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Hyperhomocysteinemia in Alzheimer's Disease: The Hen and the Egg?

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Abstract. Hyperhomocysteinemia is associated with Alzheimer's disease (AD). The causality of this association is controversial. In this study we tested the effect of a hyperhomocysteinemia-inducing diet in the ArcAβ transgenic AD mouse model. At 14 months of age, the hyperhomocysteinemia-inducing diet yielded higher plasma homocysteine levels in ArcAβ mice compared with wild-type mice. Levels of plasma 5-methyltetrahydrofolate (5-MTHF) in 14-month-old mice on hyperhomocysteinemia-inducing diet were lower in the transgenic than in the wild-type mice. The folate derivate 5-MTHF serves as cofactor in homocysteine metabolism. Oxidative stress, which occurs in the course of disease in the ArcAβ mice, consumes 5-MTHF. Thus, the transgenic mice may plausibly be more vulnerable to 5-MTHF-depleting effects of hyperhomocysteinemia and more vulnerable to hyperhomocysteinemia-inducing diet. This argues that AD pathology predisposes to hyperhomocysteinemia, i.e., as a facultative consequence of AD. However, we also observed that dietary-induced folate reduction and homocysteine increase was associated with an increase of plasma (young animals) and brain (older animals) amyloid-β concentrations. This suggests that the hyperhomocysteinemia-inducing diet worsened pathology in the transgenic mice. In conclusion, this data may argue that folate reduction and hyperhomocysteinemia may contribute to neurodegeneration and may also be triggered by neurodegenerative processes, i.e., represent both a cause and a consequence of neurodegeneration. Such a vicious cycle may be breakable by dietary or supplementation strategies increasing the availability of 5-MTHF.

Keywords: Alzheimer's disease, diet, folic acid, homocysteine, hyperhomocysteinemia, mice, transgenic, vitamin B6, vitamin B12

INTRODUCTION

Sporadic Alzheimer's disease (AD) is the most common form of dementias. Higher age, female gender, and presence of the apolipoprotein E4 allele have been identified as risk factors. In addition, hyperhomocysteinemia is independently associated with AD [1]. Factors like higher age, male gender, renal dysfunction, genetic variants, high methionine uptake via protein-rich food, and deficiencies of folate, vitamin B12, and vitamin B6 are linked to hyperhomocysteinemia [2]. The nature of the relationship between hyperhomocysteinemia and AD is controversial [3–5].

Homocysteine is a toxic intermediate of methionine metabolism. It is either irreversibly metabolized by the vitamin B6-dependent transsulfuration pathway or is recycled to methionine by the folate- and vitamin B12-dependent remethylation pathway (Fig. 1). Methionine can be activated to S-adenosylmethionine (SAM), which serves as universal methyl-group donor, e.g., for DNA, RNA, and protein methylation reactions. SAM is thereby converted to S-adenosylhomocysteine (SAH), which is further hydrolyzed to homocysteine in a reversible reaction. Although elevated plasma

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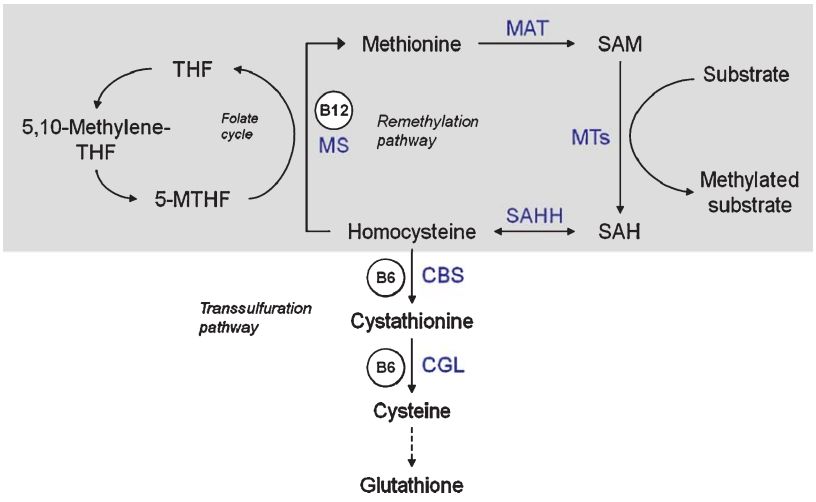

Fig. 1. The two pathways of homocysteine metabolism. In the remethylation pathway, the methyl group from 5-methyl-tetrahydrofolate (5-MTHF) is transferred to homocysteine by the vitamin B12-dependent enzyme methionine synthase (MS), producing methionine and tetrahydrofolate (THF). Methionine is activated to S-adenosylmethionine (SAM) by methionine adenosyltransferase (MAT) transferring adenosine from ATP. SAM acts as methyl group donor for a diversity of methylation reactions catalyzed by methyltransferases (MTs) producing S-adenosylhomocysteine (SAH), which is reversibly hydrolyzed to homocysteine by SAH hydrolase (SAHH). In the transsulfuration pathway, homocysteine is irreversibly degraded to cystathionine by the vitamin B6-dependent enzyme cystathionine-β-synthase (CBS) and then further catalyzed to cysteine, a precursor of glutathione, by the vitamin B6-dependent enzyme γ-cystathionase (CGL).

homocysteine levels have consistently been reported as an independent risk factor for AD [1, 6, 7] and predict cognitive decline in AD patients [8], several interventional trials have not observed beneficial effects of homocysteine-lowering strategies in AD [3, 9–11]. However, the recent VITACOG trial showed a highly significant reduction in brain atrophy as an effect of homocysteine-lowering vitamins in individuals with mild cognitive impairment [12]. In AD patients, high serum homocysteine levels are often accompanied by low serum folate levels [6, 13]. Low folate levels may be an additional independent AD risk factor [14, 15] and may independently correlate with cognitive decline in AD patients [8, 16]. Cerebrospinal fluid (CSF) folate levels were decreased in AD patients in some [17–19], but not all studies [20, 21]. CSF and brain SAM levels, which depend on the availability of folate (Fig. 1), are decreased in AD [20, 22, 23]. Whether hyperhomocysteinemia is an independent causal AD risk factor or occurs as secondary epiphenomenon of neurodegeneration, is an important unanswered question. To test the hypothesis that changes of homocysteine metabolism occur during the natural course of neurodegeneration, we analyzed plasma homocysteine and folate levels and their association with brain and plasma $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ fractions in the ArcAβ mouse model.

MATERIALS AND METHODS

The ArcAβ mouse strain overexpresses human AβPP containing the Swedish double mutation (Swe; K670 N; M671 L) and the Arctic mutation (E693 G) [24]. ArcAβ mice exhibit extracellular amyloid-β (Aβ) plaque deposition starting at 7 months of age followed by massive Aβ plaque formation and cerebral Aβ angiopathy between 9 and 15 months. These biochemical hallmarks are accompanied by progressive cognitive impairment of spatial memory starting at 6 months.

In this study, 40 ArcAβ mice (18 female; 22 male) and 51 wild-type littermates (33 female; 18 male) were fed either normal diet (3 g/kg methionine; 2 mg/kg folic acid) or methionine-rich diet (20 g/kg methionine, inducing hyperhomocysteinemia without folate depletion) from the age of three months until sacrificed at the age of 6 or 14 months, respectively. In-cage behavior and body weight were monitored. Total plasma homocysteine and 5-MTHF were measured after cardiac puncture in terminal anesthesia; other folate fractions were below the threshold of measurement [25, 26]. Brains were extracted and snap frozen. For the analysis of the Aβ fractions, the brains were homogenized in liquid nitrogen, lysed in precooled 2% SDS buffer pH = 8.0 (2% SDS, 100 mM TrisBase, 150 mM NaCl, 1x protease and phosphatase

inhibitor solutions (Roche)) and centrifuged for 30 min at 100,000 × *g* at 8°C. Supernatants (= SDS-soluble brain Aβ fraction) were stored at −80°C until further measurements. Pellets were dissolved in 70% formic acid by ultra-sonication and centrifuged for 30 min at 100,000 × *g* at 4°C. Supernatants (= SDS-insoluble brain Aβ fraction) were transferred to low-bind Eppendorf tubes and lyophilized. Pellets were resolved in 2% SDS buffer pH = 8.0 and stored at −80°C until further measurements.

Brain and plasma $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ fractions were measured with the Meso Scale Discovery System Multiplexed Aβ Ultra-Sensitive Assays (Meso Scale Diagnostics, LLC, Gaithersburg MD, USA). Plasma homocysteine levels were not normally distributed and were therefore transformed to a logarithmic scale for statistical analysis. Metabolite levels were analyzed by Pearson's correlation and by multivariate-ANOVA with the variables age, diet, genotype, and gender followed by multivariate-ANOVA of the age and diet subgroups with the variables genotype and gender. Animal experiments were approved by the Federal veterinary office and conducted according to the Swiss Law for Animal Protection.

RESULTS

In-cage behavior, food intake, and body weight were not associated with genotype or diet. As expected, the methionine-rich diet led to increased plasma homocysteine levels in both the 6- and the 14-month-old animals (log plasma homocysteine at 6 months $p = 0.022$, log plasma homocysteine at 14 months $p < 0.001$; Fig. 2A). Plasma homocysteine levels did not differ between age groups, independent from the diet (Fig. 2A). However, the 14-month-old ArcAβ mice on methionine-rich diet had higher plasma homocysteine levels than their wild-type littermates on the same diet (log plasma homocysteine $p = 0.006$, Fig. 2A).

To gather further information on the reasons for stronger methionine-induced hyperhomocysteinemia in transgenic mice at a progressed stage of pathology, we next measured the concentrations of the main biological folate derivative, 5-MTHF. As expected, the methionine-rich diet group showed reduced 5-MTHF levels (6 months $p < 0.001$, 14 months $p < 0.001$; Fig. 2B). However, at 14-months of age, 5-MTHF levels negatively correlated with homocysteine levels in the ArcAβ mice only ($r = -0.861$; $p = 0.001$).

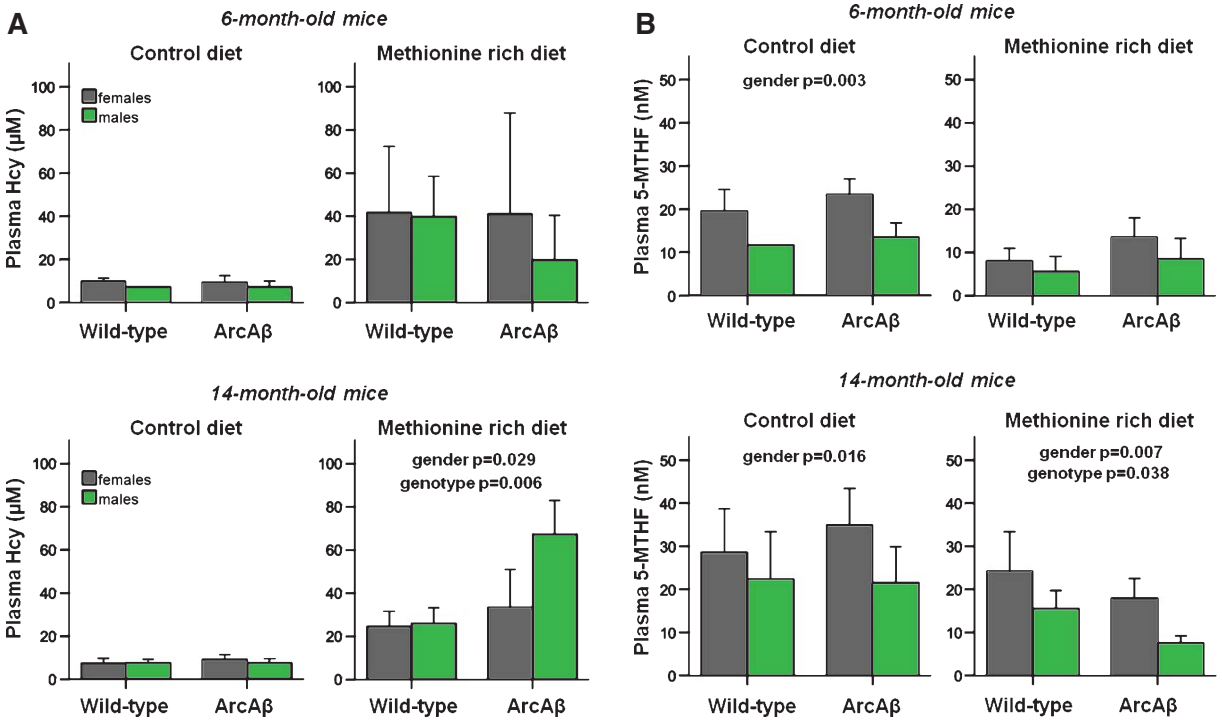

Fig. 2. Metabolite concentrations of A) plasma homocysteine and B) plasma 5-MTHF of wild-type and ArcAβ mice on either control diet or methionine rich diet at the ages of 6 months and 14 months. Values represent mean ± standard deviation. Statistically significant difference between the two genotypes or genders is indicated (analysis of variance over all groups).

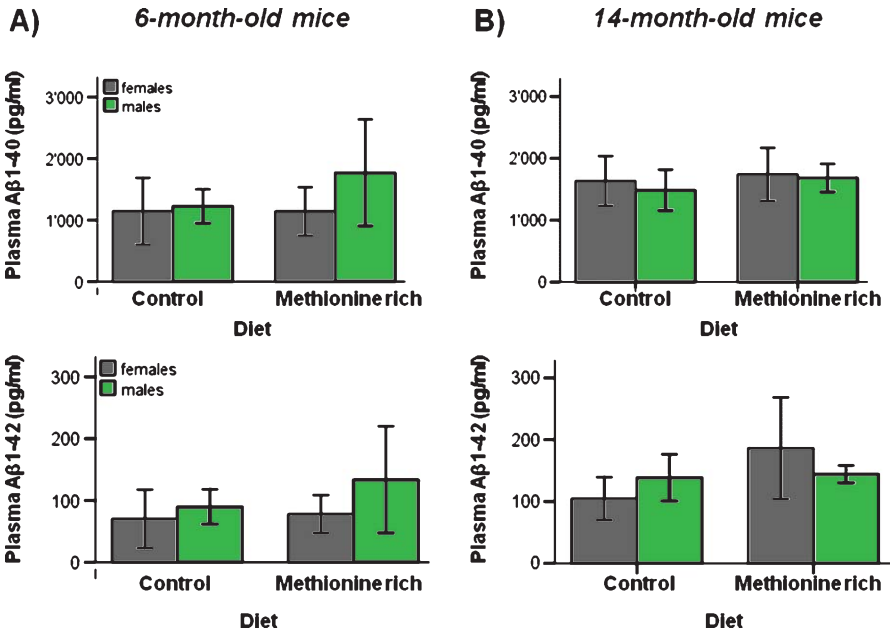

Fig. 3. Concentrations of plasma $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ in ArcAβ mice on either control diet or methionine rich diet at the ages of A) 6 months and B) 14 months. Values represent mean ± standard deviation. Statistically significant difference between the two genotypes or genders is indicated (analysis of variance over all groups).

Further, 5-MTHF was lower in males than in females ($p < 0.001$), but this was not associated with homocysteine plasma levels (Fig. 2A, B).

Aβ levels

As expected, plasma $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ levels were higher in the 14-month-old ArcAβ mice (1619 ± 350 pg/mL and 146 ± 56 pg/mL) than in 6-month-old ArcAβ mice (1288 ± 468 pg/mL and 92 ± 44 pg/mL; $p = 0.033$ and $p = 0.006$, respectively; Fig. 3). In addition, in the 6-month-old animals, $A\beta_{1\text{-}42}$ levels inversely correlated with plasma 5-MTHF levels ($r = -0.521$, $p = 0.027$, Fig. 4), which was not significant in the progressed, i.e., 14-month-old, animals ($r = -0.164$, $p = 0.465$).

Levels of both the SDS-soluble and the SDS-insoluble brain $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ fractions were below the detection limits in wild-type mice and in 6-month-old ArcAβ mice. In the 14-month-old ArcAβ mice, SDS-insoluble brain $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ were higher in animals on methionine-rich diet, which was significant for $A\beta_{1\text{-}40}$ in the female mice only ($F = 11.715$; $p = 0.027$; Fig. 5). Further explorative analyses revealed that, in these animals, SDS-insoluble brain $A\beta_{1\text{-}40}$ ($p = 0.058$ for trend) and SDS-insoluble brain $A\beta_{1\text{-}42}$ levels negatively correlated with plasma

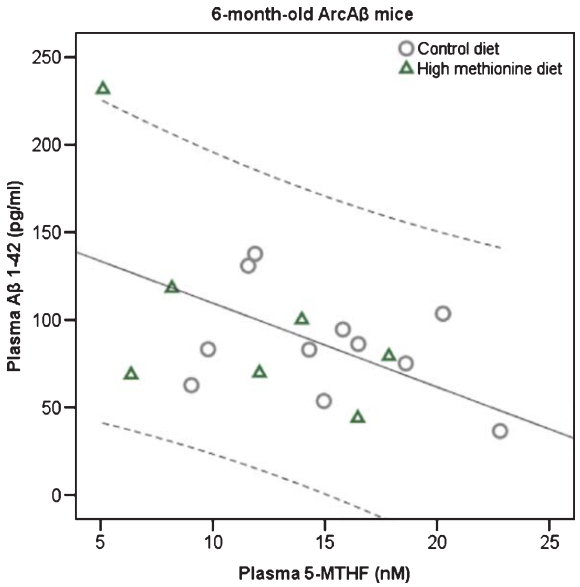

Fig. 4. Correlation between plasma 5-MTHF and plasma $A\beta_{1\text{-}42}$ in 6-month-old ArcAβ mice on either control diet (circles) or high methionine diet (triangles). Pearson correlation $r = -0.527$, $p = 0.027$, $n = 18$.

5-MTHF levels ($r = -0.905$, $p = 0.013$; Fig. 6) and with plasma homocysteine levels for trend ($r = 0.808$, $p = 0.051$).

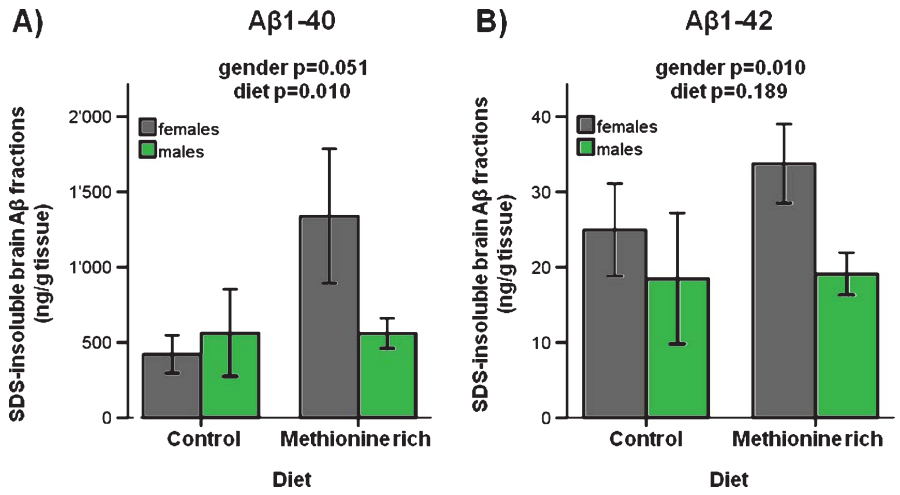

Fig. 5. Concentrations of SDS-insoluble brain A) $A\beta_{1\text{-}40}$ and B) $A\beta_{1\text{-}42}$ in 14-months-old ArcAβ mice on either control diet or methionine rich diet. Values represent mean ± standard deviation. Statistically significant difference between the two genotypes or genders is indicated (analysis of variance over all groups).

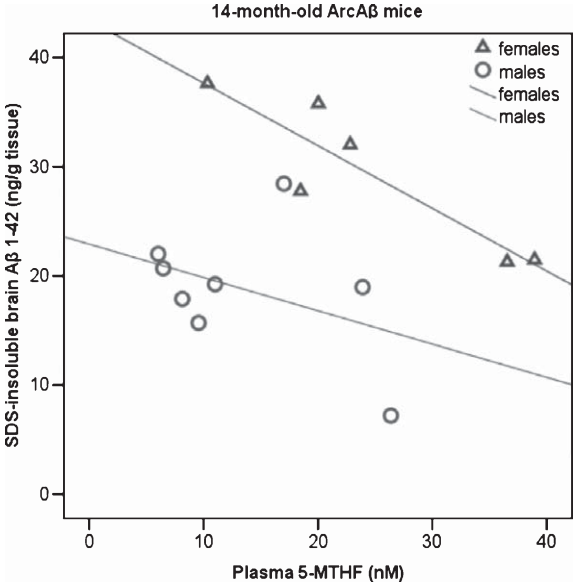

Fig. 6. Correlation between plasma 5-MTHF and SDS-insoluble brain $A\beta_{1\text{-}42}$ levels in 14-month-old ArcAβ mice of both diet groups. Pearson correlation was calculated for females (triangles; $r=-0.905$, $p=0.013$, $n=6$) and males (circles; $r=-0.404$, $p=0.321$, $n=8$).

DISCUSSION

Hyperhomocysteinemia is a risk factor for AD. In animal models, hyperhomocysteinemia induced by folate depletion promotes AD pathology [4, 34]. However, in patients low serum folate levels were observed as risk factor for cognitive decline independent from hyperhomocysteinemia questioning the role of homocysteine in folate-induced hyperhomocysteinemia in AD models [14, 15]. We analyzed homocysteine and the major biological folate derivative, 5-MTHF, in an AD mouse model, both in the natural course and under nutritional conditions inducing hyperhomocysteinemia without manipulating diet folate concentrations. As major result, we observed a reduction of plasma 5-MTHF levels and an increase of plasma homocysteine levels in higher aged transgenic animals with progressed pathology in comparison to age-matched littermates. However, these differences between transgenic and control mice were only observable in the group on hyperhomocysteinemia-inducing diet, whereas in the normal diet group, no differences between genotypes were observed. We also found negative correlations of brain (progressed pathology) and plasma Aβ levels (early pathology) with 5-MTHF and, for trend, with homocysteine (progressed pathology) levels suggesting that a possible direct or indirect interaction between folate and homocysteine metabolism with Aβ may not be restricted to the central nervous system, but may take place on a systemic level.

Importantly, oxidative stress and inflammation can deplete 5-MTHF, thereby increasing plasma homocysteine levels [27, 28]. Aβ induces oxidative stress and inflammation in the brain [29, 30]. Thus, Hoffman proposed that hyperhomocysteinemia is a consequence of folate depletion caused by oxidative stress [31]. In our study, under nutritional conditions elevating homocysteine plasma levels without folate depletion, mice with progressed neurodegeneration exhibited

stronger hyperhomocysteinemia associated with lower 5-MTHF levels in comparison to wild-type littermates. This offers room to speculate on co-acting effects of neurodegeneration and hyperhomocysteinemia leading to 5-MTHF reduction. In the course of a vicious cycle, reduced 5-MTHF increases homocysteine, which itself promotes oxidative stress, 5-MTHF reduction, and elevated Aβ levels [32]. In line with our findings, another AβPP-transgenic mouse model at a progressed stage had higher plasma homocysteine levels compared to the wild-type animals [33], whereas in mice at early phenotypic stage or on standard laboratory chow [34], no association between genotype and plasma homocysteine levels were observed [35]. In accordance to our animal data, men have higher plasma homocysteine levels than women [13]. However, we could only speculate whether there are specific differences explaining that some associations were restricted to one gender in our study, which also might be explained by chance regarding the decreased number of animals in gender subgroup analyses.

Reduction of 5-MTHF levels leads to decreased remethylation of homocysteine to the methyl group donor SAM and higher levels of the SAM antagonist SAH, with the consequence of a reduced SAM/SAH-ratio (Fig. 1). In addition to the above mentioned general aspects concerning 5-MTHF, homocysteine, oxidative stress, and neurodegeneration, such effects on the SAM/SAH-ratio affect site-specific methylation patterns of genes involved in Aβ generation in cell culture and in animal studies [4, 36, 37], e.g., leading to an increased expression of presenilin 1 and increased brain Aβ levels in both wild-type mice and AβPP-transgenic mouse models [4, 38, 39]. Such increase of Aβ can be prevented by dietary SAM supplementation [4, 38, 40, 41] underlining the relevance of methylation processes and of 5-MTHF as co-factor for SAM-synthesis in AD pathophysiology. The data of our study suggests that the relationship between low plasma folate levels, hyperhomocysteinemia, and AD is not unidirectional. In the progressed transgenic mice, hyperhomocysteinemia-inducing diet led to lower folate and higher homocysteine blood levels compared to wild-type littermates suggesting that, on the one hand, the diet had a decisive influence on folate reduction and, on the other hand, that the transgenic genotype is associated with increased vulnerability to folate reduction under such nutritional conditions. The higher increase of homocysteine observed in the progressed transgenic mice on hyperhomocysteinemia-inducing diet might well have been secondarily to the lower folate levels when compared to wild-type mice with same age and on the same diet. Speculatively, the combination of oxidative stress in neurodegeneration and of the specific diet might best explain the folate reduction and the pronunciation of hyperhomocysteinemia in the progressed transgenic mice on hyperhomocysteinemia-inducing diet. As folate levels negatively correlated with Aβ, the current experiments suggest that such changes of folate levels may be pathophysiologically relevant. We conclude that the transgenic mice are more vulnerable to develop folate depletion and hyperhomocysteinemia under respective dietary conditions and that such biochemical changes contribute to AD-like pathology. Thus, changes of folate and homocysteine metabolism may be both contributor to and consequence of neurodegeneration. This may be a vicious cycle breakable by homocysteine-lowering vitamins as suggested by the VITACOG trial [12].

DISCLOSURE STATEMENT

Authors' disclosures available online (http://www.j-alz.com/disclosures/view.php?id=1538).

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