DOI 10.1002/mnfr.201200162 *Mol. Nutr. Food Res.* 2012, *56*, 1582–1589

RESEARCH ARTICLE

Blood biomarkers of methylation in Down syndrome and metabolic simulations using a mathematical model

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Received: March 21, 2012
Revised: June 22, 2012
Accepted: July 5, 2012

Scope: The study tests the metabolites of the methylation cycle in individuals with Down syndrome (DS) and applies a mathematical model in order to change this cycle by nutritional factors.

Methods and results: We measured concentrations of the metabolites related to the methylation cycle in the blood of 35 young individuals with DS and 47 controls of comparable age. Moreover, we applied a mathematical model to learn more about the regulation of the methylation cycle in DS. Concentrations of cystathionine, cysteine, betaine, choline, dimethylglycine, *S*-adenosylhomocysteine (SAH), *S*-adenosylmethionine (SAM), and holotranscobalamin were significantly higher in DS compared to the controls. The median SAM/SAH ratio was lower in DS and that of methionine and reduced glutathione did not differ significantly between the groups. The mathematical model showed that enhanced methionine turnover and accelerated Hcy-remethylation might explain the shift in the methylation cycle in DS.

Conclusion: In addition to the DS-related excess of cystathionine beta synthase (CBS) activity, increases in the activities of MS and betaine homocysteine methyl transferase, and in methionine input were necessary to account for the changes in metabolite levels observed in DS. A low-methionine diet might offer a perspective for reversing the metabolic imbalance in DS, but this awaits clinical investigations.

Keywords:
Betaine / Choline / Down syndrome / Homocysteine / Methylation

1 Introduction

Down syndrome (DS) is the most common human aneuploidy and the major cause of metal retardation and early-onset dementia [1–3]. According to the EUROCAT network for the surveillance of congenital anomalies, 3396 live births of 7359 DS pregnancies were registered in Europe between 2004 and 2008 (http://www.eurocat-network.eu.). The majority of individuals with DS develop neuropathological features similar to those seen in patients with Alzheimer disease like amyloid plaques, neurofibrillary tangles, and degeneration of basal forebrain cholinergic neurons [4, 5]. Plasma concentrations of total homocysteine (tHcy) and low vitamin B12 status are important biomarkers that predict the development or the presence of dementia in the general population [6, 7]. A causal role for homocysteine (Hcy) in neurodegeneration is controversial but hypomethylation associated with hyperhomocysteinemia remains a possible mechanism [8, 9]. Experimental studies have shown that deprivation of B-vitamins causes low S-adenosylmethionine (SAM) and accelerates the neurodegenerative process [10–12]. Targeting the Hcy metabolic pathway has been discussed in the context of dementia prevention [13, 14].

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Abbreviations: ACh, acetylcholine; **AD**, Alzheimer disease; **BHMT**, betaine homocysteine methyltransferase; **CBS**, cystathionine beta synthase; **DMG**, dimethylglycine; **DS**, Down syndrome; **GSH**, reduced glutathione; **holoTC**, holotranscobalamin; **MMA**, methylmalonic acid; **MS**, methionine synthase; **SAH**, *S*-adenoylhomocysteine; **SAM**, *S*-adenosylmethionine; **tHcy**, total homocysteine

The gene-encoding cystathionine beta synthase (CBS) is located on chromosome 21. An increased CBS activity in DS fibroblasts [15], lower plasma concentrations of tHcy, and higher cysteine levels were reported in individuals with DS [16, 17]. Further genes on chromosome 21 [18] that are related to the methylation cycle are those encoding for formiminotransferase cyclodeaminase (FTCD), phosphoribosylglycinamide transformylase (GART) that encodes a trifunctional protein carrying out three steps of de novo purine synthesis [19], protein arginine methyltransferase (PRMT2), N-6 adenine-specific DNA methyltransferase 1 (putative) N6AMT1, reduced folate carrier (SLC19A1), and DNA methyltransferase 3-like (DNMT3L). The CBS is the main enzyme on chromosome 21 that is involved in driving the SAM production and is likely to be affected by dietary modifications (e.g. methionine load). It is not known whether the effect of an additional copy of CBS might be counterbalanced by changes in other key steps in this metabolic pathway.

To date, the choline/betaine pathway as an alternative methylation pathway has not been studied in individuals with DS. Choline is an essential micronutrient that is involved in brain development [20–22], brain function, the structural integrity of cell membranes, and lipids transport and metabolism. Choline is a precursor of acetylcholine (ACh). It is provided by the diet or produced endogenously from phosphatidylcholine [23]. Choline is oxidized to betaine, a direct methyl donor for Hcy-remethylation [24]. Betaine can be alternatively obtained from the diet and is considered an important osmolyte. The product of betaine demethylation is dimethylglycine (DMG).

The lower concentrations of tHcy observed in the blood of individuals with DS are thought to be related to enhanced transsulfuration of Hcy to cystathionine via CBS. Enhanced Hcy-transsulfuration in DS might increase the amount of cysteine available for glutathione synthesis. The oxidative stress in subjects with DS [25] might have an impact on the methylation cycle, since both CBS [26, 27] and methionine synthase (MS) [28] are sensitive to the oxidative balance. Interventions aimed at correcting the methylation potential in DS are not recommended without a complete understanding of the regulation of the methylation cycle. To date, no study has provided complete metabolic profile of the methylation cycle in DS and non-DS subjects. In the current study, we tested concentrations of markers of the methylation cycle in the blood of young individuals who were non-vitamin users with or without DS. In addition, we used a mathematical model in an attempt to study how the methylation cycle in DS can be modulated to make it more similar to that in the controls.

2 Subjects and methods

Study participants were children and adolescents with and without DS who presented for routine medical testing to the Department of Paediatrics at Saarland University Medical Centre, Homburg, Germany. Exclusion criteria were: serious clinical conditions, recent surgery, methotrexate treatment, and current vitamin usage. The group of DS consisted of 35 subjects (median (10–90th percentiles) age = 11.0 (1.9–27.0) years), three patients had congenital heart diseases, but all were in a clinically stable condition. The control group consisted of 47 healthy children and adolescents (median (10–90th percentiles) age = 13.0 (5.7–17.0) years) who were admitted to the hospital for treatment of minor injuries or presented for scheduled medical examinations. The control group individuals had no chronic diseases or infections and were not using any medications or vitamin supplements.

Fasting blood (2–8 h) was collected from all participants. Both serum and EDTA blood were obtained. Blood samples were centrifuged at 2000×g and 4°C, serum and EDTA plasma were separated within 30 min, and several aliquots were prepared and kept at −70°C until analysis. For SAH and SAM assay, 500 μL of the EDTA plasma was immediately acidified with 50 μL acetic acid and stored at −70°C. Concentrations of SAH and SAM in the acidified plasma were measured using a ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) method [29]. Concentrations of holoTC and total vitamin B12 in serum were measured using Axsym and Centaur (Abbott Diagnostics, Wiesbaden, Germany) analyzers, respectively. The concentrations of choline, betaine, and DMG were measured in EDTA plasma using UPLC-MS/MS as described earlier [30]. Serum and EDTA concentrations of tHcy, cystathionine, and serum methylmalonic acid (MMA) were measured using gas chromatography-mass spectrometry (GC-MS) [31, 32]. EDTA–plasma concentrations of methionine, cysteine, reduced glutathione (GSH), and tHcy were measured at the Department of Pharmacology, University of Oxford using liquid chromatography-tandem mass spectrometry (LC-MS/MS) [33]. Samples used for analyzing the metabolites at the Department of Pharmacology, University of Oxford were thawed twice before.

The study protocol was reviewed and approved by the Ethical Committee of the Medical Association in Saarland (Nr. 107/09). The objective of the study was explained to all participants and their parents and a written informed consent was obtained from the parents.

2.1 Mathematical model

The mathematical model we used was that described by Reed et al. [34]. In brief, this model consists of a system of coupled differential equations and kinetic equations that describe folate-mediated one carbon metabolism, including the methionine cycle and glutathione synthesis. The kinetics and parameter values of enzymes and metabolite transporters in this model are derived from experimental and clinical data. This model allows in silico investigation of the effects of genetic and nutritional variables on metabolite levels and reaction rates such as methylation capacity.

2.2 Statistical analyses

Data analyses were performed using SPSS (version 19.0; SPSS Inc, Chicago, IL, USA). Results are presented as median (10–90th) percentiles. Comparisons of medians of the two groups were performed using Mann–Whitney test. The Chi-square test was performed to test differences in categorical variables between the groups. Correlations between different variables were examined by Spearman test. Multiple backward regression analyses were applied on the log-transformed data to find predictors of concentrations of SAM in DS and non-DS individuals. All tests were two-sided and p values less than 0.05 were regarded as statistically significant.

3 Results

Table 1 shows median (10–90th) percentiles of the concentration of the main metabolic markers in plasma or serum. The median serum concentration of tHcy (GC-MS) was 29% lower in the DS group compared to the control group (7.0 versus 10.1 μmol/L). Concentrations of tHcy measured in plasma by LC-MS/MS showed no significant differences between the controls and the DS individuals (8.0 versus 7.5 μmol/L). The concentrations of cystathionine and cysteine were higher in individuals with DS compared with that in the controls (Table 1). Plasma concentrations of SAH and SAM were respectively 51 and 34% higher, but the SAM/SAH ratio was lower in the DS compared with the control group (median SAM/SAH = 8.9 versus 10.4) (Table 1). Plasma concentrations of betaine and choline were significantly higher in DS individuals compared with the controls. Concentrations of methionine and GSH did not differ significantly between the study groups. Furthermore, median concentrations of total vitamin B12 and MMA (a functional marker of vitamin B12) did not differ significantly between the study groups. However, in subjects with DS, concentrations of holoTC were significantly higher and the proportions of holoTC to total vitamin B12 were slightly higher compared to non-DS individuals ($p = 0.058$).

Multiple backward regression analyses were applied to find out significant predictors of SAM concentrations in the DS and the control groups. SAH and choline were the significant positive predictors of SAM concentrations in the controls (Table 2). In the DS group, concentrations of plasma SAM were positively predicted by that of SAH.

We used a mathematical model of the folate and methionine cycles [34] to simulate the patterns of metabolite concentrations we found in DS in an attempt to elucidate the conditions that could have led to the deviations from controls. Moreover, we looked for an approach to correct the methylation cycle by modifying certain nutritional components of this cycle (Table 1). Because CBS is expressed on chromosome 21, we increased the activity of CBS to 150% to account for its overdosage in DS. This change alone was not sufficient to reproduce the metabolite changes observed (Table 3). Our data show a better vitamin B12 status, which we modeled by increasing the activity of MS, the enzyme for which vitamin B12 is a cofactor, by 20%. The data also show an increase in betaine, which we modeled by increasing the

Table 1. Concentrations of main metabolic markers in the study population

Variable	Controls $N = 47$	Down Syndrome $N = 35$	p
Age, years	13.0 (5.7–17.0)	11.0 (1.9–27.0)	0.278
Male, *n* (%)	21 (45.7%)	20 (57%)	0.267[a)]
Serum tHcy (GC-MS), μmol/L	10.1 (4.7–16.0)	7.0 (4.0–11.6)	0.001
Plasma tHcy (GC-MS), μmol/L[b)]	8.8 (5.3–13.4)	7.5 (5.5–10.8)	0.009
Plasma tHcy (LC-MS/MS), μmol/L[c)]	8.0 (4.5–12.2)	7.5 (5.3–11.0)	0.384
Plasma methionine, μmol/L	22.1 (14.4–33.0)	22.7 (15.3–31.8)	0.957
Plasma SAM, nmol/L	89 (74–114)	119 (90–165)	<0.001
Plasma SAH, nmol/L	9.0 (5.9–15.4)	13.6 (8.8–24.6)	<0.001
Serum cystathionine, nmol/L	164 (89–321)	257 (153–765)	<0.001
Plasma cysteine, μmol/L	221 (186–279)	255 (206–311)	0.001
Plasma GSH, μmol/L	5.5 (3.3–8.5)	5.9 (3.1–8.7)	0.574
Plasma SAM/SAH ratio	10.4 (6.3–13.7)	8.9 (5.7–11.9)	0.003
Serum MMA, nmol/L	199 (108–257)	206 (97–402)	0.361
Serum holoTC, pmol/L	51 (28–105)	75 (44–114)	0.001
Total serum vitamin B12, pmol/L	308 (224–622)	378 (235–531)	0.354
% holoTC/totalB12	17.5 (5.6–29.0)	21.2 (11.3–29.0)	0.058
Plasma betaine, μmol/L	28.9 (16.4–44.1)	34.0 (24.0–52.6)	0.013
Plasma choline, μmol/L	8.8 (5.6–11.3)	10.3 (6.2–15.6)	0.013
Plasma DMG, μmol/L	3.5 (1.6–5.5)	5.9 (4.0–10.5)	<0.001

Data are median (10–90th) percentiles. p values are according to Mann-Whitney test.
a) p value is according to Chi-square test.
b) 46 controls/31 DS.
c) 47 controls/ 33 DS.

Table 2. Multiple backward regression analyses applied to find predictors of SAM

Dependent variable log-SAM			
	Variables with significant effect	Regression coefficient B (95% CI)	*p*
In Down syndrome[a)]	Log-SAH	0.517 (0.366–0.668)	<0.001
In the controls[b)]	Log-SAH	0.394 (0.199–0.589)	<0.001
	Log-choline	0.255 (0.036–0.474)	0.024

Independent variables entered in the model: methionine, choline, betaine, DMG, cysteine, serum tHcy (GC-MS), SAH, cystathionine, age, total B12, MMA, holoTC, GSH.
a) Unadjusted $R^2 = 0.69$, adjusted $R^2 = 0.67$: constant = 1.476.
b) Unadjusted $R^2 = 0.48$, adjusted $R^2 = 0.44$: constant = 1.353.

Table 3. Percent change from control of patients with DS (from Table 1). Values are obtained from simulations with a mathematical model

Metabolite[a)]	In DS % of the controls	Treatment[b)]			
		CBS - - -	CBS BHMT MS -	CBS BHMT MS metin	CBS - - metin
Hcy	69	73	66	79	84
Cystathionine	157	103	98	122	125
SAH	151	73	94	141	102
SAM	134	79	102	155	113
DMG	169	76	117	124	80
Cysteine	115	101	99	112	113
GSH	107	102	99	106	106

a) Hcy = homocysteine, SAH = *S*-adenosylhomocysteine, SAM = *S*-adenosylmethionine, DMG = dimethylglycine, GSH = reduced glutathione.
b) CBS = 1.5 × activity; BHMT = 1.75 × activity; MS = 1.2 × activity; metin = 1.2 × normal methionine input.

activity of betaine homocysteinemethyltransferase (BHMT), the enzyme that uses betaine as a substrate, by 75%. With these additional changes, the model began to approximate the changes in metabolite levels found in the controls. Finally, we found that by adding an additional 20% methionine input, the model more closely approximated the metabolite deviations we found in DS (Table 1 and 3). The need for increased methionine is in effect a prediction of the model, and could imply a diet richer in protein in DS, which is unlikely, or a higher rate of protein turnover in individuals with DS. Simply increasing methionine input alone did not produce changes in metabolite levels that resembled those found in DS suggesting that methionine turnover, rather than methionine intake, is likely to be accelerated in DS. Exceptions were cysteine and glutathione whose change in DS could be explained entirely by the effects of CBS overdosage and increased methionine input.

We used the mathematical model to investigate the effect of supplementation with several metabolites in the methionine cycle. We progressively increased the input of methionine, of SAM, and the level of betaine and measured the changes of the other metabolites relative to the normal steady-state concentrations. The results of these simulations are shown in Fig. 1. In each case, SAM and SAH responded most strongly, although their relative responses differed, resulting in different SAM/SAH ratios. Hcy increased with SAM and methionine input, but decreased with increasing betaine and levels. This response is explained by the fact that higher betaine levels increase the BHMT reaction rate, which uses Hcy. The simulations on SAM and methionine supplementation assume that betaine levels are higher in DS, as we found in this study.

Finally, we tested the effects of increasing oxidative stress on the methylation cycle in DS. DS individuals were reported to have increased levels of oxidative stress [35], and this affects the activities of CBS, MS [36], Glycine-N-methyltransferase (GNMT), γ-glutamyl-cysteine synthetase (GCS), and indirectly of methionine S-adenosyltransferase (MAT-I and MAT-III). We found that increased oxidative stress resulted in higher level of tHcy, and further depressed the level of SAM. Interestingly, the level of tHcy could be raised to normal, and even well above normal levels, depending on the severity of the oxidative stress. Thus, oxidative stress would be expected to increase tHcy and to lower SAM levels (and hence methylation capacity) in individuals with DS.

The model accounts also for potential changes in the activities of MAT I and III. We tested the effect of increasing the expression levels of both enzymes by twofold in combination with CBS 1.5-fold in DS. SAM and SAH were modestly elevated but tHcy was down compared to normal and DMG was reduced by 20% (data not shown), which is not what was found in the DS group in this study. Thus, increased expression of MATI+III in addition to a triple dose of CBS does not produce the metabolic profile we found in DS.

The difference in tHcy results obtained by GC-MS and LC-MS/MS might be related to differences in the methods, materials used for the test (plasma or serum), or preanalytical factors, as serum samples for GC-MS were not thawed before analysis, whereas plasma samples for LC-MS/MS were thawed twice before. The modeled Hcy levels are a logical outcome of the model, given the assumptions of CBS activity, vitamin B12 and betaine levels, and methionine input. Therefore, the differences in tHcy concentrations measured in the plasma or serum had no effect on the models and simulations we discussed since this model is based on increasing enzyme activity and Hcy is an output marker.

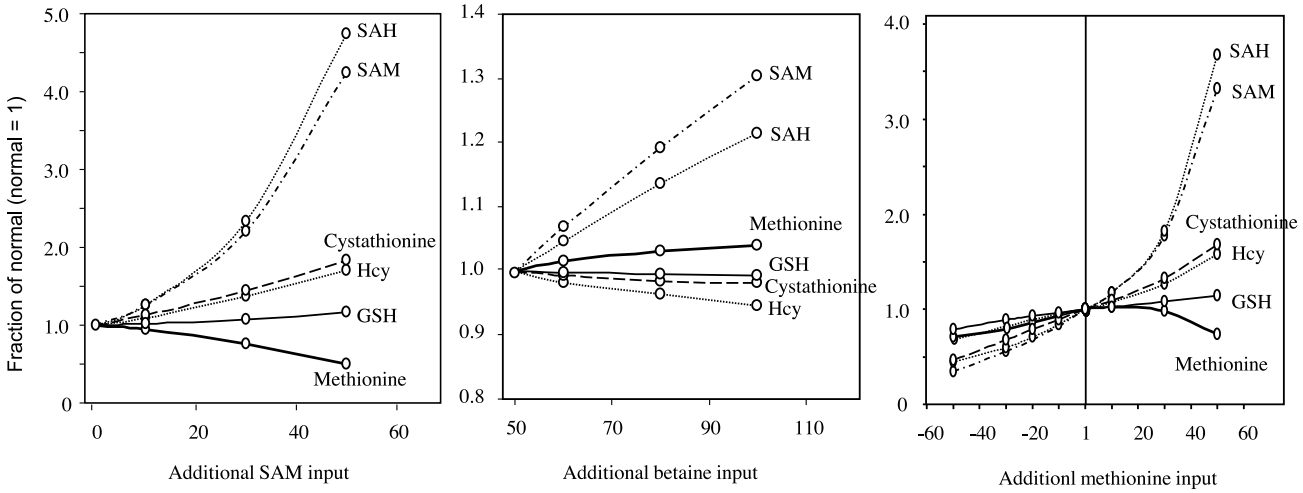

Figure 1. The theoretical effects of increasing methionine input, or the levels of SAM or betaine on the concentrations of other metabolites in the methionine cycle. The model effects of increasing or decreasing methionine input assume that betaine levels are high (as in DS; see Table 1). The model effects of betaine supplementation assume that methionine is unchanged, and that of SAM supplementation assume that betaine is high and methonine is unchanged.

4 Discussion

Targeting the methylation pathway has been repeatedly discussed in the context of several neurodegenerative diseases [13, 14]. Individuals with DS have an additional copy of the CBS gene and several other genes related to this cycle [18]. Therefore, a better understanding of the regulation of the methylation cycle is required before constructing supplements intended to slow or prevent the neurodegenerative disorders in triosomy 21.

Our study is the first to show a complete profile of the methylation cycle components in individuals with DS. The observed concentrations of tHcy, cystathionine, and cysteine are in agreement with the presence of an additional copy of the CBS gene (Table 1) causing enhanced Hcy transsulfuration. Because concentrations of SAM, betaine, and DMG were higher in DS compared to non-DS subjects, the results suggest that the methylation of Hcy via BHMT and/or MS might be accelerated in DS. Enhanced Hcy-methylation would be expected to cause higher concentrations of methionine in subjects with DS compared to the controls, which was not the case in this study. Comparable concentrations of methionine in addition to the predictions made by the mathematical model suggest that the turnover of methionine is enhanced in subjects with DS. Our study suggests that a diet low in methionine, in addition to increasing betaine and counteracting the oxidative stress might enhance SAM synthesis without increasing SAH or tHcy in individuals with DS.

One explanation for SAM elevation in DS might be that elevated SAH inhibits the utilization of SAM and cause a secondary increase in SAM. Because increased expression of MATI+III by twofold did not produce the metabolic profile we found in DS, an enhanced production of SAM from methionine cannot be assumed. Moreover, any proposed over activation of MAT-I/III in DS can not explain higher active B12, betaine, and choline that supports the synthesis of additional methionine in DS. Higher methionine input as a primary upstream precursor for CBS may reflect higher demands for glutathione in DS.

Our findings of higher plasma concentrations of SAM and SAH, and lower SAM/SAH ratios in DS are in agreement with a recent study [17]. Our results disagree with earlier results reported by Pogribna et al., who found lower concentrations of SAM and SAH in DS compared to control children [16]. The same authors reported lower methionine levels in DS that is in line with the proposed higher turn over of methionine [16]. Infantino et al. reported on hypomethylation of the mitochondrial DNA and lower SAM and methionine in cytosole of lymphoblastoid cells from DS individuals [37]. However, a direct comparison with our results is not possible since we measured extracelleular metabolites. Differences in methionine, SAH, or SAM between the current study and earlier ones [16, 37] might be related to antioxidant status or nutritional supplements usage. Moreover, analytical or preanalytical factors could be involved. For example, Pogribna et al., measured SAM and SAH using an HPLC method, and the study did not mention treating EDTA plasma samples with acetic acid or perchloric acid before freezing [16]. Infantino et al. used a LC-MS/MS method [37]. However, the assay range for intracellular metabolites [37] was much lower than ours and sufficient information on the relationship between intra and extracellular markers is not available.

The role of the methylation cycle [25] in glutathione production in the light of enhanced oxidative stress in DS seems to play an important regulatory role [26, 27].The overexpression of CBS might deliver more cysteine (which we also found). Our study showed a comparable GSH between DS

and controls despite higher cysteine in DS that might support increased consumption of GSH in DS. All results collectively suggest a complex gene-nutrient adaptive regulation in the methylation cycle in DS.

Studies on the effect of vitamin supplements on metabolic or clinical outcomes in DS showed limited, if any, evidence of improvements [38, 39]. Infants with DS showed no significant improvements in developmental scores after 18 months of treatment with folinic acid alone, folinic acid in combination with an antioxidant cocktail, or a placebo [40]. Biochemical markers related to the C1 metabolism, however, were not investigated in that study [40]. Another study reported a limited effect of a 1-year treatment with a high dose of folinic acid on the psychomotor development of children with DS receiving thyroxine treatment [39], but also did not investigate metabolic markers. Therefore, studies in individuals with DS have not demonstrated metabolic improvements after folinic acid treatment.

Available evidence of the effect of B-vitamins on the methylation pathway in other developmental disorders is also not conclusive. For instance, a study in which patients with Angelman Syndrome used L-5-methyltetrahydrofolate, creatine, betaine, and vitamin B12 [41] assessed changes in blood metabolites after 1 year [41]. DNA methylation in a subgroup of 16 participants was determined using the Infinium HumanMethylation27 BeadChip array. Compared to the placebo, the methylation regimen had no significant effect on tHcy, but did cause significantly higher methionine levels in the group that received the supplements ($p = 0.027$) [41]. The treatment with the methylation regimen did not result in any specific changes in DNA methylation in this study [41]. In a further study on patients with autistic disorders, folinic acid and methylcobalamin were supplemented for 3 months with the aim of improving the production of GSH [42]. The study observed a reduction in tHcy and an increase in cysteine and GSH [42]. However, DS individuals from the current study had cysteine concentrations (median 255 μmol/L) that were higher than those obtained in patients with Autism after folinic acid and methylcobalamin supplementation (mean 215 μmol/L) [42]. As expected, folinic acid and methylcobalamin did not raise SAM or lower SAH, which are main targets in DS [42]. Therefore, our study offers an alternative approach to modify the methylation cycle in DS.

Experimental deficiency of choline can cause developmental disorders, fetal brain damage [43], fatty liver, or muscle damage [44]. Moreover, supplementing the maternal diet of Ts65Dn mice (an established DS model) with choline during pregnancy and lactation substantially improved attention [45]. Choline supplementation was found to improve the hippocampal cholinergic system and behavioral deficits in animal models of foetal alcohol exposure [46]. The volume of the hippocampus was significantly smaller in DS and dementia in DS was associated with a decline in the volume of the hippocampus [47]. Therefore, choline metabolism might be an important target in DS or in women pregnant with a DS.

Taken together, markers of the methylation cycle showed significant differences between DS and non-DS individuals. Based on the differences in blood markers between the groups, our simulations with a mathematical model suggest that in addition to the CBS overdose caused by trisomy-21, an increase in the activities of MS and BHMT, and an increase in methionine input were necessary to account for the observed changes in metabolite levels in DS. These findings imply that some of these metabolic effects might be reversed by reducing methionine input (when assuming higher betaine levels) or betaine supplementation (assuming stable methionine), and simultaneously reducing the oxidative stress. Before dietary approaches to reducing the risk of dementia in DS can be recommended, there is a need for clinical studies to investigate the short-term metabolic effect of the approaches we suggest.

We would like to thank Professor Helga Refsum and Maria Valdivia-Garcia (Department of Pharmacology, University of Oxford, Mansfield Roa Oxford, OX1 3QT, UK) for running the blood analyses of methionine, cysteine, GSH, and Hcy.

The authors have declared no conflict of interest.

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