Folic acid supplementation does not reduce intracellular homocysteine, and may disturb intracellular one-carbon metabolism.

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Abstract

Background
In randomized trails, folic acid (FA) lowered plasma homocysteine, but failed to reduce cardiovascular risk. We hypothesize this is due to a discrepancy between plasma and intracellular effects of FA.

Methods
In a double-blind trial, 50 volunteers were randomized to received 500 µg FA daily for 8 weeks, or placebo. Plasma and peripheral blood mononuclear cell (PBMC) concentrations of homocysteine (Hcy), S-adenosylmethionine (SAM), S-adenosylhomocysteine, methionine, cystathionine and 5-methyltetrahydrofolate (bioactive folate) were measured by LC-MS/MS. PBMCs were used as a cellular model since they display the full spectrum of 1C enzymes and reactions.

Results
At baseline, plasma concentrations were a poor reflection of intracellular concentrations for most 1C metabolites, except 5-methyltetrahydrofolate (R=0.33, p=0.02), Hcy (R=0.35, p=0.01), and cystathionine (R=0.45, p=0.001). FA significantly lowered plasma homocysteine (p=0.00), but failed to lower intracellular homocysteine or change the concentrations of any of the other PBMC 1C metabolites. At baseline, PBMC homocysteine concentrations correlated to PBMC SAM. After FA supplementation, PBMC homocysteine no longer correlated with PBMC SAM, suggesting a loss of SAM’s regulatory function. In-vitro experiments in lymphoblasts confirmed that at higher folate substrate concentrations, physiological concentrations of SAM no longer effectively inhibit the key regulatory enzyme methylenetetrahydrofolate reductase (MTHFR).

Conclusion
FA supplementation does not reduce intracellular concentrations of Hcy or any of its closely related substances. Rather, FA may disturb physiological regulation of intracellular 1C metabolism by interfering with SAM’s inhibitory effect on MTHFR activity. In order to correctly assess the efficacy of therapy or diet in intervention studies on the activity of cystathionine β-synthase (CBS) a sensitive analytical method is necessary.
**Introduction**

Homocysteine (Hcy), a sulphur containing amino acid, received much attention when mildly increased plasma concentrations were linked to a variety of diseases ranging from neural tube defects to cardiovascular disease. (1-3) A number of clinical trials subsequently addressed the potentially beneficial effects of B-vitamin therapy in high-risk individuals. The main focus has been on supplementation with folic acid (FA), a synthetic analog of folates. Even though FA indeed effectively decreased plasma Hcy, it failed to lower cardiovascular risk. (4) Several explanations for this apparent lack of effect have been proposed, amongst which are methodological issues relating to study designs, as well as potential unfavorable effects of folate on atherosclerotic lesions. (5) Another possibility, however, is an adverse effect of excessive folate administration on the physiology of intracellular one-carbon metabolism. However, there are no studies investigating the impact of FA.

![Figure 1](image-url). A simplified scheme of the one-carbon metabolism.

SAM (S-adenosylmethionine), SAH (S-adenosylhomocysteine), THF (tetrahydrofolate), MTHFR (methylene tetrahydrofolate reductase), CBS (cystathionine β-reductase), SH (SAH hydrolase), MAT (methionine adenosyl transferase), MS (methionine synthase).
supplementation intracellular components of 1-carbon metabolism in humans. A simplified scheme of normal one-carbon metabolism is shown in Figure 1. S-adenosylmethionine (SAM) is a key component of one-carbon metabolism, as it provides the methyl group for almost all methylation reactions, generating S-adenosylhomocysteine (SAH) in the process. In keeping with its crucial role as a universal methyl donor, SAM regulates its own availability by regulation of methionine adenosyltransferase (MAT), cystathionine β-synthase (CBS) and, most importantly, by inhibition of methylenetetrahydrofolate reductase (MTHFR) activity, which provides 5-methyltetrahydofolate (5-methylTHF) for the remethylation of Hcy to methionine.\(^{(6)}\) MAT and CBS are variably expressed in different tissues (e.g. CBS is mainly expressed in liver and kidney), whereas MTHFR is more ubiquitously present, including in vascular cells.\(^{(7)}\) In addition to Hcy, other 1C metabolites such as SAM and SAH have been the subject of study.\(^{(8)}\) These studies provided more insight into the effects of FA supplementation, showing for example that plasma SAM and SAH concentrations were not altered.\(^{(9)}\) However, the interpretation of these studies is limited by the fact that only plasma concentrations were studied, whereas 1C metabolism effectively takes place intracellularly, and the correlations between intracellular and plasma concentrations are mostly unknown. In order to circumvent this limitation, some studies have been conducted in erythrocytes. However, no relation between plasma and erythrocyte 1C metabolite concentrations was found.\(^{(10)}\) Since erythrocytes are, in terms of 1C metabolism, relatively inactive cells (Hcy remethylation and DNA/RNA synthesis do not occur), peripheral blood mononuclear cells (PBMCs) are more suitable. A few studies investigated 1C metabolites in tissues. One was in mice, but addressed only the effects of dietary methyl deficiency on intracellular SAH and DNA methylation, and the second was an observational human study suggesting a correlation between plasma SAH with intracellular SAH and DNA methylation.\(^{(11;12)}\) Intracellular 1C metabolites have not been extensively studied, in part because of their low concentrations. In particular, measuring intracellular Hcy concentrations is an analytical challenge. Substantial progress has however been made with the introduction of increasingly sensitive LC-MS/MS systems.\(^{(13)}\) Taken together, there is a lack of insight into the correlations between plasma and intracellular components of 1C metabolism. This impairs our ability to understand the (lack of) effects of folate intervention, as well as to identify potential adverse effects of pharmacological folate administration. In this study, we investigate the effect of FA supplementation on intracellular
concentrations of three key components of 1C metabolism: Hcy (the prime focus of 30 years of 1C related diseases), SAM (the universal methyl donor and central regulator of 1C fluxes), and SAH (considered to be a key toxic metabolite due to its role as a methylation inhibitor(14)). We studied their response to FA administration in samples obtained from a randomized trial in healthy volunteers.

**Materials and methods**

**Study participants**
Healthy, moderately overweight volunteers (n=50; 11 male, 39 female) entered a double-blind randomized placebo controlled trial designed to test the effect of FA supplementation on vascular function. The participants’ median age was 49 years (range 24-55), median body mass index was 27.6 (range 25.1-31.9). We excluded individuals with a history of diabetes mellitus, cardiovascular, pulmonary, neurological, gastrointestinal or other chronic disease, use of any medication affecting cardiovascular or gastrointestinal function, use of any type of (multi-)vitamin supplement during the previous 3 months, hypertension (systolic blood pressure >160 mmHg and/or diastolic blood pressure >95 mmHg), alcohol consumption in excess of 3 units per day, anemia (hemoglobin concentration <7 mmol/l), renal insufficiency (serum creatinine >130 µmol/l), hyperglycemia (fasting plasma glucose >6.9 mmol/l), and pregnancy. Subjects were randomized to receive either placebo or 500 µg of FA daily for 8 weeks. The study was approved by the local ethics committee, and written informed consent was obtained from all study participants.

**Sample collection**
Fasting venous blood was collected in EDTA vacutainers, and immediately placed on ice. Plasma was isolated by centrifugation for 10 min at 2000 g at 4ºC. PBMCs were isolated using the Histopaque 1077 system (Sigma). PBMCs were lysed by addition of 500 µL milliQ water and 3 freeze-thaw cycles. Serum (centrifuged for 10 min at 3300 g at room temperature) was used for the determination of total folate. All samples were stored at -80ºC until analysis.

**Lymphoblast cultures**
A human control lymphoblast cell lines was grown in RPMI medium containing no FA (Invitrogen, Carlsbad, California, USA) supplemented with 20 nmol/L
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5-methylTHF, 10% (v/v) heat-inactivated fetal calf serum (Invitrogen), and 1% (v/v) penicillin-streptomycin (Invitrogen). Cultures were grown in 175 cm² culture flasks (Greiner Bio One, Frickenhausen, Germany) and maintained at 37°C in an atmosphere of 5% CO₂. The cells were harvested after 5 passages and washed twice with Hank’s buffered salt solution (Invitrogen). Cell pellets were stored at -80°C. Lymphoblast extracts were obtained by 3 freeze-thaw cycles. Subsequently the cell lysate was centrifuged (8000 g, 10 min., 4°C) to remove cell membranes and debris.

**Analytical methods**

Serum folate concentrations were determined by means of a competitive immunnoassay (Architect, Abbott Laboratories, Abbott Park, IL). Inter- and intra-assay CVs were 4% and 6%, respectively. Plasma and PBMC SAM and SAH concentrations were determined by LC-MS/MS (API3000, Applied Biosystems, Foster City, CA, USA). The intra-assay and inter-assay CVs for SAM were 6.8% and 4.2%, respectively. The intra-assay and inter-assay CVs for SAH were 6.9% and 5.5%, respectively. PBMC folate vitamers were also determined by LC-MS/MS. Intra-assay and inter-assay CVs for 5-methyltetrahydrofolate (the bioactive folate vitamer involved in Hcy remethylation) were 1.2% and 2.8%, respectively. Intra-assay and inter-assay CVs for the composite of non-methylTHF vitamers were 1.6% and 1.5%, respectively.

Plasma and PBMC total Hcy, methionine (Met) and cystathionine (Cysta) concentrations were measured by LC-MS/MS. For the PBMC lysates, the internal standard mixture contained 12.5 pmol [D₄]-Hcy (C/D/N Isotopes Inc., Quebec, Canada), 80 pmol [¹³C, D₃] Met (Isotec, Miamisburg, Ohio, USA) and 125 pmol [D₄] Cysta (C/D/N Isotopes Inc., Quebec, Canada) for the PBMC lysates. For the plasma samples, the internal standard mixture contained 250 pmol [D₄]-Hcy, 625 pmol [¹³C, D₃] Met and 125 pmol [D₄] Cysta. After the cells were lysed using 0.1% lubrol, the internal standard mixture was added, and the samples were reduced using 25 mmol/L dithiothreitol for 30 minutes at 4°C. Oasis MCX cartridges (Waters, Milford, MA, USA) were used for sample clean-up. The cartridges were conditioned with 500 µL of methanol, and 1 mL of water. After conditioning, the mixture was applied to the cartridges. The cartridges were washed with 1.5 mL of water and subsequently eluted with 500 µL of 6 mol/L ammonia into vials. The ammonia fraction was evaporated under nitrogen at 45°C, and re-dissolved in 100 µL 10 mmol/L dithiothreitol. Subsequently, positive electrospray LC-MS/MS was used for determination of concentrations of Hcy, Met and Cysta. The following transitions were used: for Hcy 136.1→90.1 and 140.1→94.1, for Met...
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150.1→104.0 and 154.1→108.0, and for Cysta 223.1→134.0 and 227.1→138.0. Using an Xterra MS C18 analytical column (2.9 x 100 mm; 3.5 µm; Waters) 20 µL of the sample was separated using 5 mmol/L nonapentanoic acid as an ion pair reagent. In 15 minutes the acetonitrile content was linearly increased from 10% to 50%. Average recovery was 98% (n=4), intra-assay CVs (n=6) were <9% for Hcy, Met & Cysta, the sample was stable for at least 24 hours at 4ºC, and 6 freeze-thaw cycles, no carry over was observed between injections, and no signal suppression was observed. Standards were stable (no homocysteic acid or homocysteinesulfinic acid was observed). Linearity ranges from 1 nmol/L – 100 µmol/L for all 3 metabolites.

DNA methylation (DNA-CH$_3$) of genomic DNA of PBMCs was measured by LC-MS/MS (API3000, Applied Biosystems). Intra-assay and inter-assay CVs were 1.7% and 3.5%, respectively. DNA-CH$_3$ was calculated using the following formula: nmol 5-methylcytosine/(nmol 5-methylcytosine + nmol cytosine)*100% (nmol mCyt/(nmol mCyt + nmol Cyt)*100%).

The effect of SAM on MTHFR activity was determined by SAM incubation of a lysate of lymphoblasts in a phosphate buffer (pH=6.5) containing 1 mmol/L NADPH and 12.5-400 nmol/L R-5,10-methyleneTHF for 30 min at 37ºC. Concentrations of SAM were varied from 50-1000 nmol/L. The amount of 5-methylTHF formed was measured by LC-MS/MS. Intra- and inter-assay variations were 2.6% and 12.1%, respectively. Details of the method will be published elsewhere.

Statistical analysis

All statistical analyses were performed using SPSS 17.0 for Windows. Spearman rank correlations were determined between 1C metabolite concentrations in PBMCs and their respective plasma concentrations. One-way ANOVA was used to determine whether FA supplementation had a significant effect on any of the studied PBMC 1C metabolite concentrations. As outlined in the introduction, the key 1C metabolites in PBMCs were considered to be Hcy, SAM and SAH. Their potential determinants were first identified in univariate regression analyses. Subsequently, multivariate regression analyses were conducted to identify independent determinants of PBMC Hcy, SAM and SAH. The same analyses were done after FA supplementation.

Results

Table 1 shows baseline concentrations of 1C metabolites in both plasma and
PBMCs. Note that PBMCs have a ~20 times higher Met/Hcy ratio compared to plasma. Another ratio that is different, although less pronounced, is the SAM/SAH ratio, which is 2-fold higher in PBMCs.

Table 1. Study population characteristics.

<table>
<thead>
<tr>
<th></th>
<th>Plasma</th>
<th>PBMC</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>median</td>
<td>range</td>
<td>unit</td>
<td>median</td>
</tr>
<tr>
<td>Hcy</td>
<td>8.3</td>
<td>4.4-22.0</td>
<td>µmol/L</td>
<td>33.3</td>
</tr>
<tr>
<td>Met</td>
<td>22.0</td>
<td>15.2-31.9</td>
<td>µmol/L</td>
<td>3.9</td>
</tr>
<tr>
<td>Cysta</td>
<td>0.10</td>
<td>0.04-0.44</td>
<td>µmol/L</td>
<td>1.8</td>
</tr>
<tr>
<td>SAM</td>
<td>81.9</td>
<td>55.5-106.4</td>
<td>nmol/L</td>
<td>528</td>
</tr>
<tr>
<td>SAH</td>
<td>13.2</td>
<td>6.8-22.5</td>
<td>nmol/L</td>
<td>49</td>
</tr>
<tr>
<td>SAM/SAH</td>
<td>6.5</td>
<td>3.9-9.8</td>
<td></td>
<td>11.0</td>
</tr>
<tr>
<td>5-methylTHF</td>
<td>9.6</td>
<td>4.5-29.8</td>
<td>nmol/L</td>
<td>1.5</td>
</tr>
<tr>
<td>DNA-CH3</td>
<td>4.6</td>
<td>3.9-5.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Correlation between plasma and PBMC concentrations of 1C metabolites

Correlations between the plasma and PBMC concentrations of the various 1C metabolites are shown in Table 2. Plasma concentrations poorly reflect intracellular PBMC concentrations for most 1C metabolites. Only Hcy, 5-methylTHF and Cysta show a significant, but still relatively weak correlation. PBMC concentrations of SAM, SAH and Hcy also did not correlate significantly.

Table 2. Correlation of plasma versus intracellular PBMC concentrations of 1C metabolites.

<table>
<thead>
<tr>
<th>1C metabolite</th>
<th>R</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homocysteine</td>
<td>0.35</td>
<td>0.01a</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.02</td>
<td>0.90</td>
</tr>
<tr>
<td>Cystathionine</td>
<td>0.45</td>
<td>0.00b</td>
</tr>
<tr>
<td>SAM</td>
<td>0.15</td>
<td>0.29</td>
</tr>
<tr>
<td>SAH</td>
<td>0.11</td>
<td>0.47</td>
</tr>
<tr>
<td>SAM/SAH</td>
<td>0.27</td>
<td>0.06</td>
</tr>
<tr>
<td>5-methylTHF</td>
<td>0.33</td>
<td>0.02a</td>
</tr>
</tbody>
</table>

Spearman correlations, aCorrelation is significant at the 0.05 level (2-tailed), bCorrelation is significant at the 0.01 level (2-tailed)
with plasma concentrations of any of the other 1C metabolites (data not shown).

**Effects of FA supplementation on PBMC 1C metabolite concentrations**

As expected, plasma Hcy significantly decreased in the FA group (from 8.3 to 4.9 µmol/L, P<0.01). However, PBMC Hcy concentrations remained unchanged (baseline median 36.4 pmol/mg protein, after FA median 36.6 pmol/mg protein (Figure 2)).

![Figure 2. Hcy concentrations before (basal) and after FA supplementation (FA).](image)

The middle line of the box represents the median. The lower and higher line represent the 25th and 75th percentile, respectively.

Also, none of the other PBMC 1C metabolite concentrations was significantly altered after FA supplementation, although a non-significant trend was observed for 5-methylTHF (baseline median 1.2 pmol/mg protein, after FA 2.3 pmol/mg protein, p=0.18) and non-methylTHF (baseline median 0.6 pmol/mg protein, after FA median 0.8 pmol/mg protein, p=0.07) (Figure 3).
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**Table 3.** Multivariate regression models of predictors of PBMC Hcy, SAM and SAH concentrations at baseline (n=50) and after folic acid supplementation (n=25).

<table>
<thead>
<tr>
<th>Dependent</th>
<th>Predictor</th>
<th>Baseline</th>
<th></th>
<th>After folic acid</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>β</td>
<td>p</td>
<td>β</td>
<td>p</td>
<td></td>
</tr>
<tr>
<td>PBMC Hcy</td>
<td>PBMC SAM</td>
<td>0.43</td>
<td>0.00^b</td>
<td>0.20</td>
<td>0.18</td>
</tr>
<tr>
<td></td>
<td>PBMC SAH</td>
<td>0.22</td>
<td>0.10</td>
<td>0.45</td>
<td>0.03^a</td>
</tr>
<tr>
<td></td>
<td>PBMC 5-methylTHF</td>
<td>-0.17</td>
<td>0.09</td>
<td>-0.09</td>
<td>0.50</td>
</tr>
<tr>
<td></td>
<td>PBMC Met</td>
<td>0.39</td>
<td>0.00^b</td>
<td>-0.32</td>
<td>0.09</td>
</tr>
<tr>
<td></td>
<td>Plasma Hcy</td>
<td>0.30</td>
<td>0.00^b</td>
<td>0.37</td>
<td>0.01^a</td>
</tr>
<tr>
<td>PBMC SAM</td>
<td>PBMC Hcy</td>
<td>0.62</td>
<td>0.00^b</td>
<td>0.32</td>
<td>0.26</td>
</tr>
<tr>
<td></td>
<td>PBMC SAH</td>
<td>0.25</td>
<td>0.03^a</td>
<td>0.10</td>
<td>0.72</td>
</tr>
<tr>
<td>PBMC SAH</td>
<td>PBMC SAM</td>
<td>0.65</td>
<td>0.00^b</td>
<td>0.06</td>
<td>0.72</td>
</tr>
<tr>
<td></td>
<td>PBMC Hcy</td>
<td>0.18</td>
<td>0.13</td>
<td>0.69</td>
<td>0.00^b</td>
</tr>
</tbody>
</table>

^aCorrelation is significant at the 0.05 level (2-tailed), ^bCorrelation is significant at the 0.01 level (2-tailed). Models were adjusted for the concentration of other 1C metabolites. Additional adjustment for gender, age and BMI yielded similar results (data not shown).

Determinants of PBMC Hcy at baseline and after FA supplementation

Multiple regression analysis showed that baseline PBMC Hcy concentrations were predicted by PBMC SAM. PBMC Hcy also correlated with PBMC Met, and plasma Hcy concentrations (Table 3). The correlation between Hcy and SAM in PBMCs is illustrated in Figure 4. After FA supplementation, PBMC Hcy concentrations were correlated with PBMC SAH and plasma Hcy concentrations, but no longer with PBMC SAM, (Table 3). ΔPlasma Hcy and ΔPBMC Hcy did not correlate (R=0.14, p=0.51).

Correlations of PBMC SAM and SAH at baseline and after FA supplementation

Multiple regression analysis showed that, at baseline, PBMC SAM concentrations were predicted by PBMC SAH and PBMC Hcy (Table 3). After FA supplementation, PBMC SAM concentrations did not correlate with any of the 1C metabolites in plasma or in PBMCs. Multiple regression analysis showed that, at baseline, PBMC SAH concentrations are only predicted by PBMC SAM (Table 3). After FA supplementation PBMC SAH concentrations are
predicted only by PBMC Hcy (Table 3).

**Figure 3.** PBMC concentrations of key 1C metabolites before (basal) and after folic acid supplementation (FA).

The middle line of the box represents the median. The lower and higher line represent the 25th and 75th percentile, respectively.

**In-vitro inhibition of MTHFR by SAM in lymphoblast cells**

The percentage MTHFR activity that persisted after addition of 1 µmol/L of its natural inhibitor SAM (which resembles physiological concentrations in lymphoblasts, different concentrations between 0.25 µmol/L and 1 µmol/L showed similar results, data not shown) for various folate substrate concentrations is shown in Figure 5. SAM-induced inhibition of MTHFR in lymphoblast cells becomes less pronounced as folate substrate concentrations rise to supraphysiological concentrations. Measurement of MTHFR activity for lower substrate concentrations was not possible due to sensitivity of the assay.
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Discussion

In this paper, we show that plasma concentrations of Hcy are not representative of intracellular Hcy concentrations, at least not in PBMCs. Moreover, although plasma Hcy is significantly lowered after FA supplementation, intracellular Hcy is not. Finally, we found evidence indicating that FA supplementation may disturb normal regulation of intracellular one-carbon metabolism by interfering with physiological MTHFR inhibition by SAM.
The lack of an in-vivo intracellular Hcy lowering effect of FA in a cell type representing the full spectrum of 1C metabolic reactions in humans is novel and provides more understanding of the effect of FA intervention trials. Many of the hypothesized adverse effects of elevated plasma Hcy have been related to intracellular mechanisms.\textsuperscript{(5;18-20)} A continuum between the extra- and intracellular space in terms of 1C metabolites has been assumed by many, but studied by few.\textsuperscript{(10;21)} Although in some animal studies intracellular concentrations of 1C metabolites have been measured in a variety of tissues\textsuperscript{(22;23)}, human studies are very scarce\textsuperscript{(22)} and the effects of folate-based interventions on intracellular concentrations are lacking. In addition, a correlation between intracellular 1C metabolites and cardiovascular disease has never been shown.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure5.png}
\caption{The inhibition of MTHFR activity in lymphoblasts by SAM (1 \textmu mol/L) at different 5,10-methyleneTHF concentrations.}
\end{figure}

Numbers above the symbols represent the amount of 5,10-methyleneTHF added. Lymphoblasts were grown on medium containing 20 nmol/L 5-methylTHF.
There are several possible explanations for the absence of intracellular Hcy lowering after FA. Plasma Hcy and its response to therapy may predominantly be a reflection of Hcy production/export by specific organs or tissues, such as the liver and kidney, rather than a reflection of Hcy concentrations in most cell types.(23) Alternatively, cellular Hcy may simply be too tightly regulated to be affected by folate administration, for example by export of excess Hcy from the cell or by regulation of Hcy transsulphuration. In any event, the intracellular concentrations of Hcy and SAH, two of the prominent candidates for explaining the adverse effects in hyperhomocysteinemia, remain unchanged after FA supplementation. The lack of change in intracellular SAM and SAH concentrations, may explain why, in our study, global DNA methylation remain unaffected after FA supplementation.(24)

Consistent with its role as a central regulator of 1C metabolic fluxes (25), PBMC SAM concentrations are, at least statistically, determinants of PBMC concentrations of SAH and Hcy. After FA administration, however, this is no longer the case. SAM has the ability to control SAH and Hcy concentrations both by inhibiting MTHFR activity and by activating CBS.(6) Since the activity of CBS is very low in this cell type, we hypothesized that SAM loses its ability to inhibit MTHFR at higher folate substrate concentrations, and investigated this in a subsequent MTHFR activity assay. In-vitro experiments in lymphoblasts indeed showed that at higher folate substrate concentrations, physiological concentrations of SAM no longer inhibit MTHFR. If Figure 5 is extrapolated to lower physiological folate concentrations (estimated 2 nmol/L), a small change in substrate concentrations is expected to account for a large change in regulation by SAM. The substrate for MTHFR is 5,10-methylenetetrahydrofolate and, ideally, the exact concentration of this substrate would be measurable in PBMCs after FA treatment. However, the measurement of individual non-methylfolates is hampered by interconversions between the folate forms in this group during analysis (i.e. 5,10-methyleneTHF, 5,10-methenylTHF, 10-formylTHF, 5-formylTHF and THF).(16) In PBMCs, total non-methylTHF concentrations increased by 20% due to FA supplementation. This 20% may, however, well be an underestimation of the increased substrate flux via MTHFR, as concentrations of 1C metabolites are often poor reflections of changes in flux rates, and for example remethylation fluxes have been shown to increase sharply after FA treatment.(26) Hence, be it via increased substrate concentrations or via increased flux via the MTHFR pathway, there is evidence of disturbed physiological regulation of MTHFR activity by SAM after FA supplementation.

A few limitations of this study merit discussion. Firstly, this study was conducted in PBMCs, because this is the only easily available cell type with
the complete spectrum of cellular one-carbon metabolic pathways to study in humans. To ascertain that the effects are applicable to cardiovascular disease, other cell types, like endothelial cells, need to be studied, but this may prove to be a major challenge and require vascular biopsy studies. Secondly, the MTHFR inhibition experiment was carried out in isolated lymphoblasts, which may not fully represent what happens in vivo, either in PBMCs or in other cell types. Finally, it would have been interesting to study the effect of 5MTHF instead of FA supplementation, since FA may directly have inhibited MTHFR. (27) Also, we showed in a previous study discrepant effects of FA and 5MTHF on human umbilical vein endothelial cells, with an apparent benefit of 5MTHF in terms of impacting on intracellular 1C fluxes, at least as suggested by changes in Hcy export from these cells. (28)

In conclusion, although FA originally has been administered to lower plasma Hcy, knowledge of how Hcy concentrations became elevated in the first place, and how plasma concentrations were related to intracellular concentrations, was lacking. We have demonstrated that plasma Hcy only weakly correlates with PBMC Hcy, and that although FA lowers plasma Hcy, intracellular PBMC Hcy concentrations remain unaffected. Moreover, we found that FA supplementation may interfere with the normal regulatory effect of SAM on Hcy metabolism, which we could confirm in-vitro. We suggest that future studies addressing the biochemical effects of interventions with folate or other B-vitamins should not just concern plasma 1C metabolite concentrations, but extend their focus to what happens within cells, not just plasma.

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