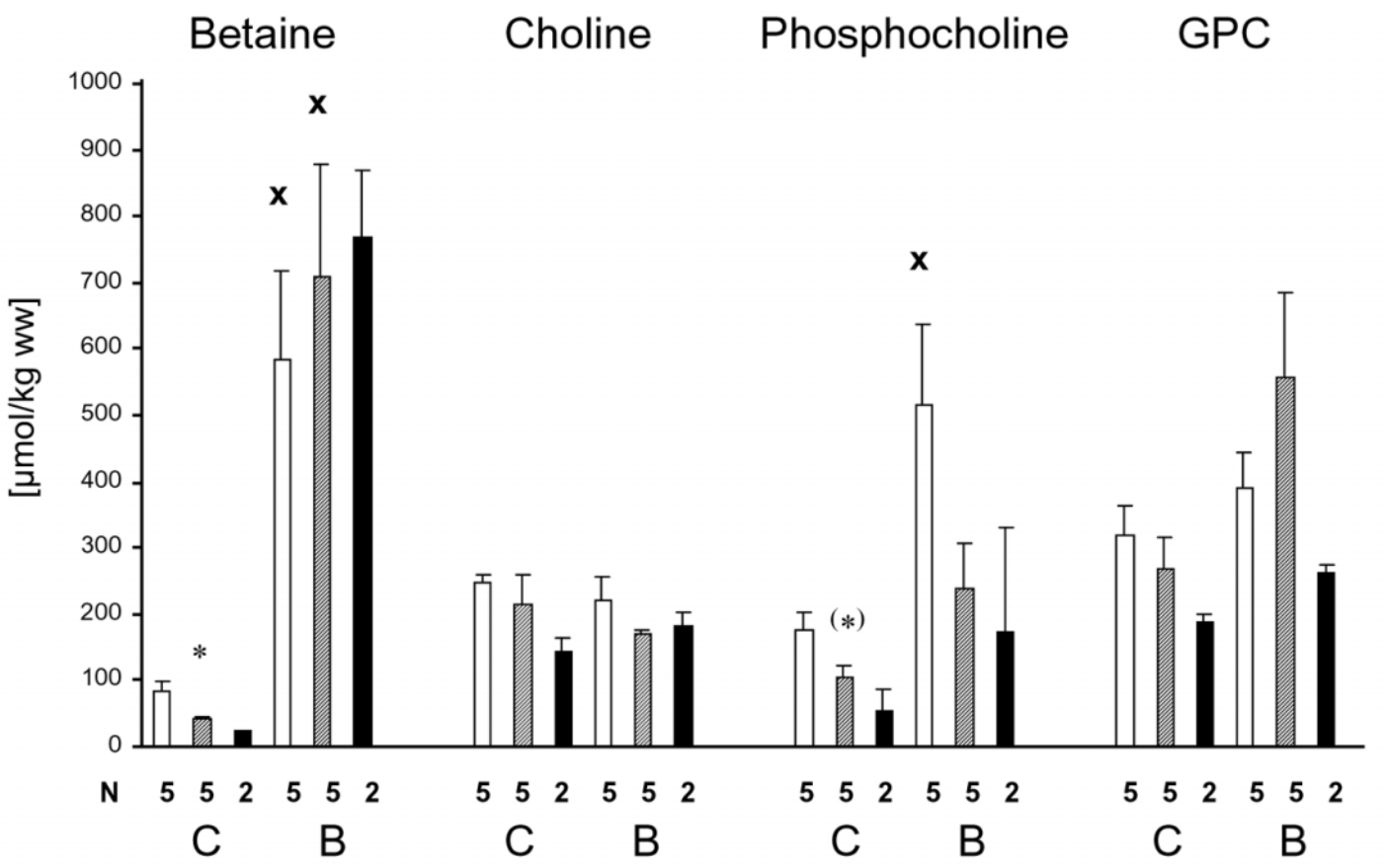

Figure 2. Liver metabolites of *Mthfr*-deficient male mice stratified by genotype and diet. C, control diet; B, betaine diet. Open bars, wild type; hatched bars, heterozygous; filled bars, nullizygous. Data are presented as mean and SE [µmol/l]. **P*<0.05 compared with wild-type mice of the same dietary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

Fig. 3

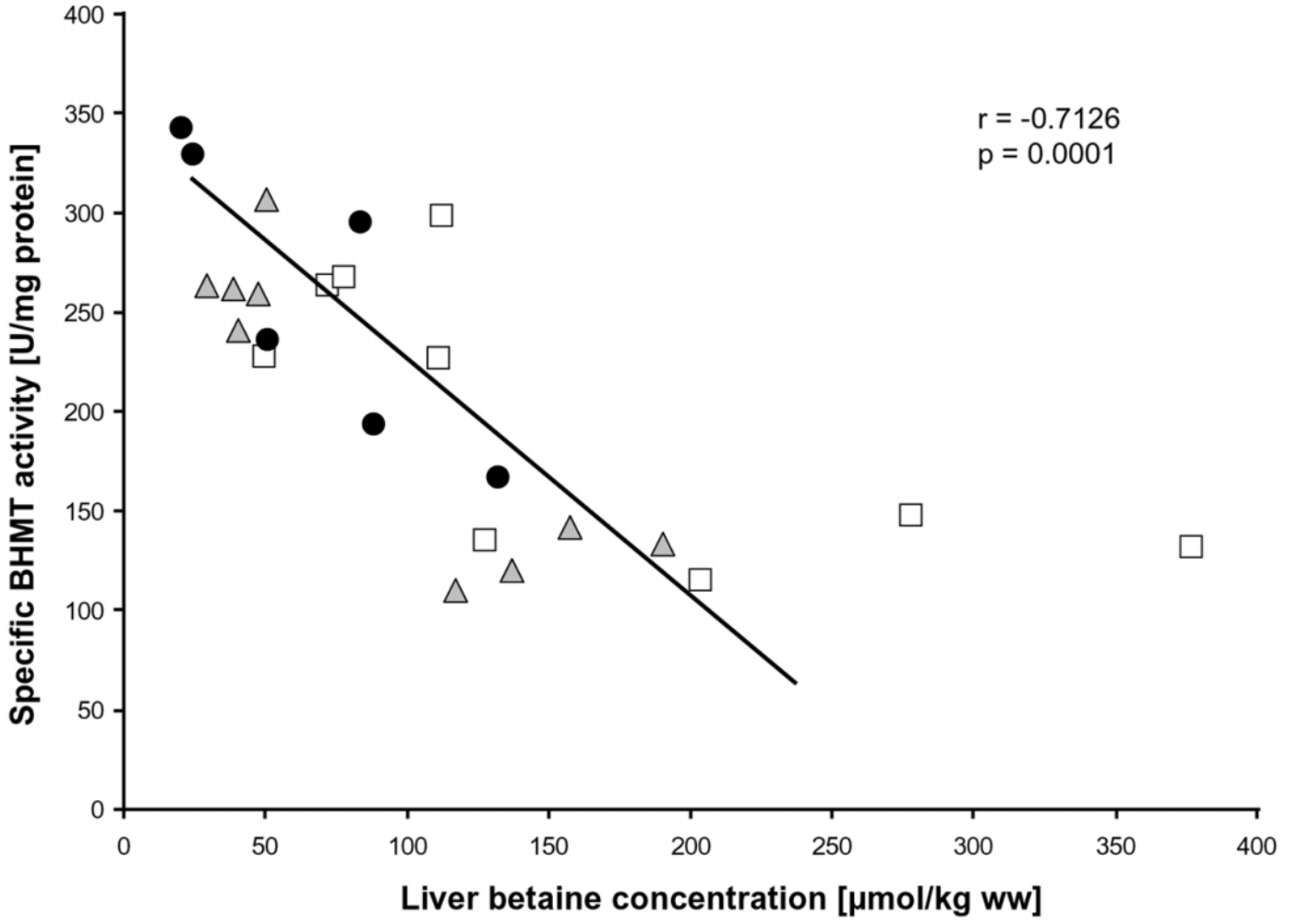

Figure 3. Linear correlation between specific BHMT activity and liver betaine concentration in 24 mice of all three genotypes on the control diet. Open rectangles, wild type; gray triangles, heterozygous; filled circles, nullizygous.

Fig. 4

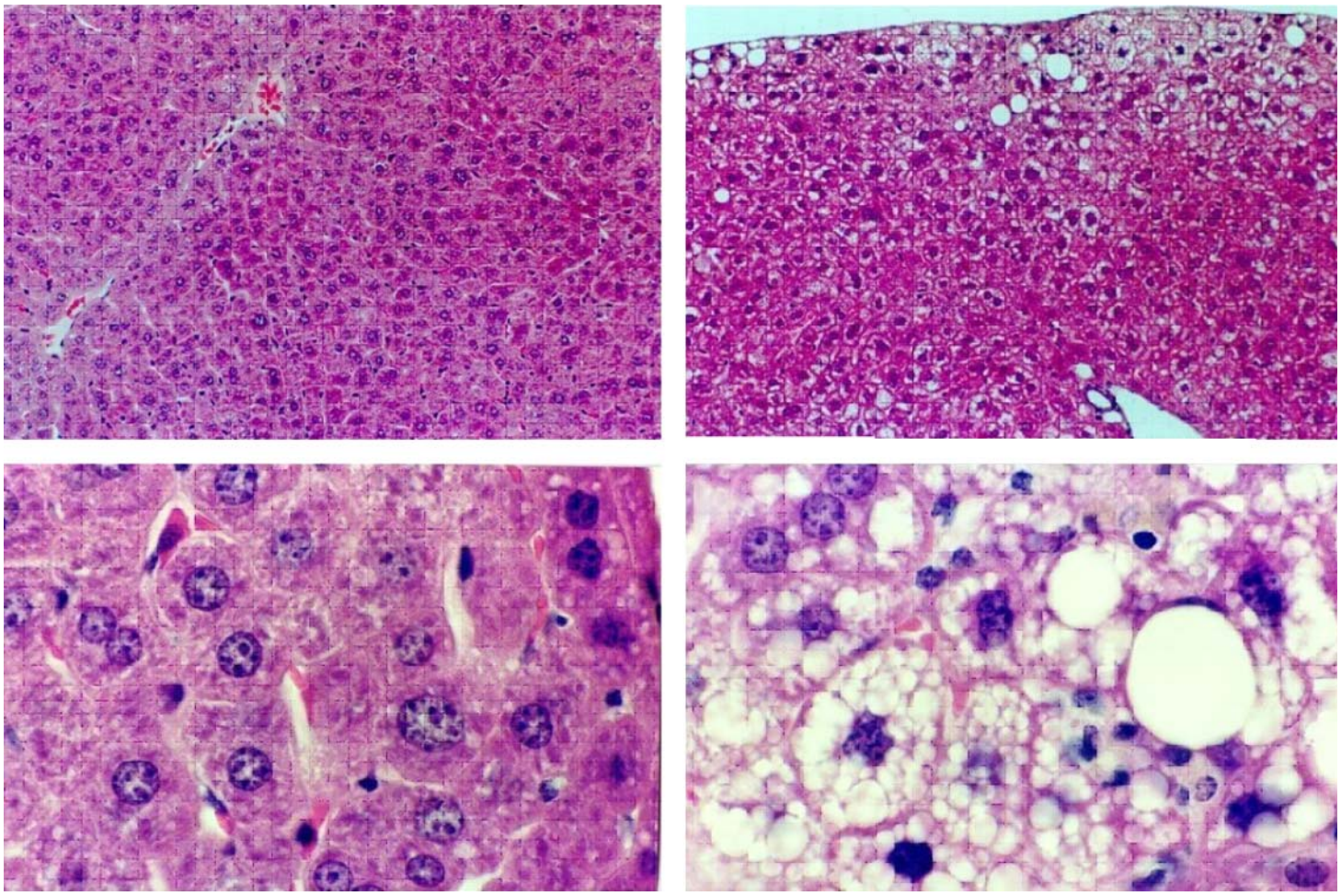

Figure 4. Liver morphology of Mthfr –/– mice on betaine (left panels) or control (right panels) diet. HE stain, upper panels 20×, lower panels 100× magnification.

Fig. 5

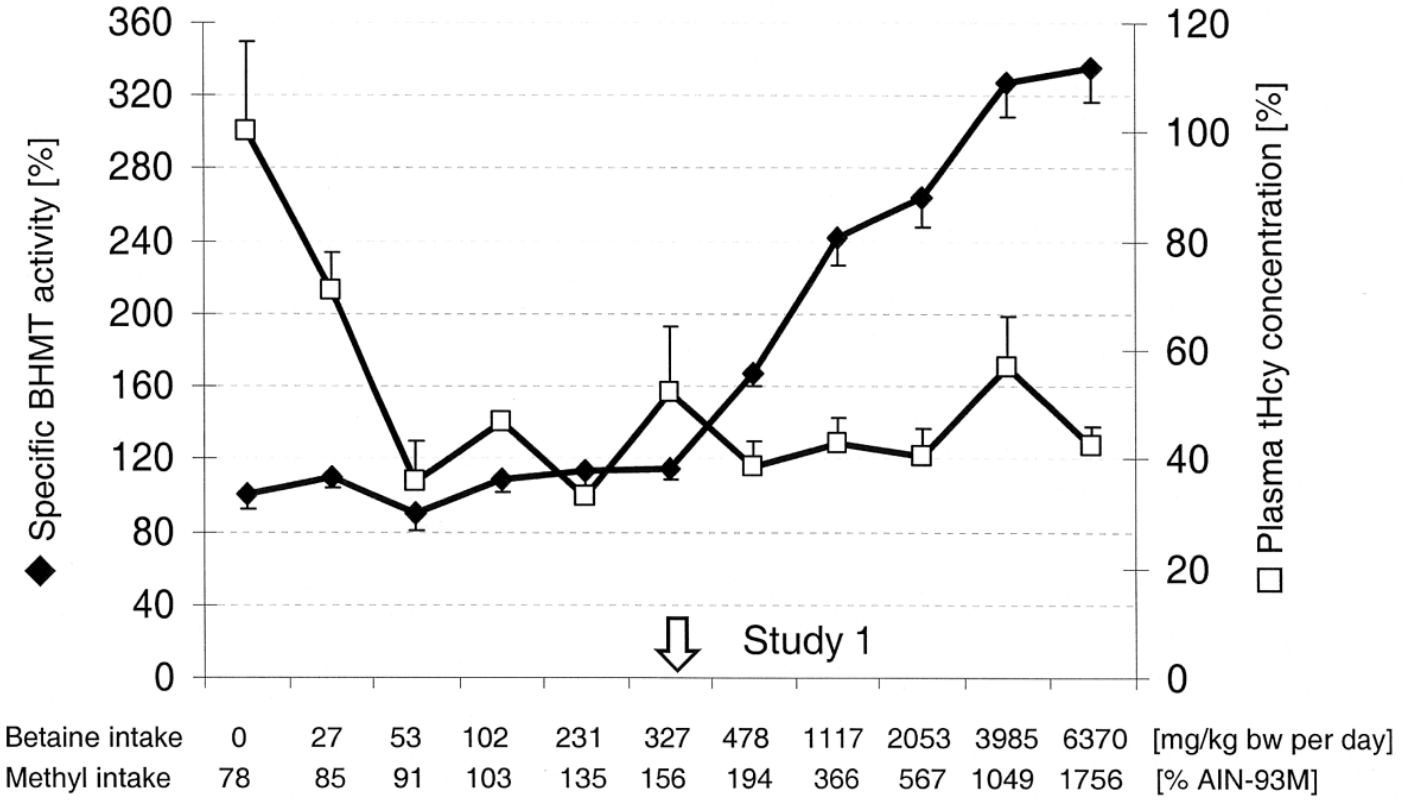

Figure 5. Effects of increasing betaine intake on plasma homocysteine and on specific activity of liver BHMT in *Mthfr* +/– mice. Values have been normalized to the group without the betaine supplement. Data are mean ±SE from four female animals. The arrow indicates the calculated level of betaine intake 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

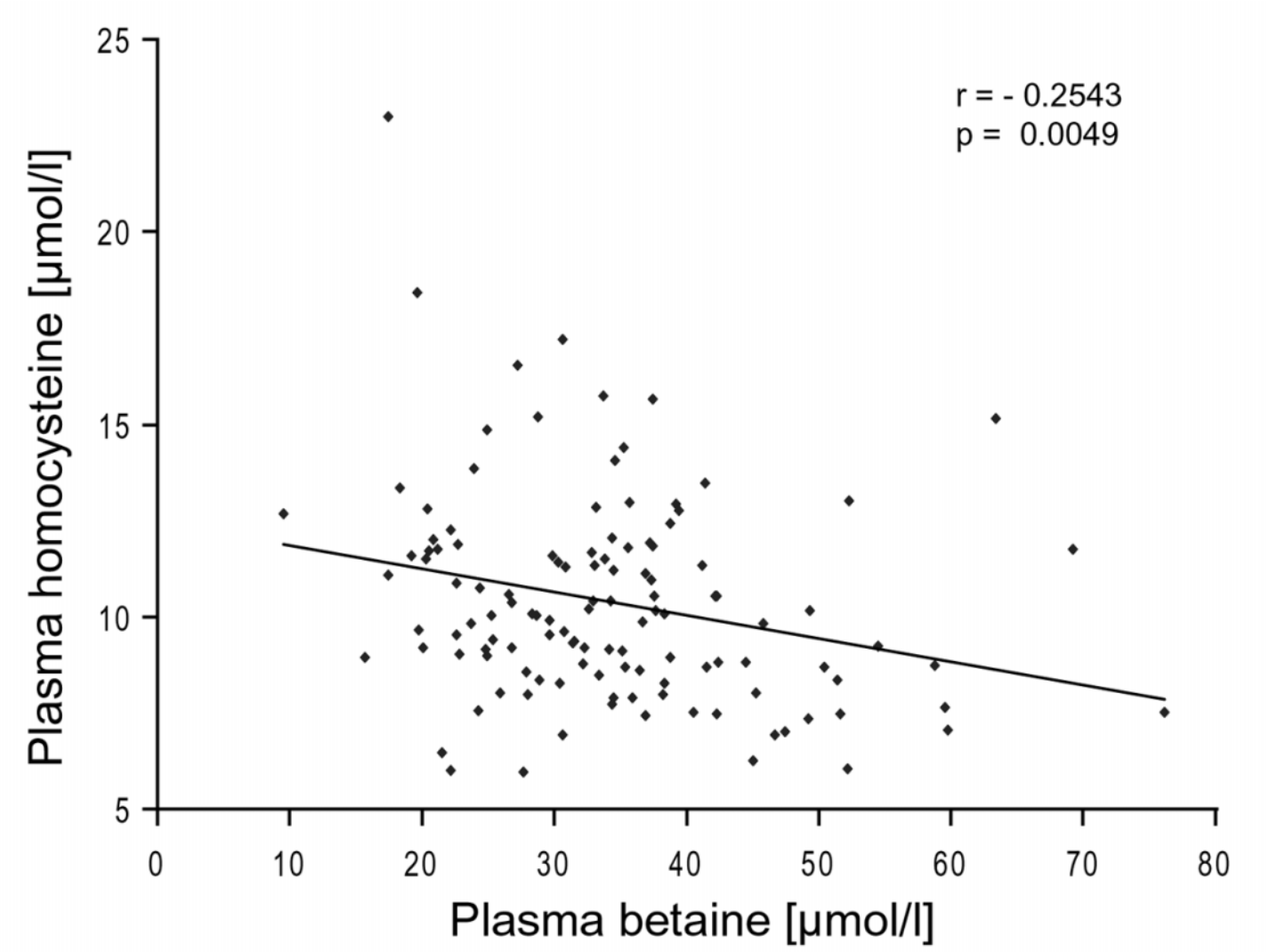

Figure 6. Linear correlation between plasma betaine and total homocysteine concentrations in 121 patients with angiographically confirmed cardiovascular disease.

Pharmacokinetics of oral betaine in healthy subjects and patients with homocystinuria

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Aims Large oral doses of betaine have proved effective in lowering plasma homocysteine in severe hyperhomocysteinaemia. The pharmacokinetic characteristics and metabolism of betaine in humans have not been assessed and drug monitoring for betaine therapy is not available. We studied the pharmacokinetics of betaine and its metabolite dimethylglycine (DMG) in healthy subjects and in three patients with homocystinuria.

Methods Twelve male volunteers underwent an open-label study. After one single administration of 50 mg betaine kg^{-1} body weight and during continuous intake of twice daily 50 mg kg^{-1} body weight, serial blood samples and 24 h urines were collected to determine betaine and DMG plasma concentrations and urinary excretion, respectively. Patients were evaluated after one single dose of betaine.

Results We found rapid absorption ($t_{1/2,abs}$ 00.28 h, s.d. 0.17) and distribution ($t_{1/2,\lambda 1}$ 00.59 h, s.d. 0.22) of betaine. A C_{max} of 0.94 mmol l^{-1} (s.d. 0.19) was reached after t_{max} 00.90 h (s.d. 0.33). The elimination half life $t_{1/2,z}$ was 14.38 h (s.d. 7.17). After repeated dosage, $t_{1/2,\lambda 1}$ (01.77 h, s.d. 0.75) and $t_{1/2,z}$ (41.17 h, s.d. 13.50) increased significantly (95% CI 0.73, 01.64 h and 19.90, 33.70 h, respectively), whereas absorption remained unchanged. DMG concentrations increased significantly after betaine administration and accumulation occurred to the same extent as with betaine. Renal clearance was low and urinary excretion of betaine was equivalent to 4% of the ingested dose. Distribution and elimination kinetics in homocystinuric patients appeared to be accelerated.

Conclusions Betaine plasma concentrations change rapidly after ingestion. Elimination half-life increased during continuous dosing over 5 days. Betaine is mainly eliminated by metabolism. More pharmacokinetic and pharmacodynamic studies in hyperhomocysteinaemic patients are needed to refine the current treatment with betaine.

Keywords: betaine, homocysteine, homocystinuria, pharmacokinetics

Introduction

Betaine ([2(N,N,N-trimethyl)ammoniumacetate]) is a zwitterionic compound at neutral pH. It occurs naturally in cells exposed to osmotic stress, such as in various plants or in mammalian kidney medulla [1, 2]. Betaine is commonly ingested in unknown amounts through intake of vegetables, cereals, and seafood [3]. More importantly, betaine is an endogenous catabolite of choline [4]. Betaine in mammals has three known functions. Firstly, as an organic osmolyte, it helps to maintain normal cell volume under osmotic stress and can accumulate to molar concentrations [5]. Secondly, it provides protection against protein denaturation and has been termed a 'chemical chaperone' [6]. Thirdly, betaine is the only molecule besides methylfolate that provides methyl groups for homocysteine remethylation [3]. The cytosolic enzyme betaine: homocysteine methyltransferase (BHMT, EC 2.1.1.5), abundantly expressed in liver and kidney, catalyses this methyl transfer, yielding methionine

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and dimethylglycine (DMG). The latter is further metabolized to sarcosine and glycine.

Betaine gains further importance as methyl donor in pathological situations associated with hyperhomocysteinaemia. Severe hyperhomocysteinaemia (or homocystinuria) is rare and caused by genetic disruption of either the oxidative breakdown of homocysteine by cystathionine-beta-synthase (CBS), or the remethylation of homocysteine to methionine by methionine synthase (MS). MS uses 5-methyltetrahydrofolate as cosubstrate that is provided by 5,10 methylenetetrahydrofolate reductase (MTHFR). Homocystinuria is associated with damage to the vascular, neural, and skeletal systems, leading to severe disability or death. Treatment aims to decrease the toxic concentrations of homocysteine by dietary intervention, supplementation of cofactors, and provision of betaine to enhance remethylation [7].

However, mild hyperhomocysteinaemia due to nutritional factors, comorbidity with renal disease, or a frequent genetic polymorphism in the *MTHFR* gene (677T) and concomitant low folate status is a common condition, which has recently been shown to be linked to several multifactorial diseases, such as vascular disease or neural tube defects [reviewed in 8]. Betaine therapy for mild hyperhomocysteinaemia has not been evaluated, but it has proved to be effective for the treatment of severe hyperhomocysteinaemia and has been used successfully since 1952 [9–13]. In spite of the long-term use of betaine, no systematic study of its pharmacokinetics has been undertaken, other than two anecdotal reports [14, 15]. This lack of information is probably due to the absence of a convenient method for the determination of betaine and its metabolite DMG in body fluids. Detailed pharmacokinetic data on betaine are required for the establishment of optimal regimens.

We have previously developed a rapid and reproducible method for the determination of betaine and DMG concentrations in human body fluids [16]. Using this technique, the present study reports the basic pharmacokinetic characteristics of betaine after oral administration.

Methods

Study design

We designed an open-label study in 12 healthy male volunteers, following the Recommendations for Biomedical Research Involving Humans (current revision of the Declaration of Helsinki of the World Medical Assembly). The study was approved by the Ethics Committee of the Heinrich Heine–University, Düsseldorf. Informed consent was obtained from each participant before they entered the study.

Three study periods, A, B and C, were defined. During period A, single-dose pharmacokinetics were determined. Baseline plasma concentrations were obtained after an overnight fast immediately prior to drug intake. All subjects ingested 0.43 mmol kg^{-1} body weight (50 mg kg^{-1} body weight) betaine. During the following 24 h, urine was collected and serial plasma samples were obtained at 5, 15, 30 and 45 min, and 1, 1.5, 2, 3 h, 4, 6, 8, 12 and 24 h after betaine intake. After a washout of 7–14 days, study period B followed. This lasted for 5 days and served to evaluate the pharmacokinetics of betaine during multiple dosing. All volunteers received betaine (0.43 mmol kg^{-1} body weight) every 12 h and venous blood was collected each morning, immediately before drug intake for determination of trough concentrations. Betaine was stopped after the last morning dose of period B. Period C started immediately after period B and involved the collection of urine and plasma samples for 24 h, as in period A. The decline of plasma concentrations was followed for an additional 3 days from plasma samples taken each morning.

Healthy subjects

All subjects were male and their age ranged from 21 to 36 years (mean 28.1 years, s.d. 4.6). The body mass index, defined as body weight divided by the square of height in metres, was between 18.5 and 30 kg m^{-2}. The subjects were in good health, based on medical history and physical examination, including electrocardiogram and standard laboratory tests (haematology, blood chemistry, hepatitis B surface antigen, HIV antigen, and urine analysis) performed within the 2 weeks prior to the study and at the end of period C. There was no history in any subject, or signs of prevailing active diseases, allergies or hypersensitivities, alcohol or drug exposure. The subjects were not allowed to consume alcohol, nicotine, caffeine or any drugs, and were advised not to undertake physical exercise or major dietary changes during the entire study period.

Patients

Two siblings, a boy and his older sister (patients 1 and 2), with severe hyperhomocysteinaemia and homocystinuria due to MTHFR deficiency were evaluated during reintroduction of betaine therapy after an accidental betaine-free interval of 1 year. Another female patient with classical homocystinuria due to pyridoxine nonresponsive CBS deficiency was also included in the study (patient 3). She had been prescribed a continuous betaine supplement, but voluntarily discontinued drug intake 8 days before the study period. All three patients followed the period A study protocol, but without urine collec-

tion. All were in good health during the study and they or their parents, respectively, gave informed consent to participation in the study.

Drug administration

Anhydrous betaine powder (Cystadane™) was kindly provided by Orphan-Europe, Paris. A dose of 0.43 mmol kg^{-1} body weight (50 mg kg^{-1} body weight) was dissolved in mineral water and swallowed immediately.

Sampling and drug analysis

Each 4-ml sample of blood was collected in appropriate vacutainers (Becton-Dickinson), anticoagulated with 5 nM K_3-EDTA and centrifuged immediately after venepuncture at 15000 *g* for 15 min at 4 °C. The supernatant plasma was kept frozen below −20 °C for less than 10 days until analysis.

Betaine and DMG plasma concentrations were determined as previously described [16]. Briefly, plasma was derivatized to produce phenacyl esters of the methylamines and compounds were analysed by an isocratic h.p.l.c. procedure with u.v. detection. This method has been shown have limits of detection of 0.005 mmol l^{-1} for betaine and 0.002 mmol l^{-1} for DMG. Inter-assay variability was low with coefficients of variation (CV) of 1.3–5.3% for betaine and of 2.0–4.4% for DMG, respectively, in both blood and urine. Intra-assay variability showed a CV of 0.4–3.8% for betaine and of 0.9–2.2% for DMG, respectively, in blood and urine [16].

Pharmacokinetic and statistical analysis

Pharmacokinetic parameters were estimated using a standard software package (TopFit 2.1: Pharmacokinetic and pharmacodynamic data analysis system for the PC; G. Fischer, Stuttgart, Jena, New York 1993). The peak plasma concentration (C_{max}) and the time to reach the peak concentration (t_{max}) were derived directly from the plasma concentration time data. The area under the plasma concentration *vs* time curve from the time of dosing to the time of the last quantified concentration (AUC(0,24 h)) was computed by the linear trapezoidal method. The theoretical accumulation ratio was calculated as $1/(1-e^{-\lambda_1 \times \tau})$, where λ_1 denotes the rate constant for the elimination phase derived from period A, where τ was the dosing interval. The following kinetic parameters were estimated using TopFit: Half-life parameters for absorption ($t_{1/2,abs}$), distribution ($t_{1/2,\lambda 1}$), and elimination ($t_{1/2,z}$); the area under the plasma-concentration-time course extrapolated to infinity towards the betaine baseline level (AUC(0,∞); and the mean residence time (MRT). A standard linear two-compartment disposition model for oral drug application with first order absorption kinetics, including a lag period, fitted best to the data. Maximal apparent total plasma clearance (CL/*F*) was calculated as dose divided by AUC(0,∞) assuming an oral bioavailability of 100%. The maximal apparent volume of distribution at steady state after a single dose was estimated using the formula $V_{ss}/F = CL/F \times MRT$. Renal clearance ($CL_R$) was evaluated as ratio of 24 h urinary excretion to the respective AUC(0,24 h).

Statistical evaluation was performed using standard software. Data were expressed as mean and standard deviation. Differences between kinetic parameters after the first single dose (period A) and the last single dose after multiple dosing (period C) were compared using Student's *t*-test for paired samples. Differences were considered significant if *P* values were less than 0.05.

Results

Healthy volunteers

Betaine was well tolerated over the whole experimental period. No adverse events were observed. All routine laboratory data remained unchanged throughout the study.

Baseline concentrations of betaine

Mean ± s.d. baseline concentrations of betaine were 0.032 ± 0.006 mmol l^{-1} in period A and 0.034 ± 0.009 mmol l^{-1} in period B, and were not significantly different. The concentrations were 0.075 ± 0.027 mmol l^{-1} after 24 h during period A, and to 0.104 ± 0.102 mmol l^{-1} 96 h after the last dose in period C.

Plasma pharmacokinetics of betaine

The results for period A are shown in Table 1. Betaine plasma concentrations increased sharply after oral dosing. Betaine was rapidly distributed into a relatively large apparent volume of distribution. The plasma concentration–time curve showed a biexponential decline according to a two-compartment model (Figure 1). Elimination of the drug was rather slow.

The pharmacokinetics after multiple doses are shown in Figure 2 and Table 2. Whereas t_{max} and $t_{1/2,abs}$ remained unchanged, C_{max}, AUC(0,24 h), $t_{1/2,\lambda 1}$, and $t_{1/2,z}$ increased significantly after multiple doses ($P < 0.01$). The mean experimental accumulation ratio clearly exceeded the theoretical accumulation ratio.

Urinary excretion of betaine

Baseline 24 h excretion of betaine before drug administration was measured in only four individuals and gave a mean value 0.159 ± 0.103 mmol. Table 3 shows the

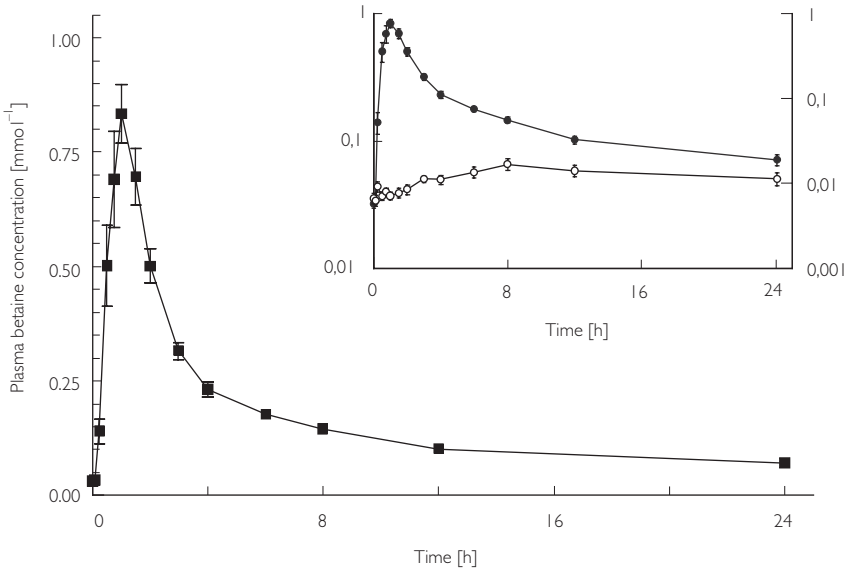

Figure 1 The mean ± s.e.mean betaine plasma concentration *vs* time profile following a single oral dose of 50 mg kg^{-1} in 12 healthy subjects. Insert: semilogarithmic plot of mean betaine (●) and DMG (○) plasma concentrations.

Table 1 Pharmacokinetic parameters after a single dose of betaine in 12 healthy subjects.

Parameter	*Mean ± s.d.*	*Range*
C_{max} (data) (mmo l^{-1})	0.939 ± 0.194	0.663–1.300
C_{max} (model) (mmol l^{-1})	0.906 ± 0.191	0.652–1.320
t_{max} (data) (h)	0.90 ± 0.33	0.50–1.50
t_{max} (model) (h)	0.92 ± 0.29	0.52–1.38
AUC(0, 24 h) (mmol l^{-1} h)	3.974 ± 0.731	2.747–5.240
AUC(0, ∞) (mmol l^{-1} h)	5.518 ± 1.919	3.730–11.000
t_{lag} (h)	0.43 ± 0.19	0.21–0.72
$t_{1/2,abs}$ (h)	0.28 ± 0.17	0.09–0.61
$t_{1/2,\lambda 1}$ (h)	0.59 ± 0.22	0.35–1.01
$t_{1/2,z}$ (h)	14.38 ± 7.17	6.04–31.64
MRT (h)	17.50 ± 9.26	7.19–40.90
CL/F * (l h^{-1} kg^{-1})	0.084 ± 0.021	0.039–0.115
V_{SS}/F* (l kg^{-1})	1.324 ± 0.382	0.728–1.896

C_{max}, maximum plasma concentrations; t_{max}, times of C_{max}; AUC, areas under the plasma concentration/time curve; t_{lag}, lag time; $t_{1/2,abs}$, half-life of absorption; $t_{1/2,\lambda 1}$, half-life of distribution; $t_{1/2,z}$, half-life of elimination; MRT, Mean residence time; CL/F, total oral plasma drug clearance; V_{SS}/F, volume of distribution at steady-state after oral application. *Assuming 100% bioavailability.

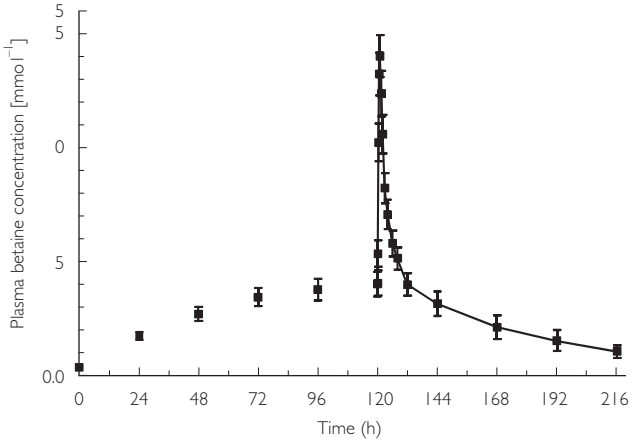

Figure 2 The mean ±s.e.mean betaine plasma concentration *vs* time profile after multiple dosing of 50 mg kg^{-1} to 12 healthy volunteers.

results for renal betaine excretion after one single dose in period A and C, respectively. Betaine excretion was low in period A and increased threefold after multiple doses. Renal clearance was only 5.3% of apparent total plasma clearance in period A and remained essentially the same during the study period.

Plasma pharmacokinetics of DMG

The mean ± s.d. initial concentrations of DMG were 0.007 ± 0.003 mmol l^{-1} for period A and 0.008 ± 0.005 mmol l^{-1} for period B. DMG rose significantly after 3 h, to a peak concentration of 0.019 ± 0.008 mmol l^{-1} at t_{max} 09.72 h ± 7.24 in period A. During periods B and C, DMG concentration rose significantly by 0.033 mmol l^{-1} (95% CI 0.017, 0.049) to a peak concentration C_{max} of 0.052 ± 0.034 mmol l^{-1} after 126 ± 5 h, corresponding to 06.45 h after the last betaine dose. The AUC(0,24 h) for period A was 0.308 ± 0.147 mmol l^{-1} h, whereas the AUC(0,24 h) for period C was 0.999 ± 0.662 mmol l^{-1} h. The accumulation ratio of 3.31 ± 1.25 was comparable with that of betaine.

Urinary excretion of DMG

The mean 24 h urinary excretion of DMG after single dose (period A) was 0.339 ± 0.362 mmol (range 0.055–1.253). After multiple dosage (period C), the 24 h excretion of DMG in urine rose significantly by 1.079 mmol

Table 2 Pharmacokinetic parameters after multiple dosing of betaine in 12 healthy subjects (values were evaluated for the time 0–96 h after the final dose (period C).

Parameter	*Mean ± s.d.*	*Range*	*95% CI Δ*	P
C_{max} (data) (mmol l^{-1})	1.456 ± 0.308	1.050–2.178	0.369–0.665	<0.01
t_{max} (data) (h)	0.90 ± 0.25	0.34–1.00	−0.19–0.18	0.97
AUC(0,24 h) (mmol l^{-1} h)	12.528 ± 4.496	8.134–24.118	6.244–10.862	<0.01
AUC(0,96 h) (mmol l^{-1} h)	26.298 ± 15.664	14.918–71.403		
t_{lag} (h)	0.36 ± 0.10	0.21–0.48	−0.20–0.05	0.27
$t_{1/2,abs}$ (h)	0.68 ± 0.25	0.32–1.06	−0.07–0.21	0.35
$t_{1/2,\lambda1}$ (h)	1.77 ± 0.75	0.60–2.68	0.73–1.64	<0.01
$t_{1/2,z}$ (h)	41.17 ± 13.50	27.81–74.32	19.89–33.70	<0.01
Accumulation ratio (theoretetical)	2.29 ± 0.84	1.34–4.33		
Accumulation ratio (experimental)	3.14 ± 0.80	2.22–4.60		

*C*max, maximum plasma concentrations; *t*max, times of *C*max; AUC, areas under the plasma concentration/time curve; *t*lag, lag time; $t_{1/2,abs}$, half-life of absorption; $t_{1/2,\lambda1}$, half-life of distribution; $t_{1/2,z}$, half-life of elimination. 95% confidence intervals and *P* values are given for differences between results of period A and period C.

Table 3 Urinary excretion of betaine after a single dose (period A) or multiple doses (period C) of oral betaine in 12 healthy subjects.

Parameter	*Mean ± s.d.*	*Range*
Period A		
24 h urinary excretion (mmol)	1.338 ± 1.111	0.153–4.324
% of oral dose (%)	4.0 ± 3.4	0.4–13.1
CL_R (l h^{-1} kg^{-1})	0.0044 ± 0.0037	0.0004–0.0139
% of total plasma CL (%)	5.3 ± 3.9	0.5–14.3
Period C		
24 h urinary excretion (mmol)	4.361 ± 2.105	0.756–7.444
CL_R (l h^{-1} kg^{-1})	0.0045 ± 0.0022	0.0006–0.0086

CL_R, renal clearance.

(95% CI 0.543, 1.616) to 1.407 ± 1.150 mmol (range 0.252–4.139), compared with period A. The renal clearance of DMG was calculated as 0.014 ± 0.018 l h^{-1} kg^{-1} (range 0.004–0.017) for period A and 0.017 ± 0.007 l h^{-1} kg^{-1} (range 0.005–0.027) for period C ($P > 0.05$).

Patients with homocystinuria

Baseline concentrations of betaine and DMG

Baseline concentrations of betaine were 0.004, 0.012, and 0.011 mmol l^{-1} in patients 1, 2, and 3, respectively, before reintroduction of betaine. Betaine concentrations were 0.006 and 0.031 mmol l^{-1} after 24 h, 0.003 and 0.012 mmol l^{-1} after 48 h in patients 1 and 2, respectively. Patient 3 had a betaine concentration of 0.050 mmol l^{-1} 24 h after dosing. DMG baseline concentrations were below the detection limit (<0.001 mmol l^{-1}) in all three patients.

Plasma pharmacokinetics of betaine

Absorption was rapid in patients 1 and 3, whereas patient 2 had a much longer $t_{1/2,abs}$ than all the healthy volunteers and the two other patients (Table 4). Distribution and elimination half-lives were somewhat shorter in patients 1 and 3 and apparent total plasma clearance appeared to be increased in all three patients compared with the healthy volunteers.

Plasma kinetics of DMG

DMG rose to a mean peak concentration of 0.011 and 0.010 mmol l^{-1} 4–5 h after administration of betaine in patients 1 and 2, respectively. In patient 3, DMG rose to a maximum of 0.140 mmol l^{-1} after 6 h. Twenty-four hours after the dose, plasma concentrations returned to their initial values in patients 1 and 2, whereas in patient 3 DMG remained elevated at 0.102 mmol l^{-1}.

Discussion

Despite its long-standing use for treatment of severe hyperhomocysteinaemia, the pharmacokinetics of betaine have not been explored thoroughly in animals or humans. Previously published values of plasma betaine concentrations in blood samples taken randomly from

Table 4 Pharmacokinetic parameters after a single dose of betaine in patients with homocystinuria

Parameter	*Patient 1*	*Patient 2*	*Patient 3*
Age (years)	5.8	10.2	25.5
Body weight (kg)	22	40	91
Height (cm)	114	142	183
C_{max} (data) (mmol l^{-1})	0.506	0.556	1.176
C_{max} (model) (mmol l^{-1})	0.466	0.506	1.070
t_{max} (data) (h)	1.20	2.00	0.88
t_{max} (model) (h)	1.00	2.06	0.93
AUC(0, 24 h) (mmol l^{-1} h)	1.321	3.491	4.215
AUC(0, ∞) (mmol l^{-1} h)	1.450	4.230	4.810
$t_{1/2,abs}$ (h)	0.46	1.22	0.37
$t_{1/2,\lambda1}$ (h)	0.46	1.22	0.37
$t_{1/2,z}$ (h)	6.63	23.23	9.70
MRT (h)	5.81	18.70	11.10
CL/F* (l h^{-1} kg^{-1})	0.297	0.102	0.089
V_{SS}/F* (l kg^{-1})	1.726	1.907	0.992

C_{max}, maximum plasma concentrations; t_{max}, times of C_{max}; AUC, areas under the plasma concentration/time curve; $t_{1/2,\lambda1,}$ half,life of absorption; $t_{1/2,\lambda1}$, half,life of distribution; $t_{1/2,z}$, half,life of elimination; MRT, Mean residence time; CL/F, total oral plasma drug clearance; V_{SS}/F, volume of distribution at steady,state after oral application. *Assuming 100% bioavailability.

healthy individuals show similar results to our study. Allen and coworkers [17] found serum concentrations of 0.046 ± 0.014 mmol l^{-1} ($n = 60$), which were not affected by age and gender. One group [18] found mean plasma concentrations of 0.034 ± 0.011 mmol l^{-1} in females ($n = 37$), and of 0.047 ± 0.018 mmol l^{-1} in males ($n = 35$). In a previous study [16], we reported a mean plasma concentration of 0.027 mmol l^{-1} ($n = 12$). In the present study our baseline concentrations are consistent with these previous findings.

After oral uptake of 0.43 mmol kg^{-1} body weight betaine in 12 healthy volunteers, betaine was rapidly absorbed with a sharp plasma concentration peak. Distribution half-life was short after the first dose. Assuming 100% oral bioavailability, a relatively high apparent distribution volume of 1.32 l kg^{-1} was calculated, indicating extensive uptake of betaine into tissue. Of more relevance for the use of betaine in hyperhomocysteinemia, its pharmacokinetics were also studied after a therapeutic repeated dose regimen of 0.43 mmol kg^{-1} every 12 h for 5 days, with a final single dose on day 6. Whereas absorption kinetics did not change over this period, distribution half-life was significantly longer compared with that after single dose, indicating that transport and redistribution between different compartments might have been partially saturated.

Betaine is exclusively and irreversibly catabolized by the zinc metalloenzyme betaine: homocysteine methyltransferase (BHMT) [20]. The accumulation of betaine during the 5-day study period B and the increased elimination half-life measured in period C indicate limited capacity for betaine metabolism as a result of either saturation or inhibition.

The K_m of BHMT for betaine metabolism is 2.2 mmol l^{-1} [21]. Given that betaine is known to be present in liver and kidney and because it fits the high apparent volume of distribution, a concentration of betaine in hepatocytes in the molar range and thus near to or even above the K_m of BHMT can be expected, and would be compatible with saturation of metabolism.

We also found a considerable increase in DMG plasma concentration and urinary excretion during multiple betaine dosing. This provides evidence for an enhancement of betaine metabolism by exogenous betaine in healthy subjects, which has also been suggested by others [22]. In contrast, it has been shown that DMG inhibits BHMT activity *in vitro* with a low K_i of approximately 0.010 mmol l^{-1} [17]. Increasing concentrations of DMG during periods B and C of the study could have led to increased inhibitory effects on BHMT activity.

The mean ratio between AUC(0,24 h) of betaine and DMG in period A was 12.90 compared with 12.54 for period C. Thus, both betaine and DMG appear to accumulate to the same extent. This observation argues against saturation of the BHMT-mediated reaction as the sole cause for the increased elimination half-life of betaine. Taking into account an unchanged renal clearance and the plasma accumulation of DMG, our data provide also evidence for a limited capacity of healthy subjects to metabolize DMG by oxidative demethylation to sarcosine, which seems to be the rate-limiting step in betaine metabolism and might indirectly influence the latter through inhibition of BHMT by DMG. In conclusion, increased DMG formation and saturation of DMG metabolism with subsequent product inhibition of BHMT would best explain our results.

Increasing availability of betaine enhances metabolic flow through BHMT until saturation occurs. Animal studies have shown that enzyme induction can then occur, particularly when methionine availability is low [20]. All subjects in the present study were on normal western diets with a high protein content, when methionine availability should be high. BHMT induction should therefore be negligible in this study.

Betaine is not a xenobiotic but an endogenous metabolite of choline. In an attempt to correct for the contribution of endogenous betaine, we subtracted the baseline plasma betaine pool, defined by the product of betaine concentrations at t_0 multiplied by the study period observation, from total AUC(0, 24 h). The same percentage of total area was subtracted from AUC(0, ∞). We observed

a decrease in AUC(0, 24 h) of 19% and a corresponding increase in total plasma clearance and distribution volume of 24% after correction in period A. We consider this to be a minor confounder in the interpretation of results and did not perform such a correction for period C or on the patient data, where baseline betaine plasma pools were even smaller relative to the AUC after betaine dosage.

Betaine normally accumulates in human kidney medulla [2]. Its release into urine is dramatically augmented after water diuresis [5]. There is evidence that plasma concentrations are under homeostatic control, and the urinary excretion of betaine shows marked variability. Betaine transporter gene (*BGT1*) expression is directly regulated by tonicity of the intracellular fluid [19], therefore different intakes of water and salts during the study could account for some of the interindividual variability in the elimination kinetic data. However, renal clearance of betaine did not change during the study and contributed to only a minor extent to the elimination of betaine. A fractional clearance of less than 6% in male healthy subjects has been demonstrated for betaine [17, 18]. In the present study, renal clearance corresponded to 5.3% of total oral plasma clearance and 4.0% of the ingested dose was excreted as betaine in the 24-h urine samples during period A.

Betaine baseline concentrations in the three patients with severe hyperhomocysteinaemia before reintroduction of betaine therapy were decreased compared with healthy subjects. Plasma betaine concentrations in patients undergoing continuous high-dose betaine treatment range from 0.02 to 2.68 mmol l^{-1} [14, 17]. Obviously, concentrations depend heavily on the interval between dosing and time of measurement. Betaine kinetics after the first dose in the three patients were similar to those in healthy volunteers in period A. The results of patient 2 were distorted by delayed absorption. Distribution and elimination half-lives of betaine in patients 1 and 3 were decreased and total plasma clearance increased in all three individuals compared with healthy subjects. The apparent volume of distribution was increased in patients 1 and 2, and the lower value in patient 3 might also relate to their obesity. A short report on betaine kinetics in five homocystinuric patients described absorption half-lives of between 0.3 and 1.4 h and oral total clearances ranging from 0.06 to 0.21 l h^{-1} kg^{-1} [15], which are comparable with our results. In spite of the small number of observations, the turnover of betaine seems to be accelerated in patients with homocystinuria. From these findings it can be hypothesized that the endogenous betaine pool is diminished in severe hyperhomocysteinaemia, where increased remethylation of homocysteine via BHMT takes place.

The dosing interval selected for the present study (12 h), which corresponds to current practice in the treatment of hyperhomocysteinaemia, appears not to be adequate for maintaining a constant high concentration of betaine in plasma. However, we have no data on intrahepatocytic betaine concentrations, which are likely to be more constant. Our results might provide a basis for further pharmacokinetic and pharmacodynamic studies of betaine in patients with mild or severe hyperhomocysteinaemia with the aim of optimizing treatment modalities.

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Effects of Betaine in a Murine Model of Mild Cystathionine-β-Synthase Deficiency

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Cystathionine-β-synthase (CBS) is required for transsulfuration of homocysteine, an amino acid implicated in vascular disease. We studied homocysteine metabolism in mice with mild hyperhomocysteinemia due to a heterozygous disruption of the *Cbs* gene. Mice were fed diets supplemented with betaine or dimethylsulfonioacetate (DMSA); betaine and DMSA provide methyl groups for an alternate pathway of homocysteine metabolism, remethylation by betaine:homocysteine methyltransferase (BHMT). On control diets, heterozygous mice had 50% higher plasma homocysteine than did wild-type mice. Betaine and DMSA had similar effects in both genotype groups: liver betaine increased dramatically, while plasma homocysteine decreased by 40% to 50%. With increasing betaine supplementation, homocysteine decreased by 75%. Plasma homocysteine and BHMT activity both showed a strong negative correlation with liver betaine. Homocysteinemia in mice is sensitive to a disruption of *Cbs* and to methyl donor intake. Because betaine leads to a greater flux through BHMT and lowers homocysteine, betaine supplementation may be beneficial in mild hyperhomocysteinemia.

HOMOCYSTEINE IS the endogenous product of all transmethylation reactions that use S-adenosylmethionine (SAM) as a methyl donor. Homocysteine can be remethylated to methionine by methionine synthase (MS, EC 2.1.1.13), using 5-methyltetrahydrofolate as cosubstrate. Alternatively, betaine: homocysteine methyltransferase (BHMT, EC 2.1.1.5) can catalyze methyl transfer from betaine to homocysteine, yielding methionine and N,N-dimethylglycine (DMG). Cystathionine-β-synthase (CBS, EC 4.2.1.22) catalyzes the only catabolic pathway for homocysteine, through transsulfuration to cystathionine and subsequently cysteine. The metabolism of excessive SAM due to high dietary methionine can lead to increased production of homocysteine. CBS is activated by high SAM availability, whereas the enzyme providing methyl groups for folate-dependent homocysteine remethylation, 5,10-methylenetetrahydrofolate reductase (MTHFR, EC 1.5.1.20), is inhibited by SAM; CBS activity becomes particularly important under these conditions.[1] In homocystinuria due to severe deficiency of CBS (MIM 236200), this regulatory system fails, with consequent severe hyperhomocysteinemia and life-threatening vascular complications. Betaine, or N,N,N-trimethylglycine, is an endogenous product of choline oxidation and is present in some foods.[2] In humans with homocystinuria due to pyridoxine nonresponsive severe CBS deficiency, betaine therapy has proven effective in decreasing homocysteine by approximately 75% and in improving outcome with respect to the vascular complications.[3] Usually, a dose of 100 to 200 (up to 400) mg/kg body weight (BW) per day is used. With the recognition that moderate hyperhomocysteinemia is associated with vascular disease, elucidation of homocysteine regulatory mechanisms is of increasing importance.[4]

Here, we studied the regulation of homocysteine metabolism by means of methyl donor supplementation in a mouse model of moderate hyperhomocysteinemia due to a heterozygous disruption of the *Cbs* gene.[5]

MATERIALS AND METHODS

Mice

Animal experimentation was approved by the Animal Care Committee of the Montreal Children's Hospital in compliance with guidelines of the Canadian Council for Animal Care. Mice with a heterozygous disruption of the *Cbs* gene (C57BL/6J-*Cbs*^{tm1Unc})[5] were obtained from the Jackson Laboratory and bred with C57BL/6J mice to obtain heterozygous and wild-type mice. These littermates were used for all studies. Mice were housed in our animal facility with free access to food and water. The mean starting age was 189.4 (6.7) days in study 1 and 225.1 (17.6) days in study 2, with mean BWs of 23.3 (0.6) g and 28.4 (0.6) g, respectively. There was no significant difference for these parameters between genotype or treatment groups in study 1 or study 2, respectively.

Genotyping

Cbs genotypes were determined by a polymerase chain reaction (PCR)-based method. Genomic DNA was extracted from mouse tail biopsies. A 0.4-kb fragment of *Cbs* intron 2 was amplified on a Perkin-Elmer (Boston, MA) TC1 using 0.25 μg sense Primer 1 (5′-TAC TAC CAC TGC CCA GCT TT-3′) and 0.05 μg antisense Primer 2 (5′-CCG AGC CAA CTT AGC CCT TA-3′) to identify the wild-type allele. The *Cbs* disruption was identified by amplifying a 0.2-kb fragment of intron 2 and the inserted *neo* gene using 0.25 μg sense Primer 1 and 0.25 μg antisense Primer 3 (5′-GAG GTC GAC GGT ATC GAT A-3′). Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA) was used with the buffer recommended by the manufacturer with PCRx

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Enhancer Solution (Invitrogen) at 1 × concentration in a final volume of 50 μL. DNA was amplified during 35 cycles with the following PCR conditions: 94°C for 1 minute, 62°C for 1 minute, and 72°C for 2 minutes. Genotypes were deduced by electrophoretic analysis of the amplification products.

Dietary Experiments

Study 1. Four female wild-type mice and 4 female mice, heterozygous for the *Cbs* disruption, were fed for 2 weeks with an amino acid–defined control diet (TD 00310; Harlan Teklad, Madison, WI). This control diet had essentially the same composition, including folate content, as the reference rodent diet, AIN-93M,[6] 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). Our control diet thus contained only 20% less labile methyl groups than the AIN-93M reference diet, and similar diets have been considered to be methyl-sufficient in other dietary studies in rodents. The lower choline concentration was chosen to avoid saturating conditions for the choline-dependent remethylation pathway.

Another 2 groups, each with 4 wild-type and 4 heterozygous female mice, were fed the same diet for 2 weeks, containing either a betaine (Sigma, St Louis, MO) or a dimethylsulfonioacetate (DMSA or DMAT (NutriQuest, Chesterfield, MO)) supplement of 25 mmol/kg diet. Anhydrous betaine was purchased from Sigma. DMSA was obtained by custom synthesis for T. Garrow. BW and food intake were recorded weekly.

After 2 weeks, mice were killed in a CO_2 chamber. Blood was collected by heart puncture, anticoagulated with EDTA (MicrovetteR 500, Sarstedt, Germany) and immediately put on ice. Plasma was quickly separated by centrifugation at 10,000 × *g* for 5 minutes and immediately frozen at −70°C until analysis. Livers were dissected and liver aliquots were frozen on dry ice and stored at −70°C until analysis.

Study 2. Four groups, each comprising 4 adult heterozygous male mice, were placed on control diet TD 00310 and supplemented with increasing amounts of anhydrous betaine in drinking water, ranging from 0 to 100 mmol/L, for 2 weeks. Water was changed twice weekly. BW and food and water consumption were monitored. Betaine intake by drinking water was calculated. After 2 weeks, mice were processed as in study 1.

Metabolites

Total homocysteine and total cysteine concentrations were measured after chemical reduction of a plasma sample by high-performance liquid chromatography (HPLC) as previously described.[7] Plasma amino acid concentrations were measured by HPLC using a previously described method[8] in 3 of the 4 groups of study 2. Choline compounds in tissues were extracted by the method of Bligh and Dyer.[9] Choline, glycerophosphocholine, phosphocholine (PCho), betaine, and phosphatidylcholine (PtdCho) were then measured using liquid chromatography-electrospray ionization-isotope dilution mass spectrometry (LC-ESI-IDMS).[10]

BHMT activity. BHMT activity in crude liver extracts was analyzed as previously described.[11]

Statistical Analyses

Results are provided as mean ± SEM. Metabolite levels between groups were compared using the Kruskall-Wallis nonparametric test. If a significant test result was found, single parameters were compared with the 2-sided Wilcoxon test. Linear correlation between 2 parameters for mice on control diet was calculated and Spearman's linear regression coefficient provided. The nonparametric Spearman's rank test was used to correlate betaine and homocysteine for the mice on varying betaine supplements, and Spearman's rank correlation coefficient was calculated. For all analyses, a *P* level of .05 was considered significant. For the correlation studies of mice on the control diet, the 8 female mice from study 1 and 4 male heterozygous mice from study 2 were analyzed together.

RESULTS

Food and Drug Intake

Study 1. Supplementing the diet with 25 mmol/kg betaine led to a slightly, but significantly, increased food intake (118.3 [2.7]) compared with the control group (108.6 [2.9] g/kg BW per day) in the second week. Betaine intake was calculated as 346 [8] mg/kg BW per day. The treatment group receiving DMSA voluntarily decreased food intake during the first days of the study by 25% as previously described[12]; this resulted in a drug intake of 246 [2] mg/kg BW per day. During the first week, mice on the DMSA diet showed a weight loss of 13.3% [0.7], which was significantly higher than that of mice on the control (5.5% [0.5]) or betaine diet (5.9% [0.7]). Weights did not change significantly in week 2 of the study.

Study 2. Food intake amounted to 120.0 [2.6] g/kg BW per day in week 1 and 107.6 [1.4] g/kg BW per day in week 2 and was not significantly different between treatment groups in week 2. Betaine intake by water was not different between weeks 1 or 2 and amounted to 14 [1], 220 [13], and 1,549 [60] mg/kg BW per day for the 3 treatment groups.

Metabolite Concentrations and BHMT Activities

Study 1. On the control diet, heterozygous mice had significantly (49.8% [18.4]) higher plasma homocysteine than did wild-type mice, whereas cysteine concentrations in plasma were not significantly different (Table 1). Liver concentrations of betaine, choline, and PCho were each moderately, but not significantly, decreased in heterozygous mice compared with wild-type mice. Liver BHMT activity was not genotype-dependent. Plasma homocysteine (Fig 1) and BHMT activity (Fig 2) both showed a strong negative correlation with liver betaine.

The effects of betaine treatment were similar in both genotype groups: in wild-type mice plasma homocysteine was 37.5% [18.2] lower and in heterozygous mice, 48.8% [7.0] lower compared with mice on the control diet (Table 1). Liver betaine was much higher in betaine-treated animals compared with mice on the control diet. Other choline metabolites did not show consistent changes. BHMT activity in liver was similar in the control and treated groups.

DMSA supplementation had similar effects to those of betaine. Plasma homocysteine was significantly lower in both genotype groups compared with mice on control diet: by 55.2% [4.5] in wild-type mice and by 50.4% [4.2] in heterozygous mice; homocysteine thus remained 1.6-fold elevated in DMSA-treated heterozygous mice compared with DMSA-treated wild-type mice. Liver betaine was much higher in both genotypes. Other choline metabolites and BHMT activity in liver were similar to those of the control group.

Study 2. At the maximum level of betaine supplementation in this study, homocysteine was decreased to 25.2% [2.3] of the control level, while methionine and serine were 49.2% [5.3] and 41.0% [6.0] elevated, respectively (Fig 3). BHMT activity

Table 1. Plasma Total Homocysteine and Total Cysteine (μmol/L), Liver Choline Metabolite Concentrations (μmol/kg wet weight), and Specific Activity of BHMT in Liver (U/mg protein) of Female *Cbs*-Mice Stratified for Genotype and Diet

	Control		Betaine		DMSA	
	Wild-Type	Heterozygous	Wild-Type	Heterozygous	Wild-Type	Heterozygous
t.Homocysteine	15.4	23.0*	9.6†	11.8†	6.9†	11.4†
	0.4	2.8	2.8	1.6	0.7	1.0
t.Cysteine	149.2	154.9	168.1	167.8	158.8	157.2
	7.7	10.5	7.1	11.3	4.8	6.74
Betaine	183.5	142.8	1,031.9†	672.8†	1287.9†	1,555.9†
	15.8	22.1	373.4	154.9	250.3	697.7
Choline	119.4	86.3	80.7†	128.5†	96.5	116.9†
	13.7	5.7	7.7	7.4	13.1	4.9
PCho	239.9	172.6	224.7	697.3	235.8	276.6
	27.5	26.8	44.1	409.3	62.3	49.6
GPC	538.5	517.8	499.7	364.7	457.9	411.2
	39.6	19.2	27.9	92.8	26.6	44.4
PtdCho	17,087	17,033	16,568	17,123	17,449	17,619
	157	302	297	504	694	359
BHMT	135.3	126.2	149.5	118.7	120.9	100.0
	8.4	12.9	14.5	12.2	17.0	10.1

NOTE. Results are presented as mean and SEM of 4 mice per group.

Abbreviations: PCho, phosphocholine; GPC, glycerophosphocholine; PtdCho, phosphatidylcholine.

*$P < .05$ compared with wild-type genotype, †$P < .05$ compared with control diet of same genotype.

gradually decreased to 76.5% [3.1], whereas liver betaine concentration increased up to 150-fold (45 [3] μmol/kg wet weight (ww) with no betaine, compared with 6,645 [1,770] μmol/kg ww at the maximum level, $P < .05$). Other choline metabolites did not show consistent changes. Plasma homocysteine and liver betaine showed a highly significant negative correlation in this study, as well (Fig 4). Plasma methionine and liver betaine showed a significant positive correlation (Fig 4).

DISCUSSION

We used mice with a heterozygous knockout of the *Cbs* gene as a model of disrupted homocysteine metabolism. These mice showed frank, although moderate, hyperhomocysteinemia compared with wild-type littermates, as reported in the original description of this animal model.[5] Mean levels of tissue metabolite concentrations were not particularly informative due to high variability. However, the observations in individual mice were highly informative and demonstrated a significant negative correlation between plasma homocysteine and liver betaine, as well as between BHMT activity and liver betaine. These results indicate an increased consumption of betaine in hyperhomocysteinemia due to enhanced flux through the betaine-dependent remethylation pathway. In another mouse model of hyperhomocysteinemia, due to a disruption of the *Mthfr* gene, we also found a clear correlation between the extent of hyperhomocysteinemia and the degree of betaine depletion. However, in the *Mthfr* mice, betaine depletion was also associated with choline deficiency as indicated by low concentrations of liver PCho, the intracellular storage form of choline.[13] Addition of betaine to the diet in *Cbs* mice led to increased liver betaine and lower plasma homocysteine concentrations without affecting the concentrations of other choline compounds or specific BHMT activity. Betaine supple-

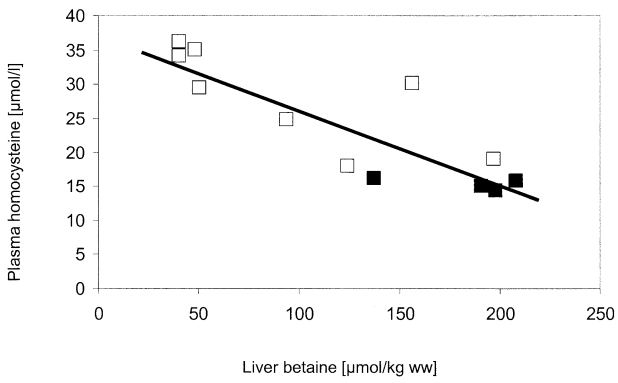

Fig 1. Linear correlation between plasma homocysteine and liver betaine concentrations in *Cbs* mice on the control diet. Wild-type (■), heterozygous (□); $r = -.8558$, $P = .0004$, n = 12.

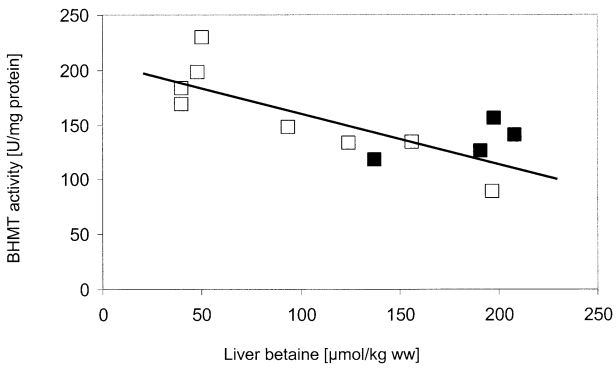

Fig 2. Linear correlation between specific BHMT activity and liver betaine concentrations in wild-type (■) and heterozygous (□) *Cbs* mice on the control diet; $r = -.7494$, $P = .0050$, n = 12.

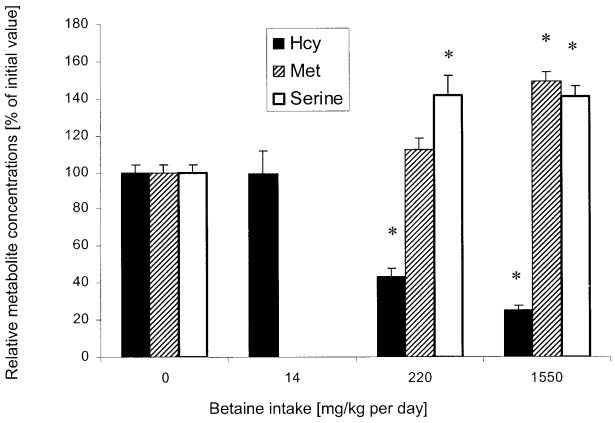

Fig 3. Mean [SEM] relative concentrations of homocysteine, methionine, and serine in plasma in heterozygous male *Cbs* mice on increasing betaine supplements. Values for methionine and serine were not obtained at the lowest dose of betaine (14 mg/kg), but it is unlikely that they would differ from the 0 betaine value at this low dose, particularly because plasma homocysteine was unchanged. *$P < .05$ *v* 0, n = 4 per group.

mentation decreased homocysteine levels in wild-type and heterozygous mice, demonstrating the sensitivity of rodents towards betaine (or choline) intake, independent of the *Cbs* genotype. Betaine intake at approximately 350 mg/kg BW per day, in study 1, lowered homocysteine levels in *Cbs* heterozygotes to values that were below those of wild-type mice on the control diet. However, despite the greatly elevated betaine concentrations in liver, *Cbs* heterozygotes on the betaine supplement maintained homocysteine concentrations that were still clearly above those of their wild-type littermates on the same diet. This indicates that betaine status is a strong determinant of plasma homocysteine, but that repletion of hepatic betaine stores cannot completely compensate for the effects of heterozygous CBS deficiency on plasma homocysteine. The same was true in our study of mice with MTHFR deficiency.[13]

Using an alternate methyl donor, DMSA, we were able to mimic the effects of betaine. DMSA is also exclusively metabolized by BHMT, but has some theoretical advantages, because its demethylated product has a higher K_i towards BHMT than the demethylated product of betaine, dimethylglycine, and may therefore be less likely to cause feedback inhibition.[12] Interestingly, the homocysteine-lowering effect of DMSA was very similar to that of betaine in study 1, and the DMSA supplement led to greatly increased liver betaine concentrations, without significantly affecting other choline metabolites or BHMT activity. Augmentation of betaine stores could be due to decreased catabolism or increased synthesis of betaine. Betaine can be generated via 3-fold methylation of phosphatidylethanolamine (PtdEth) to PtdCho, which is converted to choline, the precursor of betaine. DMSA catabolism yields 1 SAM molecule that could be used to methylate PtdEth. Liver PtdCho concentration remained unchanged in our study, but its large cellular pool could have masked a small increase. Although we cannot exclude an augmentation of methylation-dependent synthesis of PtdCho and hence betaine due to the DMSA supplement, the increase in liver betaine is more likely due to a betaine-sparing effect of DMSA, because DMSA is preferentially bound to the catalytic domain of BHMT compared with betaine.[12]

To determine whether an increase in betaine intake would lead to further homocysteine-lowering, we exposed heterozygous *Cbs* mice to betaine doses up to 1,550 mg/kg BW per day. We observed an additional decrease of plasma homocysteine to 25% of the initial level to an extent that corresponds very well to the described effects in humans.[3] In heterozygous *Mthfr* mice, we had observed a maximum homocysteine-lowering to 40% of the initial level. In parallel with the dose-dependent decrease in homocysteine levels at betaine intakes from 0 to 220 and 1,550 mg/kg BW per day, we observed a dose-dependent increase in plasma methionine. An examination of the correlation between plasma homocysteine or plasma methionine with liver betaine (Fig 4) reveals that there is only a small, if any, benefit in terms of homocysteine-lowering beyond a liver betaine concentration of 240 μmol/kg ww, which was largely achieved with a supplement of 220 mg/kg BW per day. In contrast, in *Mthfr*-deficient mice, we found no additional effect above a dose of 53 mg/kg BW.[13] It appears, therefore, that betaine may be more effectively used in CBS deficiency than in remethylation defects, such as MTHFR deficiency. Another possible explanation is that betaine stores were more depleted in *Cbs* mice than in *Mthfr* mice before the study period. Because both homocystinuria mouse models had been backcrossed to different genetic backgrounds, direct comparison of absolute metabolite concentrations has to be performed with caution. However, plasma concentrations of homocysteine and cysteine and hepatic BHMT activity were not significantly different between wild-type mice of the *Mthfr* strain (on a BALB/c background) and wild-type mice of the *Cbs* strain (on a C57BL/6 background) on the control diet, whereas liver concentrations of Cho, PCho, and PtdCho were significantly lower, and betaine concentrations showed a trend toward lower values in *Cbs* mice compared with *Mthfr* mice (not shown).

In heterozygous CBS deficiency in mice, plasma serine in-

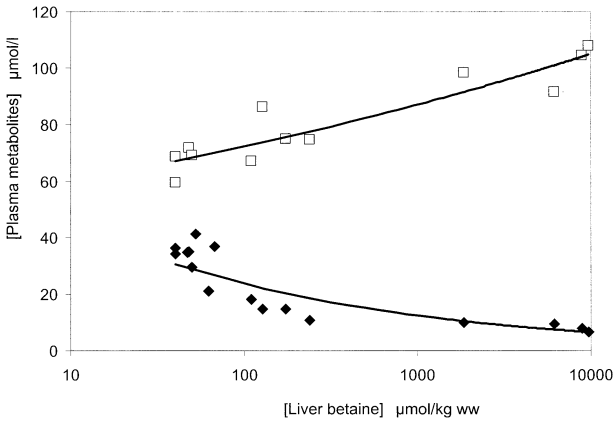

Fig 4. Nonlinear correlation between plasma homocysteine and liver betaine (■, $r = -.5441$, $P = .0351$) and between plasma methionine and liver betaine concentrations (□, $r = .7273$, $P = .0159$) in 16 male heterozygous *Cbs* mice on various betaine supplements.

creased with betaine supplementation (Fig 3). Serine plasma concentrations were shown to be depressed in 16 humans with CBS deficiency and were normalized with betaine supplementation, whereas serine concentrations in patients with remethylation defects were normal.[14] There may be an increased requirement for serine in CBS deficiency due to its role as a 1-carbon donor for folate-dependent remethylation. These results are consistent with earlier work in humans, in which it was observed that dietary choline supplementation decreased the de novo production of 1-carbon units used to support SAM-dependent transmethylation reactions.[15,16]

The dramatic increase of liver betaine, following betaine supplementation, demonstrated that betaine was well absorbed and stored. However, other choline compounds remained unchanged, and specific BHMT activity decreased slightly. Liver betaine concentrations with the highest dose (mean = 6.6 mmol/kg ww with 1,550 mg betaine per kg BW) were probably beyond substrate saturation of BHMT, whose K_m was determined to be 2.2 mmol/L[17] or even less.[18] In this study, we did not observe an increase of BHMT activity in wild-type or heterozygous *Cbs* mice with increasing betaine doses. In our previous study, however, BHMT activity was induced in heterozygous *Mthfr* mice receiving betaine supplementation. These data are consistent with previous work on methionine and methyl donor intake on hepatic BHMT expression in rats.[12] In these studies, BHMT induction was observed to require some degree of methionine deficiency, along with betaine or DMSA supplementation. Induction was found to be directly correlated to the level of dietary methyl donor supplementation and the degree of concomitant methionine deficiency. The exact molecular signals that mediate this response are not known, although we have shown that BHMT induction may be due to low intracellular SAM levels, because we observed that SAM inhibits BHMT transcription in HepG2 cells.[19] Because dietary methionine was not restricted in our dose-response study in heterozygous *Cbs* mice, the hepatic concentrations of methionine and SAM would be expected to be either normal or even slightly elevated. Therefore, the combination of the dietary conditions and genetically impaired CBS activity used in this study would not be expected to result in the induction of BHMT activity in wild-type mice, but rather in a slight repression, as observed. In contrast, *Mthfr* mice have reduced liver homocysteine remethylation rates and low methionine and SAM levels.[21] These conditions are favorable for the induction of BHMT expression following betaine supplementation, as observed.[13] As an alternative explanation for any effects intracellular SAM may have had on BHMT expression in this study, the small decrease in BHMT activity observed at the highest betaine dose in *Cbs* mice could have been caused by increasing DMG levels.[21]

In conclusion, increased plasma homocysteine can be viewed as an indicator of limited betaine supply for remethylation, a condition that readily occurs in mice, especially when homocysteine metabolism is impaired. It is intriguing to assume that the same could be true in humans, particularly because we recently demonstrated a negative correlation between plasma homocysteine and plasma betaine in human subjects.[13] Although the *Cbs* mouse is a suitable model to study the pharmacologic action of betaine in moderate hyperhomocysteinemia, human studies are warranted to clarify the role of betaine in hyperhomocysteinemic conditions.

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Betaine rescue of an animal model with methylenetetrahydrofolate reductase deficiency

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MTHFR (methylenetetrahydrofolate reductase) catalyses the synthesis of 5-methyltetrahydrofolate, the folate derivative utilized in homocysteine remethylation to methionine. A severe deficiency of MTHFR results in hyperhomocysteinaemia and homocystinuria. Betaine supplementation has proven effective in ameliorating the biochemical abnormalities and the clinical course in patients with this deficiency. Mice with a complete knockout of MTHFR serve as a good animal model for homocystinuria; early postnatal death of these mice is common, as with some neonates with low residual MTHFR activity. We attempted to rescue *Mthfr*−/− mice from postnatal death by betaine supplementation to their mothers throughout pregnancy and lactation. Betaine decreased the mortality of *Mthfr*−/− mice from 83 % to 26 % and significantly improved somatic development from postnatal day 1, compared with *Mthfr*−/− mice from unsupplemented dams. Biochemical evaluations demonstrated higher availability of betaine in suckling pups, decreased accumulation of homocysteine, and decreased flux through the trans-sulphuration pathway in liver and brain of *Mthfr*−/− pups from betaine-supplemented dams. We observed disturbances in proliferation and differentiation in the cerebellum and hippocampus in the knockout mice; these changes were ameliorated by betaine supplementation. The dramatic effects of betaine on survival and growth, and the partial reversibility of the biochemical and developmental anomalies in the brains of MTHFR-deficient mice, emphasize an important role for choline and betaine depletion in the pathogenesis of homocystinuria due to MTHFR deficiency.

Key words: betaine supplementation, brain development, hyperhomocysteinaemia, methylenetetrahydrofolate reductase (MTHFR), remethylation.

INTRODUCTION

Homocysteine is a sulphur amino acid which serves as the carbon backbone in methyl group metabolism (via the remethylation pathway) and as a precursor for the synthesis of cysteine, taurine and glutathione (via the trans-sulphuration pathway). The enzyme MTHFR (5,10-methylenetetrahydrofolate reductase; EC 1.5.1.20) catalyses the irreversible reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, which functions as a methyl donor for the remethylation of homocysteine to methionine by 5-methyltetrahydrofolate–homocysteine *S*-methyltransferase (methionine synthase; EC 2.1.1.13). The flux through MTHFR determines the amount of newly formed one-carbon units that are directed towards nucleotide synthesis or towards the *de novo* synthesis of transferable methyl groups, following the activation of methionine to SAM (*S*-adenosylmethionine). Other sources of transferable methyl groups are the dietary preformed donors – methionine, betaine and choline [1]. Choline can be metabolized to betaine, which serves as an alternative methyl donor for the remethylation of homocysteine to methionine, by the enzyme BHMT (betaine–homocysteine methyltransferase; EC 2.1.1.5) [2].

Bi-allelic defects in the human *MTHFR* gene cause severe MTHFR deficiency, the most common inherited disorder of folate metabolism, with residual enzyme activities between 0 % and 20 % of those of controls in cultured fibroblasts [3]. This enzymic defect is one of the contributors to the inborn error of metabolism, homocystinuria. Although homocystinuria is a relatively rare disorder, the features seen in these patients with marked impairment of enzyme function can provide some insight into the clinical consequences of the frequently encountered milder MTHFR deficiency due to homozygosity for the C → T substitution at cDNA position 677 [4,5]. The phenotype of severe MTHFR deficiency has two major features: (1) various neurological and psychiatric symptoms caused by encephalopathy with neuronal loss and demyelination; and (2) recurrent thrombotic or thromboembolic events. Other symptoms relate to connective tissue anomalies and liver pathology [6]. Newborns with MTHFR deficiency are homocystinuric but have non-specific symptoms or are asymptomatic. The diagnosis of MTHFR deficiency is usually made in infants or older children, after the onset of symptoms such as psychomotor developmental delay [6].

Betaine supplementation has proven effective in ameliorating the biochemical abnormalities and the clinical course in homocystinuria due to severe MTHFR deficiency. It lowers the elevated plasma homocysteine levels associated with this disease and increases plasma methionine concentrations [7,8]. Betaine supplementation is believed to directly enhance homocysteine remethylation and, consequently, to increase the availability of methionine for protein synthesis and transmethylation. However, high-dose betaine treatment does not normalize homocysteine metabolism in homocystinuric patients, as indicated by plasma homocysteine levels that remain 5–10-fold elevated [7].

We have created a murine model for severe and mild MTHFR deficiency by generating mice with homozygous and heterozygous knockout of *Mthfr* alleles [9]. The *Mthfr*−/− and

Abbreviations used: BHMT, betaine–homocysteine methyltransferase; DMG, dimethylglycine; E18, embryonic day 18 (etc.); MTHFR, methylenetetrahydrofolate reductase; P0, postnatal day 0 (etc.); SAH, *S*-adenosylhomocysteine; SAM, *S*-adenosylmethionine.

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Mthfr+/− mice are good animal models for human homocystinuria and moderate hyperhomocysteinaemia respectively, based on residual enzyme activities and degree of hyperhomocysteinaemia. We recently reported the effects of betaine supplementation on homocysteine and choline metabolism in adult *Mthfr*+/+ (wild-type) mice and in their littermates with mild (*Mthfr*+/−) or severe (*Mthfr*−/−) deficiency. We demonstrated that MTHFR deficiency in mice is associated with a higher demand for betaine-dependent remethylation, and that betaine can lower plasma homocysteine levels in mice with all three *Mthfr* genotypes. However, remethylation through BHMT could compensate only partly for MTHFR deficiency, even with high-dose betaine treatment [10].

Homocystinuria due to severe MTHFR deficiency in humans can cause early lethality in the first year of life. There is a good correlation between the degree of residual enzyme activity and the clinical course in these patients [3]. It is not surprising, therefore, that there is considerable early postnatal loss of *Mthfr*−/− mice. In this paper, we report the results of our attempts to rescue nullizygous *Mthfr* mice from early postnatal death by betaine supplementation of their mothers, and describe the impact of this treatment on homocysteine metabolism and brain development of the pups.

EXPERIMENTAL

Animal husbandry and experiments

All mice were produced in our own breeding facility and housed in adequate shoebox cages with free access to food and water. The animal experiments were 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, heterozygous for a disruption of the *Mthfr* gene (*Mthfr*+/−) and from F6 or F7 generations of backcrosses to the BALB/cAnNCrlBR strain, were used for breeding. Female *Mthfr*+/− mice on regular rodent chow (Purina laboratory rodent diet 5001; Purina Mills) were supplemented with anhydrous betaine (Sigma) at 2 % (w/v) in the drinking water before mating them to *Mthfr*+/− males. Water intake was monitored. Betaine supplementation was continued until weaning of the pups at 3 weeks of age. Untreated matings served as controls. Cages were checked daily. The day of birth was designated as P0 (postnatal day 0). Newborn mice were examined daily for viability and body weight. The body weights of homozygous mutant (*Mthfr*−/−) mice were compared with those of their wild-type (*Mthfr*+/+) and heterozygous (*Mthfr*+/−) littermates. A few mice who died before P2 were excluded from weight and mortality calculations. This mortality, due to non-specific causes, was gender- and genotype-independent.

A total of 15 *Mthfr*+/+ and *Mthfr*+/− mice of betaine-supplemented mothers were supplemented with 2 % betaine in the drinking water from weaning until 7–9 weeks of age for measurements of betaine and DMG (dimethylglycine) in plasma and tissues. Results were compared with those from 20 untreated mice of the same age whose mothers had not been supplemented with betaine.

At different prenatal [E18 (embryonic day 18)] and postnatal (P6, P9, P12 and adult) time points, mice were killed. The blood of older mice was collected by heart puncture, anticoagulated with EDTA (MicrovetteR 500; Sarstedt) and immediately placed on ice. Plasma was separated by centrifugation at 10 000 ***g*** for 5 min and frozen immediately at − 70 °C until analysis. Livers and brains were dissected and weighed. They were then frozen on solid CO_2 and stored in aliquots at − 70 °C, or fixed by immersion in 10 % (v/v) formalin and processed for 5 μm paraffin sections and haematoxylin/eosin staining.

Genotyping

Mice were genotyped for the *Mthfr* disruption by PCR of genomic DNA extracted from tail biopsies, as described previously [9].

Metabolites

Plasma total homocysteine concentrations were measured after reduction by a HPLC method, as described previously [11]. Betaine and DMG concentrations in plasma and tissue extracts were analysed by HPLC as reported in [12]. Betaine and DMG were extracted from tissues by adding 1 ml of 100 mM KH_2PO_4 (pH 4.5) to 100–150 mg of freeze-dried tissues in 2 ml Eppendorf tubes. After addition of an equal volume of clean glass beads, the tubes were mixed vigourously to homogenize the contents. The mixture was incubated for 10 min with intermittent mixing and vortexing. At the end of the incubation time, the tubes were centrifuged (5000 ***g***, 10 min) and the supernatant was collected. The extraction procedure was repeated twice and the combined supernatants were frozen until analyses.

Tissue aminothiols were measured as reported [13], and tissue SAM and SAH (*S*-adenosylhomocysteine) concentrations were measured according to the method described by Melnyk et al. [14]. Global DNA methylation was assessed by a cytosine-extension assay as described by Pogribny et al. [15]. Adenosine in tissues was measured according to Nithipatikom et al. [16] after adaptation for electrochemical detection.

BHMT activity

BHMT activity in crude liver extracts was analysed as reported previously [17].

Statistical analyses

Standard software was used for all analyses. Arithmetic means ± S.E.M. are provided. Genotype and gender distributions were compared using the χ^2 test. Weights or metabolite levels were compared between groups using one-way ANOVA and, if a significant difference was found, single parameters were compared with the unpaired, two-sided *t* test. If values were not normally distributed, the Kruskal–Wallis test and the non-parametric Wilcoxon test were used as appropriate. Linear correlations between two parameters were calculated and expressed using Spearman's coefficient. Throughout all analyses, a *P* value of < 0.05 was considered significant.

RESULTS

Betaine supplementation increases the survival rate of *Mthfr*−/− mice

A total of 314 mice in 50 litters (mean litter size 6.28 ± 0.31) were born after mating of untreated *Mthfr*+/− mice. *Mthfr* genotype frequencies were 25.1 % *Mthfr*+/+, 48.0 % *Mthfr*+/− and 26.9 % *Mthfr*−/−. This genotype distribution was not significantly different from expected values using the χ^2 test. Gender proportions showed a small but significant preponderance of female (56.4 %) compared with male (43.6 %) mice for the *Mthfr*+/+ and *Mthfr*+/− genotypes. The genders of the 45 *Mthfr*−/− mice were evenly distributed (23 female, 22 male;

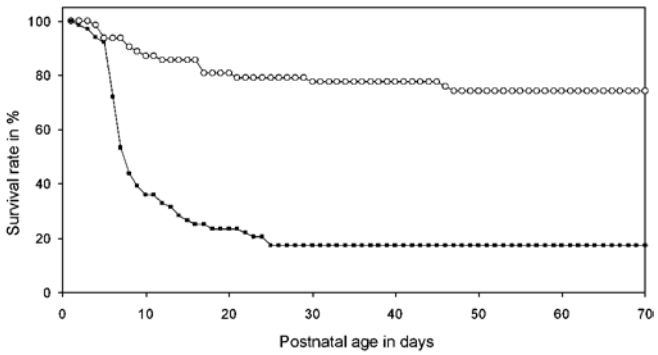

Figure 1 Survival of mice homozygous for a disruption of the *Mthfr* gene (−/−)

Data are derived from 64 *Mthfr*−/− mice without (■) and 62 *Mthfr*−/− mice with (○) a betaine supplement to their mothers.

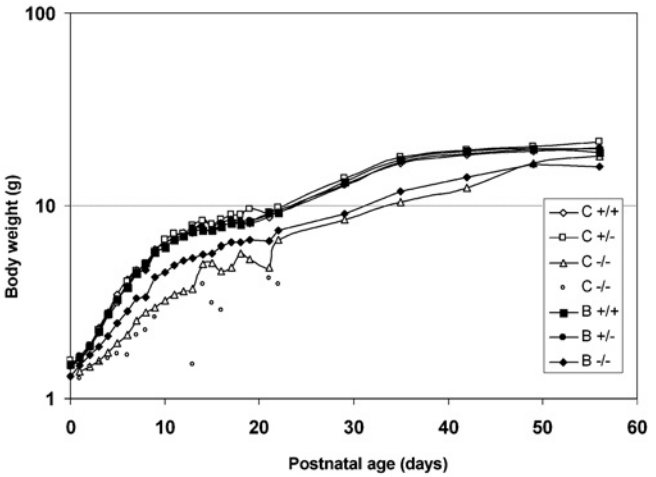

Figure 2 Postnatal weight development of mice wild-type (+/+), heterozygous (+/−) or homozygous (−/−) for a disruption of the *Mthfr* gene and stratified for treatment with (B) or without (C) a betaine supplement

Body weights are depicted as arithmetric means. The numbers of *Mthfr*−/− pups evaluated were 62 from mothers with a betaine supplement and 55 from mothers without a supplement. Pups from the latter group of untreated mothers were separated into two groups: survivors ($n = 12$; Δ) and non-survivors ($n = 43$; ○). The number of the latter group decreased with age due to their high postnatal mortality.

with five surviving females and six surviving males). One-third of the *Mthfr*−/− mice could not be classified by gender because of early death and/or incomplete recovery of their bodies. Of the homozygous mutants, 83 % died, at an average age of 9.1 ± 0.7 days (median 7.0 days; $n = 53$). Only 11 out of 64 (17 %) *Mthfr*−/− mice survived (Figure 1).

Betaine supplementation in the drinking water was tolerated without apparent side effects. A mean daily betaine intake in water of approx. 3 g/kg body weight was observed during mating and pregnancy, which increased to a variable extent during lactation. Matings of betaine-treated heterozygotes yielded a total of 249 mice in 38 litters. The litter size (mean 6.38 ± 0.44) was not different from that of untreated matings. Genotype frequencies were 27.3 % *Mthfr*+/+, 44.9 % *Mthfr*+/− and 27.8 % *Mthfr*−/−. Gender (51.1 % female, 48.9 % male) and genotype distributions were not significantly different from expected values. The mortality of homozygous mutant pups from betaine-treated mothers decreased significantly compared with that of pups from untreated mothers, to 26 % (average day of death 16.3 ± 3.4; median 11.0 days; $n = 16$). Of 62 *Mthfr*−/− mice, 46 (74 %) survived. Withdrawal of betaine supplementation after weaning of homozygous mutant pups, at the age of 3 weeks, had no adverse effects on survival (Figure 1).

Betaine supplementation improves the somatic development of *Mthfr*−/− mice

Body weight

Body weights were followed for 480 pups, 253 from untreated dams and 227 from betaine-supplemented dams. These included 55 untreated and 62 betaine-supplemented *Mthfr*−/− mice (Figure 2).

The weight development of *Mthfr*+/− and *Mthfr*+/+ pups of untreated mothers was not different. The mean body weight of untreated *Mthfr*−/− mice at P0 was 87.9 % of that of heterozygous and wild-type littermates, corresponding to 1.00 ± 0.34 S.D.s. Starting from P1, the body weights of untreated *Mthfr*−/− mice were significantly decreased. The trough of the weight development curve occurred at P9–P13, together with a peak of mortality at this age.

Starting from P5, the body weights of untreated non-surviving *Mthfr*−/− mice were significantly lower than those of surviving nullizygotes. Surviving nullizygotes regained weight until weaning. Their relative weight decreased during adolescence, then recovered, with the adult weight being 0.97 ± 1.12 S.D.s below that of their heterozygous and wild-type littermates.

In the betaine-supplemented group, the weight development of *Mthfr*+/− and *Mthfr*+/+ offspring was not different. Fur appearance, which is usually delayed by about 5 days in *Mthfr*−/− mice [8], was delayed by only approx. 2 days in *Mthfr*−/− mice from betaine-supplemented mothers. The body weights of *Mthfr*−/− pups at P0 were 91.4 ± 3.1 % (-1.02 ± 0.34 S.D.s) of those of age- and sex-matched *Mthfr*+/− and *Mthfr*+/+ littermates. From P1, their weights were significantly decreased compared with those of their heterozygous and wild-type littermates. Weight development showed decelerations during the first 2 weeks of life and during the first 2 weeks after weaning. The adult weight of *Mthfr*−/− mice was 1.18 ± 0.23 S.D.s below that of their heterozygous and wild-type littermates.

The body weight development of *Mthfr*+/− and *Mthfr*+/+ offspring was not distinguishable between litters of untreated and betaine-supplemented mothers. The mean body weights of *Mthfr*−/− mice were significantly higher when their mothers had been treated, compared with those from untreated mothers, starting from P1. Although the weights of untreated *Mthfr*−/− mice remained lower than those of betaine-treated mice throughout somatic development, statistical significance was not achieved after P16, mostly due to high inter-individual variation. Adult body weights did not differ between treatment groups.

Brain weight and morphology

Brain weights were evaluated in a subgroup of 6-day-old pups (Figure 3). At this age, *Mthfr*+/− mice in the untreated group had slightly but significantly lower body and brain weights than *Mthfr*+/+ pups. Untreated *Mthfr*−/− pups with severely decreased body weights also showed a severe reduction in brain weight, with a significant increase in the brain/body weight ratio compared with *Mthfr*+/+ mice (0.076 ± 0.008 and 0.054 ± 0.003 respectively, $n = 10$, $P < 0.01$); this observation indicates that, at P6, somatic dystrophy was more pronounced than the disturbance in brain development.

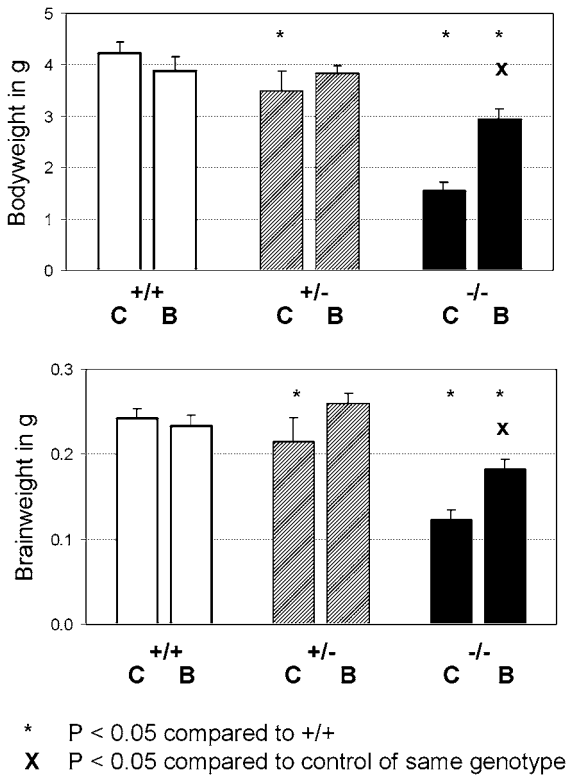

Figure 3 Influence of a betaine supplement on body weights and brain weights at P6 of wild-type (+/+) mice and their littermates with a heterozygous (+/−) or homozygous (−/−) disruption of the *Mthfr* gene

Data are means and S.E.M. of eight (body weight) or five (brain weight) mice per genotype and treatment group. C, pups of control untreated mothers; B, pups of betaine-supplemented mothers.

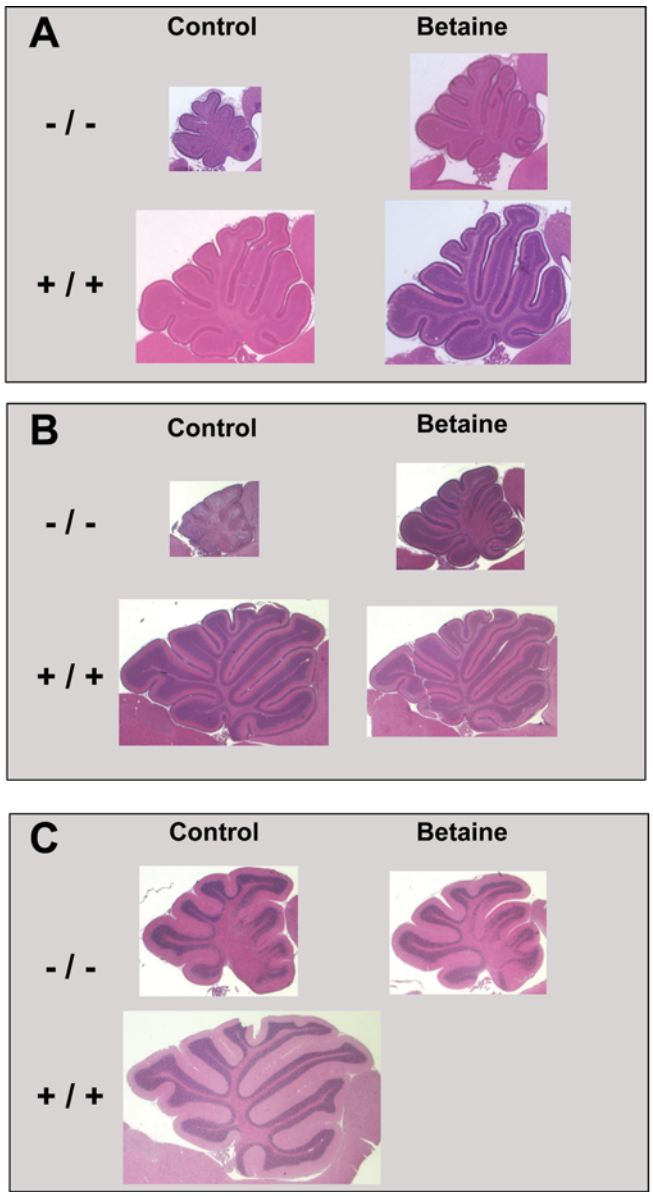

Figure 4 Impact of betaine supplementation to mothers on cerebellar development of their *Mthfr*−/− pups

Depicted are parasagittal cerebellar sections of mice at age P9 (**A**), P12 (**B**) and adult (**C**). A haematoxylin/eosin stain was used, and size proportions are conserved for each age group. The slides are oriented with rostral to the right.

The brain and body weights of *Mthfr*+/+ and *Mthfr*+/− pups from betaine-supplemented dams were not different from those of the corresponding untreated pups. However, *Mthfr*−/− pups with the betaine supplement had significantly higher body and brain weights than untreated *Mthfr*−/− pups, resulting in a significantly lower brain/body weight ratio (0.057 ± 0.001), a value that was comparable with that of wild-type mice. However, the body and brain weights of treated *Mthfr*−/− animals each still remained significantly below those of wild-type mice. When a group of 18 adult mice (three females and three males per genotype) from litters rescued by betaine supplemention of their mothers were evaluated at age P200, we found that the brain weights of the *Mthfr*−/− mice (395 ± 13 mg, $n = 6$) were still significantly lower than those of heterozygous (477 ± 8 mg, $n = 6$) or wild-type (487 ± 2 mg, $n = 6$) littermates, whereas the body weights of these 18 mice were not significantly different.

Analysis of the gross anatomy of the brains of *Mthfr*−/− mice revealed proportionately smaller cerebella (Figure 4). Pups from betaine-supplemented dams had a much larger cerebellar size compared with untreated mice. Histological examination showed that cerebellar development was disturbed in *Mthfr*−/− mice. Foliation and differentiation of the cerebellar cortex in *Mthfr*−/− mice at P9 and P12 appeared immature compared with *Mthfr*+/+ mice (Figures 4A and 4B). The internal granule cell layer was not well developed and a single Purkinje cell layer was not clearly distinguishable. These defects were observed in both anterior and posterior lobules, but were more pronounced in anterior segments. In adult *Mthfr*−/− mice, the defects were restricted to the anterior region of the cerebellum (Figure 4C), as observed in our previous report [9].

We also identified structural abnormalities in the hippocampus of *Mthfr*−/− mice (Figure 5). In addition to hypotrophy of the hippocampus with dilated ventricular spaces, the neuroepithelial layers of the dentate gyrus were only loosely assembled in comparison with wild-type or heterozygous animals. Both cerebellar and hippocampal histological abnormalities were ameliorated, but not abolished, by the betaine supplement. Maternal betaine supplementation improved the foliation defect in the anterior lobules of *Mthfr*−/− cerebella at P9 and P12, and led to a reduced number of affected lobules in the adult *Mthfr*−/− cerebellum (Figure 4). Cerebellar changes persisted until adulthood (Figure 4C); the hippocampus was not examined in adult animals.

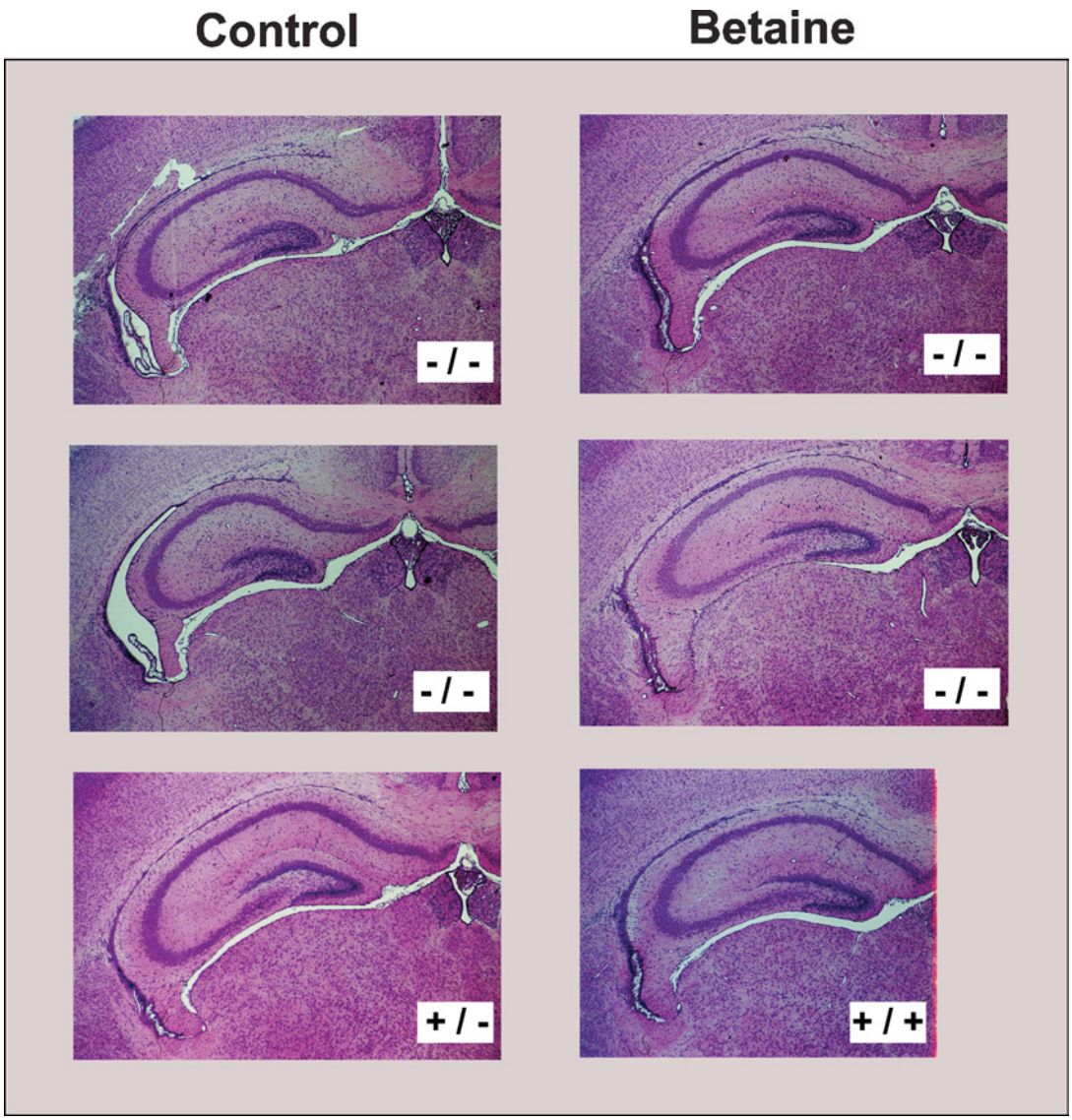

Figure 5 Impact of betaine supplementation to mothers on hippocampal development of their *Mthfr*−/− pups

Depicted are left coronar forebrain sections from mice at age P9. A haematoxylin/eosin stain was used; original magnification × 4.

Liver morphology

Mthfr−/− mice are prone to develop fatty infiltration of the liver, especially when the choline supply is limited [9]. The livers of nine untreated *Mthfr*−/− mice at age P6–P9 were evaluated by histology; six of the livers showed severe fatty infiltration, while three livers did not. The livers of eight *Mthfr*−/− pups from betaine-supplemented mothers were evaluated at age P6–P12; five of the livers showed fatty infiltration (results not shown). There was no correlation between the degree of general dystrophy of the mice, measured as body weight relative to that of *Mthfr*+/− or *Mthfr*+/+ littermates, and the extent of fatty infiltration.

Biochemical consequences of betaine supplementation

Betaine metabolism in adult mice

The effects of a 2 % (w/v) betaine supplement on betaine metabolism in adult *Mthfr*+/+ and +/− mice are shown in Table 1. Compared with *Mthfr*+/+ mice, untreated *Mthfr*+/− mice had lower betaine concentrations in plasma, liver and brain. Betaine concentrations in plasma and tissues were increased with a 2 % betaine supplement in both groups of mice, the only exception being the brains of *Mthfr*+/+ mice.

The concentrations of the demethylated metabolite of betaine, DMG, were variable in different organs and did not show consistent changes by genotype. Betaine supplementation increased DMG levels in plasma and liver, and decreased them in brain. The specific activity of the liver betaine-metabolizing enzyme, BHMT, increased significantly with betaine supplementation in both genotype groups.

Betaine metabolism in lactating dams and suckling pups

Plasma betaine concentrations in betaine-supplemented lactating dams ranged from 190 to 1835 μmol/l ($n = 3$), compared with 34–124 μmol/l ($n = 3$) in untreated dams. The corresponding plasma DMG concentrations in dams on betaine supplementation ranged

Table 1 Concentrations of betaine and DMG in plasma (μmol/l) and tissues (nmol/g wet weight), and specific activity of the betaine-metabolizing enzyme BHMT, in adult wild-type mice and heterozygous *Mthfr* mice with and without a betaine supplement of 2% (w/v) in their drinking water from birth

The dietary groups of the two genotypes comprised littermates of equally mixed genders. Results are provided as means ± S.E.M.; *$P < 0.05$ for difference between dietary groups of same genotype; †$P < 0.05$ compared with wild-type genotype in the same dietary group.

		Wild type		Heterozygous	
	Tissue	Control ($n = 10$–14)	Betaine ($n = 6$–8)	Control ($n = 6$–8)	Betaine ($n = 7$)
Betaine	Plasma	101.7 ± 15.1	556.9 ± 190.6*	56.4 ± 4.2†	229.7 ± 93.8*
	Liver	2139 ± 257	9420 ± 3275*	727 ± 43†	3993 ± 2227
	Brain	61 ± 10	56 ± 19	22 ± 4†	134 ± 30*†
	Kidney	1853 ± 103	4716 ± 679*	1645 ± 213	3274 ± 647*†
DMG	Plasma	13.5 ± 2.0	60.7 ± 13.4*	8.2 ± 0.9†	38.3 ± 12.7*
	Liver	88 ± 13	250 ± 67*	65 ± 15	160 ± 62
	Brain	15 ± 5	2 ± 1*	8 ± 3	3 ± 1
	Kidney	176 ± 81	81 ± 29	23 ± 10	71 ± 30
BHMT (units/mg of protein)	Liver	149 ± 15	244 ± 29*	184 ± 23	287 ± 26*

Table 2 Liver concentrations of total thiols and of SAH, SAM and adenosine in MTHFR-deficient pups at P6 stratified for genotype and maternal diet

Controls received standard rodent chow, whereas the betaine group received an additional betaine supplement of 2% (w/v) in the drinking water. The dietary groups of the three genotypes comprised littermates of mixed genders. Results are means ± S.E.M. for eight individuals; *$P < 0.05$ for difference between dietary groups of same genotype; †$P < 0.05$ compared with wild-type genotype in the same dietary group; symbols in parentheses indicate borderline significance ($P = 0.06$–0.09).

	Wild type		Heterozygous		Nullizygous	
Metabolite	Control	Betaine	Control	Betaine	Control	Betaine
Homocysteine (nmol/mg)	0.406 ± 0.030	0.387 ± 0.022	0.559 ± 0.024†	0.512 ± 0.031†	1.907 ± 0.112†	1.255 ± 0.076*†
Methionine (nmol/mg)	24.0 ± 1.4	25.0 ± 0.9	16.4 ± 0.9†	20.8 ± 0.8*†	12.4 ± 0.7†	15.9 ± 0.7*†
SAH (pmol/mg)	89 ± 4	121 ± 4*	124 ± 12†	101 ± 3$^{(*)}$†	290 ± 28†	144 ± 13*
SAM (pmol/mg)	467 ± 38	551 ± 24$^{(*)}$	385 ± 27(†)	473 ± 28*(†)	391 ± 39	440 ± 28†
Cysteine (nmol/mg)	45.8 ± 1.3	43.9 ± 1.0	49.5 ± 1.7	52.4 ± 1.4†	66.5 ± 2.2†	64.4 ± 1.5†
Cystathionine (nmol/mg)	1.40 ± 0.10	1.23 ± 0.06	2.38 ± 0.09†	2.10 ± 0.13†	4.57 ± 0.25†	2.77 ± 0.27*†
Glutathione (nmol/mg)	54.8 ± 1.5	52.8 ± 1.2	49.4 ± 1.5†	49.4 ± 1.9	47.4 ± 1.7†	48.9 ± 1.0†
Cysteinylglycine (nmol/mg)	31.6 ± 1.3	33.0 ± 1.0	32.5 ± 0.9	34.0 ± 1.5	26.6 ± 1.9†	25.1 ± 1.3†
Adenosine (pmol/mg)	487 ± 29	344 ± 12*	615 ± 46(†)	723 ± 25$^{(*)}$†	1308 ± 136†	820 ± 57*†

from 46 to 124 μmol/l, compared with 5 to 13 μmol/l in untreated dams.

At age P12, the plasma betaine concentrations of suckling *Mthfr*+/− and *Mthfr*+/+ pups from betaine-supplemented dams were slightly but significantly higher (110.5 ± 18.9 μmol/l, $n = 10$, 5 female, 5 male) than those of corresponding untreated pups (73.8 ± 4.9 μmol/l, $n = 16$, 8 female, 8 male). Plasma DMG was almost two times and significantly higher in pups from betaine-supplemented dams (29.2 ± 2.7 μmol/l) than in those from untreated dams (17.7 ± 0.9 μmol/l). In this group of mice, we found no differences in concentrations of betaine or DMG between genotypes or genders (results not shown).

The specific activity of liver BHMT at age P12 was 259 ± 10 units/mg of protein in pups from untreated dams ($n = 16$) and 260 ± 10 units/mg in pups from betaine-supplemented dams ($n = 10$). No differences between genders were observed. At 3 days before birth (age E18), BHMT activity in fetal liver was 73 ± 13 units/mg of protein ($n = 6$).

These experiments indicate that suckling pups received adequate amounts of betaine from their mothers and were able to metabolize substantial amounts to DMG.

Liver and brain thiols and DNA methylation in pups at age P6

The impact of betaine supplementation on biochemical parameters in liver (Table 2) and brain (Table 3) was examined in littermates of the three genotypes at age P6. Several genotype effects were seen in untreated groups. These included a significant increase in total homocysteine concentration in heterozygotes (1.4-fold in liver) and nullizygotes (5-fold in liver and 10-fold in brain), and moderately decreased methionine concentrations in the livers of heterozygotes and nullizygotes and in the brains of nullizygotes. SAH was increased in livers of heterozygotes and nullizygotes and in brains of nullizygotes.

Examination of homocysteine trans-sulphuration metabolites showed increased cysteine and cystathionine levels in livers and brains of nullizygotes. Cystathionine was also increased in livers, but not in brains, of heterozygous mice. Glutathione levels were lower in livers of heterozygotes and nullizygotes, whereas brain did not show any differences. Cysteinylglycine was decreased in livers of nullizygotes and in brains of heterozygotes and nullizygotes. Adenosine was increased in livers of heterozygotes and nullizygotes, but not in brain.

With betaine supplementation to lactating dams, homocysteine concentrations were decreased in *Mthfr*−/− pups in liver and brain compared with untreated mice. Methionine increased significantly in heterozygotes and nullizygotes in liver, but not in brain. SAH increased in livers and brains of wild-type mice, whereas it showed a decrease in livers of heterozygotes and nullizygotes, and in brains of nullizygotes.

The trans-sulphuration metabolites, cysteine and cystathionine, decreased in brains of nullizygotes from betaine-treated dams.

Table 3 Brain concentrations of total thiols and of SAH, SAM and adenosine in MTHFR-deficient pups at P6 stratified for genotype and maternal diet

Controls received standard rodent chow, whereas the betaine group received an additional betaine supplement of 2 % (w/v) in the drinking water. The dietary groups of the three genotypes comprised littermates of mixed genders. Results are means ± S.E.M. for five individuals (only four for wild-type controls); *$P < 0.05$ for difference between dietary groups of same genotype; †$P < 0.05$ compared with wild-type genotype of same dietary group; symbols in parentheses indicate borderline significance ($P = 0.06$–0.09).

	Wild type		Heterozygous		Nullizygous	
Metabolite	Control	Betaine	Control	Betaine	Control	Betaine
Homocysteine (nmol/mg)	0.265 ± 0.025	0.271 ± 0.028	0.293 ± 0.017	0.261 ± 0.021	2.662 ± 0.395†	1.991 ± 0.347*†
Methionine (nmol/mg)	30.3 ± 1.0	31.8 ± 2.4	28.8 ± 1.4	26.2 ± 1.2 (†)	25.2 ± 1.7†	31.7 ± 3.8
SAH (pmol/mg)	176 ± 35	317 ± 22*	229 ± 19	181 ± 12(*)†	357 ± 55†	277 ± 27*
SAM (pmol/mg)	579 ± 31	624 ± 78	691 ± 51	543 ± 21*	474 ± 27†	583 ± 63†
Cysteine (nmol/mg)	4.9 ± 0.2	4.8 ± 0.4	6.0 ± 1.2	4.4 ± 0.3	9.6 ± 1.0†	5.7 ± 0.3*
Cystathionine (nmol/mg)	4.2 ± 0.1	4.6 ± 0.1*	3.1 ± 0.2†	3.0 ± 0.1†	10.9 ± 1.3†	4.4 ± 0.2*
Glutathione (nmol/mg)	22.3 ± 1.1	24.1 ± 1.3	20.6 ± 1.1	21.8 ± 1.3	23.4 ± 1.2	23.1 ± 1.0
Cysteinylglycine (nmol/mg)	5.7 ± 0.1	7.3 ± 0.2*	2.6 ± 0.2†	4.4 ± 0.2*†	2.8 ± 0.3†	2.6 ± 0.2†
Adenosine (pmol/mg)	557 ± 126	555 ± 124	448 ± 28	447 ± 53	455 ± 30	572 ± 97

In liver, only cystathionine decreased with betaine supplementation in nullizygotes, whereas cysteine remained unchanged. Cysteinylglycine increased in brains of wild-type and heterozygous mice, but not in nullizygotes. Adenosine levels decreased with a betaine supplement in livers of wild-type and nullizygous mice.

In untreated pups, we noticed a trend towards increased hypomethylation of DNA with impaired MTHFR function. Due to high inter-individual variation, there were no significant differences betweeen genotypes in liver or brain. Betaine supplementation did not change the extent of DNA methylation in liver. However, in brain, when the genotype groups were combined, there was a small but significant decrease in hypomethylation in the betaine-supplemented group (4352 ± 131 d.p.m./0.5 μg of DNA for 14 untreated mice compared with 3840 ± 119 d.p.m./0.5 μg of DNA for 15 betaine-supplemented pups; $P < 0.01$).

DISCUSSION

Betaine supplementation improves the survival and somatic development of MTHFR deficient mice

Severe MTHFR deficiency due to homozygous disruption of the mouse *Mthfr* gene leads to a distinct pathology that was described in the original study of these mice when they were on a mixed genetic background [9]. In contrast with the lower mortality reported in that study (survival of 76 % of nullizygotes), the majority of *Mthfr*−/− mice in the present study, after multiple rounds of backcrosses to the BALB/cAnNCrlBR strain, died during the first two postnatal weeks. The mixed genetic background of the initial colony presumably alleviated some of the deleterious effects of severe MTHFR deficiency in the homogeneous BALB/c background.

The expected genotype distribution of offspring from *Mthfr*+/− matings was observed in the present study, as well as in the study of Chen et al. [9], indicating an absence of fetal losses. However, the lower birth weights of the *Mthfr*−/− pups point to some degree of intrauterine onset of pathology. Nonetheless, we were not able to distinguish surviving from non-surviving nullizygotes during the first few days of extrauterine life. The most reliable indicator of imminent death was poor weight gain over a few days and eventually weight loss, due to insufficient food intake as indicated by empty stomachs. Another typical feature of nullizygotes was pronounced deceleration of weight gain after weaning.

In another dietary animal model, severe folate deficiency during pregnancy and lactation led to increased fetal resorption, small litter size, low birth weight, postnatal death and depressed food consumption and weight gain, with significantly lower body and brain weights at weaning [18]. Some of these symptoms resemble those of the nullizygous pups of our present study, although we did not observe any intrauterine mortality.

The major pathology in *Mthfr* deficiency appears to occur after birth, suggesting that maternal folate pools may protect mutant pups *in utero*. Standard rodent chow contains very high levels of folate and preformed methyl groups, which are transferred from maternal to fetal tissues. However, the early postnatal period, with its increased need for methyl groups to maintain rapid cellular proliferation and differentiation, presents a particular challenge for *Mthfr*-deficient pups. Primary pathogenic factors in MTHFR deficiency may be toxicity of elevated homocysteine or its derivatives, impaired methylation or a disruption in the balance of folate species. Since betaine has not been reported to correct imbalances in folate derivatives, and betaine supplementation clearly improved survival and growth in *Mthfr*-deficient pups, the primary pathogenic factors presumably relate to the accumulation of homocysteine or its metabolite, SAH, and the consequent inhibition of transmethylation reactions.

Betaine supplementation of dams had a dramatic effect on the survival and growth of their nullizygous pups. With this simple nutritional intervention, most of the nullizygotes could be rescued from death. Nullizygotes of treated mothers had significantly higher body weights compared with untreated nullizygotes during the first two postnatal weeks, but not at birth. The lack of adverse effects of withdrawal of betaine 3 weeks after birth also demonstrates the importance of an adequate supply of transferable methyl groups during the critical first 2 weeks of postnatal life. The primary reason for the high mortality of untreated *Mthfr* nullizygotes may therefore be an insufficient intake of preformed methyl groups, leading to developmental disturbances and, beyond a point of no return, to starvation and death.

Betaine supplementation to lactating dams increases betaine availability in their pups and ameliorates biochemical anomalies in nullizygotes

Betaine-supplemented dams and their suckling offspring had elevated plasma betaine levels, demonstrating increased betaine availability in pups from treated lactating mothers. These mothers are heterozygotes for the *Mthfr* gene disruption, a condition that is

associated with elevated plasma homocysteine [9,10], decreased 5-methyltetrahydrofolate [9] and decreased liver choline and betaine [10]. These anomalies are likely to be aggravated by pregnancy and lactation, conditions that are associated with choline and betaine depletion in rats [19].

In a previous study on the effects of 2 weeks of betaine supplementation to adult *Mthfr*-deficient mice [10], we observed some disturbances in choline metabolism in untreated animals. We suggested that the fatty infiltration of livers in adult nullizygous mice might be due to choline deficiency. In the present study, most of the livers of nullizygotes at age P6 also had clear fatty infiltration. Neonatal rats have a 2-fold higher intake of choline than weanling animals. Rat and human milk do not contain measurable amounts of betaine [20,21]. However, since choline can be oxidized to betaine, choline can replace betaine as a source of transferable methyl groups. Consequently, betaine supplementation could increase choline availability in the livers of nullizygous mice either by a choline-sparing effect or by provision of methyl groups for the methylation of phosphatidylethanolamine to phosphatidylcholine, a precursor of choline. However, betaine supplementation did not decrease the extent of fatty infiltration of the liver in mutant pups in the present study. This could be a dose-dependent effect, particularly if the preformed methyl groups are utilized preferentially in maintaining brain methylation reactions at the expense of the liver in early postnatal life. On the other hand, since SAH levels were still elevated compared with control levels despite a betaine-lowering effect, phosphatidylethanolamine methyltransferase might have been inhibited.

When we investigated betaine metabolism and the consequences of high-dose betaine supplementation in adult *Mthfr*+/+ and +/− mice in the present study, we identified betaine depletion in the plasma, liver and brain of *Mthfr*+/− animals. Depletion of betaine in liver is attributable to the decreased capacity of *Mthfr*+/− mice to meet the demands for folate-dependent homocysteine remethylation, which leads to lower SAM concentrations. The compensatory increase in betaine catabolism by BHMT to restore homocysteine remethylation results in a decrease in betaine levels in liver. Betaine concentrations in other tissues are dependent on betaine export by hepatocytes and are probably reduced because betaine availability is decreased through lowered plasma concentrations. Betaine supplementation leads to greatly enhanced betaine storage and further induction of betaine metabolism, as indicated by increased concentrations of its metabolite DMG and increased BHMT activity (Table 1). These findings are in agreement with our previous study [10].

BHMT activity in fetal rat liver is only approx. 25 % of that found in adult liver, whereas the activity in the neonate is equal to or greater than that in adult rats [22]. In agreement with these earlier findings, fetal mice in our study had low BHMT activity a few days before birth, with a 3-fold increase at P12, up to adult levels. The dramatic effect of betaine on the survival of *Mthfr*−/− mice provides strong evidence for an important role for BHMT activity during the first days of postnatal life in mice.

SAH is the product of transmethylation reactions, which occur primarily in liver [23]. When homocysteine is elevated, the SAH hydrolase reaction (EC 3.3.1.1), which usually catalyses the breakdown of SAH to homocysteine and adenosine, can operate in the reverse direction, to generate SAH [2]. Betaine supplementation caused marked decreases in both homocysteine and SAH, and an increase in methionine, in heterozygotes and nullizygotes. In contrast, the administration of betaine to wild-type mice caused an increase in SAM and SAH levels, an observation that was also reported in another rodent study [24] and that could be interpreted being due to increased turnover of homocysteine by enhanced remethylation.

Adenosine was increased in the livers in heterozygotes and nullizygotes. This increase was diminished by betaine supplementation. Adenosine levels appeared to correlate strongly with the concentrations of homocysteine and SAH in liver; this finding may relate to the fact that SAH generates homocysteine and adenosine, and that adenosine elimination by adenosine deaminase may not have been enhanced.

Homocysteine can also be eliminated by trans-sulphuration, in which homocysteine condenses with serine to form cystathionine and subsequently cysteine. In the livers of nullizygous P6 pups, the most striking anomalies were elevations of homocysteine and SAH. In addition, cystathionine was markedly increased, whereas cysteine was only moderately elevated. This demonstrates the failure of co-ordinate regulation of homocysteine metabolism and, in particular, the inability of the two remaining pathways, betaine-dependent remethylation and homocysteine trans-sulphuration, to cope with a disruption of folate-dependent remethylation.

Homocysteine metabolism in the developing mouse brain and the impact of betaine supplementation

Studies in different species have shown that the prenatal brain utilizes homocysteine preferentially for folate-dependent remethylation and not for trans-sulphuration. Remethylation enzymes have higher activities during fetal life than postnatally [22,25,26]. During the first 2 weeks of postnatal life, there is an exponential decrease of folate in the mouse brain, with lower levels of polyglutamylated folates, indicating high turnover and demand for folates in early extrauterine life [27]. In contrast with the postnatal decreases in activity of the remethylation enzymes, the rate-limiting enzyme of the trans-sulphuration pathway, cystathionine β-synthase, shows decreased activity in the cerebrum during late fetal stages, increases after birth and peaks during cerebellar development [28]. The activity of the last enzyme of the trans-sulphuration pathway from methionine to cysteine, cystathionase, increases gradually in rat brain over 4 weeks after birth [29]. As a consequence of these temporal regulatory mechanisms, trans-sulphuration in fetal brain is limited.

In contrast with the liver, the brain does not use betaine as a methyl donor. There are low concentrations of betaine in brain [30], and BHMT is not expressed in this tissue [31]. When folate-dependent remethylation is disturbed, the brain becomes dependent on preformed methyl groups delivered as SAM. Furthermore, the only means of homocysteine elimination is trans-sulphuration, a pathway that is limited in brain, as discussed above. Consequently, we found more severe biochemical disruption in the brain than in the liver of 6-day-old pups from untreated dams. Most prominent were dramatic increases of homocysteine and clear elevations of cystathionine and cysteine; these findings demonstrate the activation of the trans-sulphuration pathway, but also its inability to cope with the increased homocysteine load.

Brain GSH was unchanged in nullizygotes, despite the elevation of its precursor cysteine, whereas cysteinylglycine was decreased in heterozygotes and nullizygotes. Cysteinylglycine is a product of extracellular GSH degradation, but also provides cysteine and glycine as precursor molecules for GSH synthesis in neurons [32]. Cysteinylglycine in brain was inversely correlated with homocysteine and cysteine concentrations, which could indicate a preference for cysteinylglycine over cysteine for GSH synthesis.

With betaine supplementation, biochemical parameters in brain indicated increased remethylation of homocysteine to methionine and markedly decreased flux through the trans-sulphuration pathway. As a consequence, global DNA hypomethylation was

decreased. Decreased homocysteine and SAH levels in mutant pups may have contributed to the phenomenon; DNA methylation status in another model of hyperhomocysteinaemia, the cystathionine β-synthase-deficient mouse, was largely determined by SAH concentrations [33].

The cerebella of *Mthfr*−/− mice are abnormally small and cerebellar development is disrupted, as first shown by Chen et al. [9]. We also identified some hypotrophy in the hippocampus, with abnormal formation of the neuroepithelium in the dentate gyrus. Betaine supplementation had a beneficial effect on the pups in both areas of the brain, but could not prevent residual structural abnormalities in the cerebellum in adult nullizygotes. Of note is the fact that a compartmentation defect (i.e. a difference in the degree of disturbance between the anterior and posterior cerebellar regions) was still evident.

The cerebellum is one of the late developing brain regions. Shortly after birth until the second week of life, the cerebellar granule neuroblasts proliferate extensively in the external granular layer of the cerebellar cortex, and undifferentiated Purkinje cells migrate to form a single cell layer underneath the external granular layer [34]. This postnatal proliferative and migratory period might be particularly vulnerable to the disruption in folate-dependent remethylation and supply of methyl groups. Other early postnatal insults to rodent brain also result in more severe cerebellar damage than later exposure and produce a pathological pattern that is similar to that in *Mthfr*−/− mice. Irradiation and methylazoxymethanol have been shown to be especially harmful before P8 and P4 respectively. Both cause foliation abnormalities, misalignment of Purkinje cells and continued multiple innervation of climbing fibres [35].

Intrauterine choline deficiency has been shown to result in structural defects in the hippocampus of rats [36] and mice [37–39]. Choline availability altered the timing, genesis, migration and differentiation of progenitor neuronal-type cells in the fetal hippocampus. Since we have shown previously that choline metabolites are decreased in brains of adult *Mthfr*−/− mice, and that this decrease can be ameliorated by betaine supplementation [10], it is likely that choline or betaine depletion may have played a role in the structural brain defects in the *Mthfr*−/− pups.

Conclusions

In summary, our findings demonstrate the importance of epigenetic factors, such as the intake of preformed methyl groups, for a favourable clinical outcome in severe MTHFR deficiency in mice. Very few humans with severe MTHFR deficiency have been identified prenatally; these were siblings of previously identified index patients. One such published case is remarkable in demonstrating that immediate postnatal betaine treatment may prevent symptoms until adulthood ([40]; F. Skovby, personal communication). Our results in mice emphasize the importance of early (prenatal) betaine supplementation in homocystinuria and suggest that newborn screening for hyperhomocysteinaemia would allow for the immediate therapeutic use of betaine to improve the clinical course.

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