Reduction of the genomic damage level in haemodialysis patients by folic acid and vitamin B12 supplementation

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Abstract

Background. Cancer incidence and genomic damage of peripheral lymphocytes are elevated in patients with end-stage renal failure. Among other uraemic toxins, homocysteine (Hcy) levels are increased in most of these patients. In healthy individuals, plasma Hcy correlates with the degree of genomic damage observed in peripheral blood lymphocytes (PBL). The accumulation of Hcy can be reduced by supplementation with folic acid and vitamin B12. The aim of this study was to analyse whether this supplementation can also lower the genomic damage in PBL of haemodialysis patients. This may ultimately help to reduce cancer incidence in renal patients.

Methods. In a prospective study with 27 patients, we analysed the genomic damage in dialysis patients before and at different time points after the initiation of folate/vitamin B12 supplementation. Genomic damage was measured by the frequency of micronuclei, a subset of chromosomal aberrations, in PBL.

Results. Supplementation with folic acid and vitamin B12 (more marked with both) reduced the micronucleus frequency in PBL of dialysis patients. This was not mediated by altered lymphocyte proliferation capacity or changes in DNA cytosine-methylation. Plasma-Hcy was lowered more efficiently by the combined folic acid/vitamin B12 supplementation, and lymphocyte DNA of this group exhibited a nonsignificant trend for a reduction of 1,N6-etheno-2′-deoxyadenosine, a marker for oxidative stress.

Conclusions. A reduction of the genomic damage in PBL can be achieved in dialysis patients by supplementation with folic acid and vitamin B12. This may be mediated by Hcy reduction.

Keywords: dialysis; folic acid; homocysteine; micronuclei; vitamin B12

Introduction

End-stage renal failure (ESRF) is characterized by a high incidence of cardiovascular disease and cancer [1–3]. Suppression of the immune system, chronic inflammation, viral-associated factors, increased levels of oxidative stress, reduced antioxidant levels and accumulation of uraemic toxins are among the factors thought to contribute to both types of health consequences.

In ESRF patients, whether pre-dialysis or on dialysis, elevated genomic damage in peripheral blood leukocytes compared to healthy controls has been demonstrated by the micronucleus frequency assay [4–5], analysis of sister-chromatid exchanges [6], single-cell gel electrophoresis [7–8] and by measuring the 8-hydroxy 2′-deoxyguanosine (8-OHdG) content in leukocyte DNA by many authors [e.g. 8–12]. Assays of DNA damage in peripheral blood lymphocytes (PBL) are widely used in occupational and environmental settings as biomarkers of early effects of genotoxic carcinogens. Cohort studies have shown an association between the frequency of cells with micronuclei in PBL and cancer risk [13].

A prominent uraemic toxin is the sulfur-containing amino acid homocysteine (Hcy), which is a normal product in the metabolism of the essential amino acid methionine, a methyl donor for DNA cytosine-methylation. In healthy individuals Hcy is remethylated to methionine (predominantly in the kidney) or can be broken down to cysteine and glutathione by the trans-sulfuration pathway. Normal levels of fasting plasma Hcy range between 5 and 15 µM, while moderate, intermediate and severe hyperhomocysteinaemia are defined as between 16 and 30 µM, 31 and 100 µM and >100 µM. ESRF is associated with moderate-intermediate hyperhomocysteinaemia [e.g. 14]. Hcy has often been considered a strong predictor of both cardiovascular and non-cardiovascular mortality in the general population [15] and of cardiovascular mortality in ESRF patients [16]. However, recent publications found no association between Hcy levels and cardiovascular events [17–18] and no lowering of major cardiovascular complications after the reduction of Hcy [19–20].
Additionally, hyperhomocysteinemia has been discussed as a risk factor for cancer and as a potential tumour marker [21]. This is in line with studies revealing a positive correlation between plasma Hcy and genomic damage (micronuclei) in healthy individuals [22]. The DNA of dialysis patients exhibiting hyperhomocysteinemia has been found to be hypomethylated [23], which may be due to the inhibition of methyltransferases by the accumulation of Hcy or the Hcy precursor S-adenosylhomocysteine [24]. Hypomethylated DNA tends to become more instable [25], which might be a mechanistic explanation for the enhanced genomic damage observed in dialysis patients.

Folic acid, which is required to metabolize Hcy to methionine, lowers plasma Hcy in ESRF patients. Adding vitamin B12 further enhances the reduction of plasma Hcy further [18,26–27]. It has been shown that folic acid can lower the micronucleus frequency in PBL in vitro [28] and that folic acid and vitamin B12 are important modulators of the micronucleus frequency in PBL of healthy individuals [29–31]. For example, micronucleus frequencies were lowered by a 6-month folic acid supplementation [31].

In the present study, we investigate for the first time the effects of folic acid and vitamin B12 supplementation on genomic damage levels in peripheral blood lymphocytes of dialysis patients. The ultimate goal would be to lower cancer incidence in renal failure patients.

### Materials and methods

**Chemical**

The standards 5-methyl-2′-deoxycytidine (5-mdCyt), 2′-deoxyguanosine (dGuo) and 1,N6-etheno-2′-deoxyadenosine (εdAdo) were from Sigma-Aldrich (Taufkirchen, Germany). Alkaline phosphatase (calf intestine) was from Calbiochem (Darmstadt, Germany), Nuclease P1 from MP Biomedicals, Inc. (Aurora, OH, USA), and HPLC-grade acetonitrile, methanol and water for liquid chromatography were purchased from Roth (Karlsruhe, Germany).

**Participants**

The study was approved by the Ethics Committee of the University of Wuerzburg and conducted in accordance with the current version of the Declaration of Helsinki. All participants gave their written informed consent prior to participation in the study.

Twenty-seven stable long-term dialysis patients of the haemodialysis programme of the KfH-Nierenzentrum (Kuratorium für Dialyse und Nierentransplantation Würzburg e.V.) in Wuerzburg were included in the study. Exclusion criteria were bacterial or viral infections and malignancies. All patients were treated with synthetic, bio-compatible, non-complement-activating polysulfone membranes (FX60 dialyzer, Fresenius Medical Care, Bad Homburg, Germany).

The patients’ demographic characteristics, time on haemodialysis, primary kidney disease and medications are summarized in Table 1. First, basal micronucleus frequencies were determined three times in 1-week intervals. Then, triplets of patients with similar average micronuclear frequencies were formed. Of these, one patient was assigned to the control group (CO group) and the others to the treatment groups randomly. Whether the treated patients received folic acid (FA group) or folic acid + vitamin B12 (FA/B12 group) was chosen according to dialysis group (week day). Later drop out yielded unequal patient numbers in the groups. Patients received Folgamma i.v. (15 mg folic acid) thrice weekly after the dialysis session. Patients of the FA/B12 group received an additional amount of vitamin B12 in i.v. (vitamin B12 forte Hevert inject; 1000 µg hydroxocobalamin) once a week. Folic acid was donated by Wörwag Pharma GmbH (Böblingen, Germany) and vitamin B12 depot by Hevert (Nussbaum, Germany).

**Collection of blood samples**

Blood samples (9 ml) were collected before the dialysis session in heparin-containing tubes. The samples were transported at room temperature to the Department of Toxicology for immediate isolation of peripheral blood

<table>
<thead>
<tr>
<th>Patient group</th>
<th>CO</th>
<th>FA</th>
<th>FA/B12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients</td>
<td>8</td>
<td>9</td>
<td>10</td>
</tr>
<tr>
<td>Gender (female/male)</td>
<td>4/4</td>
<td>1/8</td>
<td>3/7</td>
</tr>
<tr>
<td>Age (years)</td>
<td>60.3 ± 8.6</td>
<td>64.4 ± 10.9</td>
<td>68.2 ± 16.4</td>
</tr>
<tr>
<td>Smoker/non-smoker</td>
<td>1/7</td>
<td>1/8</td>
<td>1/9</td>
</tr>
<tr>
<td>Time on haemodialysis (years)</td>
<td>5.1 ± 5.5</td>
<td>9.0 ± 7.5</td>
<td>6.6 ± 2.9</td>
</tr>
<tr>
<td>Causes of chronic renal failure</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Glomeronephritis</td>
<td>4</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Shrunken kidney</td>
<td>1</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Polycystic kidney disease</td>
<td>1</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Chronic pyelonephritis</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Diabetic nephropathy</td>
<td>1</td>
<td>1</td>
<td>2</td>
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<tr>
<td>Nephrosclerosis</td>
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<tr>
<td>Other</td>
<td></td>
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</tbody>
</table>

Medication with folic acid and vitamin B12 is described in the Materials and methods section. CO: control group; FA: supplementation with folic acid; FA/B12: supplementation with folic acid and vitamin B12. Group averages are given as means ± standard deviation. ACEI: angiotensin II-converting enzyme inhibitor; ARB: angiotensin II receptor type 1 blocker; hHu EPO: recombinant human erythropoietin; Multivitamins: Carena® (ε-dAdo), containing vitamins B1 (2.4 mg), B2 (3 mg), B6 (10 mg), B12 (6 µg), vitamin C (100 mg), niacin (30 mg), folic acid (1000 µg), pantothenic acid (18 mg), biotin (150 µg), vitamin E (50 mg) and trace elements: selenium (50 µg) and zinc (25 mg) (Fresenius Medical Care, Bad Homburg, Germany).

### Table 1. Patient characteristics
mononuclear cells (PBMC). To determine the basal micronucleus frequencies, a total of three blood samples of each patient were taken in 1-week intervals. After initiation of vitamin supplementation, samples were taken 4, 12 and 17 weeks later for the determination of most markers and 20 weeks later for DNA isolation.

**Cell isolation**

PBMC were isolated by standard density gradient centrifugation using FicoLite H (Linaris-H, Wertheim, Germany). In brief, the blood was layered (1:1) onto FicoLite and centrifuged at 1600 rpm (370 g) for 30 min at room temperature. The PBMC layer was removed and the cells were washed twice (1300 rpm; 250 g; 10 min) with RPMI-1640.

**Culture conditions**

PBMC were cultured at a cell density of 1 Mio/ml in 5 ml of culture medium at 37°C in a humified, 5% CO₂ incubator. The culture medium consisted of RPMI-1640, supplemented with 15% fetal bovine serum, l-glutamine, N-acetylcycteine, antibiotics (penicillin/streptomycin/tylosine) and non-essential amino acids. For mitotic activation of lymphocytes, phytohaemagglutinin (PHA, final concentration 1 μg/ml) was added.

**Micronucleus assay**

Micronuclei develop in dividing cells and contain chromosome fragments or whole chromosomes that are unable to travel to the spindle poles during mitosis. They are observed in cells with completed nuclear division and are counted in a binucleated stage of the cell cycle after using the cytokinesis inhibitor, cytochalasin B (final concentration 5 μg/ml), which was added to the cultures at 44 h after stimulation with PHA. At 72 h after incubation the cells were spun on glass slides with a cytocentrifuge (Shandon, CytoSpin 3; 1000 rpm; 145 g; 5 min). Subsequently, fixation in cold (−20°C) methanol was performed for at least 2 h. Slides were stored at −20°C in sealed boxes. Before evaluation, slides were stained with acridine orange (0.00625% in Sörensen buffer, w/v; pH 6.8) for 5 min, and washed twice in Sörensen buffer.

From each blood sample, 1000 binucleated lymphocytes were screened for the presence of micronuclei with fluorescence microscopy at ×400 magnification. Objects were classified as micronuclei if they appeared separated from the nucleus, were round or oval, showed staining characteristics similar to those of the nuclei and had an area <1/4 of the area of the average normal nucleus. In addition, the percentage of binucleated lymphocytes was assessed on the same slides (from 1000 cells) as a marker for proliferation capacity. Frequencies of apoptotic cells were also registered and were always <1%.

**Homocysteine analysis**

Plasma Hcy concentrations were determined at the beginning of the study and after 12 weeks of vitamin supplementation according to the method described in [32]. From one patient of the FA/B12 group no sample was available. Hcy represents total, protein-bound and non-protein-bound Hcy. Briefly, the procedure involves a preliminary step of reduction and release from plasma proteins, using tri-n-butyl-phosphine in dimethylformamide, followed by precolumn derivatization with ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulphonate (SBD-F). Separation was accomplished on a C18, 5 μm, 250 × 4.6 mm, reverse-phase column (‘Luna’, Phenomenex, Torrance, CA, USA). The mobile phase was 0.1 M KH₂PO₄, pH 2.1, containing 4% acetonitrile, flow rate 1.0 ml/min. Micromolar concentrations of Hcy are referred to 1 l of plasma. Fluorescence intensities were measured with excitation at 385 nm and emission at 515 nm, using a Shimadzu RF-535 fluorescence detector (Shimadzu Co., Kyoto, Japan), equipped with a Shimadzu Chromatopac C-R6A data processor. Data are shown as means ± standard deviations of the groups.

**LC-MS/MS analysis of DNA cytosine-methylation and 1,N6-etheno-2′-deoxyadenosine (εdAdo)**

DNA was isolated from whole blood at the beginning of the study and after 20 weeks of supplementation (end of study) from all patients of the CO group, eight patients of the FA group and nine patients of the FA/B12 group. A DNA isolation kit (Nucleobond® AXG, Macherey-Nagel, Düren, Germany) was used following the users manual for ‘Isolation of genomic DNA from blood and cell cultures’. The DNA pellet was then dissolved in H₂O. Concentration and purity were determined by measuring the OD at 230, 260 and 280 nm. Ratios of 260/280 and 230/260 between 1.8 and 2.0, and 0.35 and 0.45, respectively, were considered acceptable.

The quantification of 5-methyl-2′-deoxycytidine (5-mdCyt) in samples of 10 μg of DNA was performed as described previously [33]. From the 20-week samples, DNA was additionally used for quantification of 1,N6-etheno-2′-deoxyadenosine (εdAdo; representing an oxidative stress-induced DNA alteration mediated by lipid peroxidation) to be analysed by LC-MS/MS as described recently in detail [34] for seven patients of the CO group, seven patients of the FA group and nine patients of the FA/B12 group. Levels of εdAdo were expressed as adducts per 10⁹ normal nucleotides (nt). Since analysis of εdAdo requires larger amounts of DNA (>50 μg), the remaining DNA allowed for only one measurement per sample. Means ± standard deviations of the groups are given.

**Statistics**

The Wilcoxon matched-pairs test was used for comparing a sampling time with the basal value before supplementation for a particular group and the Wilcoxon two-sample test for comparison between different groups. P values <0.05 were considered significant.

**Results**

To investigate the effects of folic acid and vitamin B12 supplementation on the genomic damage in PBL of dialysis patients with CRF.
patients, we recruited 27 patients and grouped them according to similar basal micronucleus frequencies, derived from three sampling time points in 1-week intervals (Figure 1) to receive either no study treatment (control/CO), folic acid (FA) and folic acid plus vitamin B12 (FA/B12). Despite some dropouts and therefore slightly uneven patient numbers per group, there was no significant deviation in the mean micronucleus frequency (Figure 1), mean age and time on dialysis, plasma Hcy, CRP and albumine levels, as well as dialysis efficiency measured as Kt/V (Table 1) between the groups (\( P > 0.05 \); Wilcoxon two-sample test). In their basal values (before supplementation), the 19 male patients exhibited a significantly lower micronucleus frequency of 31.4 ± 6.7 micronucleated cells/1000 binucleate cells (MNC/1000) than the 8 female patients (50.8 ± 23.9; \( P < 0.01 \); Wilcoxon two-sample test). No difference was observed for the basal micronucleus frequencies between the 17 individuals who had been medicated with multivitamins (Carenal®) (37.6 ± 16.9 MNC/1000) and the remaining 8 (36.3 ± 16.2 MNC/1000) (\( P > 0.05 \); Wilcoxon two-sample test).

When the sampling time points of 4 weeks, 12 weeks and 17 weeks were compared to the basal micronucleus frequencies (Figure 2), a significant reduction of genomic damage was detected at 12 and 17 weeks in the FA/B12 group (\( P = 0.037 \) for 12 weeks and \( P = 0.004 \) for 17 weeks), at all time points in the FA group (\( P = 0.02 \) for 4 weeks, \( P = 0.008 \) for 12 weeks, \( P = 0.008 \) for 17 weeks) and at 17 weeks in the CO group (\( P = 0.04 \) (Wilcoxon matched-pairs test). Folic acid and vitamin B12 (FA/B12 group) reduced the genomic damage to 89, 72 and 59% (4, 12, 17 weeks) of before, while folic acid alone (FA group) reduced it to 81, 72, 75% at the respective time points. Alteration in the CO group was 95, 104, 91% of before.

To investigate whether an altered lymphocyte proliferation capacity may have influenced the results, the percentage of binucleated lymphocytes upon PHA-stimulation was analysed (Table 2). There was no significant difference between groups at any time point and no clear trend over time (\( P > 0.05 \); Wilcoxon matched-pairs and Wilcoxon two-sample test), but an inverse correlation with donor age (not shown).

Plasma Hcy concentrations (Table 1) were elevated to 24–25 μM (normal level: 5–15 μM), but not different between groups at the beginning of the study (\( P > 0.05 \); Wilcoxon two-sample test). The 12-week values exhibited a significant reduction of plasma Hcy in the FA/B12 group (\( P = 0.008 \)), while the FA group experienced a lesser but still significant (\( P = 0.039 \)) decrease. In the control group, the difference to the values at the first time point was not significant (\( P > 0.05 \)) (Figure 3; Wilcoxon matched-pairs test).

To detect potential anti-inflammatory effects of the vitamin supplementation, C-reactive protein (CRP) was measured during routine patient treatment. CRP was slightly elevated (compared to the reference value of <6 mg/l) in the patients of this study (mean concentrations in the groups between 7.4 and 14.0 mg/l), but not significantly different (\( P > 0.05 \); Wilcoxon two-sample test) between the groups before supplementation (Table 1). Analysis after the initiation of supplementation (between weeks 12 and 17) showed...
values of 12.3 ± 14.7 mg/l (CO group), 23.4 ± 35.5 mg/l (FA group) and 8.6 ± 7.4 mg/l (FA/B12 group), which was not significantly different among the groups (P > 0.05; Wilcoxon two-sample test) or compared to before supplementation (P > 0.05; Wilcoxon matched-pairs test). Albumine levels were also analysed at the beginning (Table 1) and at the end of the study. With 38.4 ± 4.5 g/l (CO group), 35.9 ± 4.1 g/l (FA group) and 37.1 ± 3.8 g/l (FA/B12 group) at the end, the only statistically significant alteration during the study was a reduction in the FA/B12 group (P = 0.004; Wilcoxon matched-pairs test). However, this did not lead to a significant difference from the other treatment groups at this time (P > 0.05; Wilcoxon two-sample test).

The potential confounder dialysis efficiency (Kt/V) was compared at one time point before (Table 1), during (1.6 ± 0.3 for the CO group, 1.7 ± 0.3 for the FA group, 1.7 ± 0.2 for the FA/B12 group) and after the supplementation phase of the study (1.7 ± 0.3 for the CO group, 1.7 ± 0.2 for the FA group, 1.8 ± 0.2 for the FA/B12 group) and it did not differ significantly between groups at any of the time points, or over time for any of the groups (P > 0.05).

Since Hcy and its metabolic product S-adenosylhomocysteine are thought to cause DNA-hypomethylation, mass spectrometric analysis served to determine global DNA cytosine-methylation at the beginning and at the end of the study (Table 3). The percentages of methylated cytosine bases were not different between the groups (P > 0.05; Wilcoxon two-sample test) and did not change during the study (P > 0.05; Wilcoxon two-sample test), thus indicating no detectable influence of the supplementation on DNA cytosine-methylation.

Since Hcy has also been described to cause oxidative stress, Hcy concentrations were lowered due to the supplementation, and oxidative stress is known to cause DNA damage, oxidative stress may offer an explanation for reduction of genomic damage in the treatment groups. Therefore, we attempted at this point to assay the amount of oxidative stress present in the lymphocytes. Therefore, 1,N°-etheno-2′-deoxyadenosine (εdAdo), which reflects lipid peroxidation-mediated effects of oxidative stress on the DNA, was measured in the available DNA samples at the end of the study. Although a trend for a reduction of oxidative stress in the FA/B12 group—but not in the FA group—might be detectable, the differences between the groups did not reach significance (P > 0.05; Wilcoxon two-sample test).

### Discussion

A micronucleus is formed during cell division, expressing previously induced chromosomal damage and contains mostly chromosomal fragments, and to a lesser extent, whole chromatids or chromosomes. Recent cohort studies reported that an increased micronucleus frequency in PBL predicts the risk of cancer in humans [13]. Therefore, the micronucleus frequency may be a useful early biomarker to investigate protective effects of therapeutic alterations in dialysis patients, who exhibit an elevated cancer incidence [1–2].

We investigated the effects of supplementation with folic acid and vitamin B12 in a prospective study. Patients were assigned to three groups: (1) controls (CO), (2) patients treated with folic acid (FA) and (3) patients treated with folic acid and vitamin B12 (FA/B12). The groups were similar in several important characteristics, such as age, time on dialysis, basal micronucleus frequency, serum albumine, Hcy levels and DNA cytosine-methylation. The lower micronucleus frequency in male patients and the negative correlation of lymphocyte proliferation with donor age are in line with the literature [35,36]. The potential confounders CRP and lymphocyte proliferation capacity did not change during the course of the study. CRP values were above the reference values, a finding that is well known for dialysis patients [37].

Micronucleus frequencies were reduced compared to before in both treatment groups (at all time points in the FA group and two time points in the FA/B12 group) and to a small extent in the CO group at the latest time point. The maximal reduction was 9% in the CO group (17 weeks), 28% in the FA group (12 weeks) and 41% in the FA/B12 group. 5-Methyl 2′-deoxycytidine reflects the extent of global DNA cytosine methylation; 1,N°-etheno-2′-deoxyadenosine (εdAdo) reflects lipid peroxidation mediated effects of oxidative stress on the DNA. %DNA methylation: percentage of methylated cytosine bases. εdAdo/10^9 nt: number of εdAdo adducts per 10^9 DNA normal nucleotides (nt). N.d.: not determined.

### Table 3. Mass spectrometric analysis of DNA samples

<table>
<thead>
<tr>
<th>Patient group (number of patients)</th>
<th>Beginning of study</th>
<th>End of study</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>% DNA methylation</strong></td>
<td><strong>CO (8)</strong></td>
<td>5.9 ± 0.3</td>
</tr>
<tr>
<td></td>
<td><strong>FA (8)</strong></td>
<td>5.6 ± 0.5</td>
</tr>
<tr>
<td></td>
<td><strong>FA/B12 (9)</strong></td>
<td>5.6 ± 0.4</td>
</tr>
<tr>
<td>εdAdo/10^9 nt</td>
<td><strong>CO (7)</strong></td>
<td>N.d.</td>
</tr>
<tr>
<td></td>
<td><strong>FA (7)</strong></td>
<td>N.d.</td>
</tr>
<tr>
<td></td>
<td><strong>FA/B12 (9)</strong></td>
<td>N.d.</td>
</tr>
</tbody>
</table>

Fig. 3. Plasma homocysteine concentrations before and at 12 weeks of supplementation. *P < 0.05 (Wilcoxon matched-pairs test) compared to basal level. CO: control group (dialysis patients with no study treatment); FA: supplementation with folic acid; FA/B12: supplementation with folic acid and vitamin B12. Medication with folic acid and vitamin B12 is described in the Materials and methods section. Only samples from 9 of the 10 patients were available for the FA/B12 group.
group (17 weeks). Folic acid has been reported to reduce elevated plasma Hcy levels by about one third [38], but only few of the patients with CRF reached normal levels. This is in agreement with our observations, where the reduction of the average Hcy concentrations was 32.7% in the FA group. The better reduction of Hcy upon additional supplementation with vitamin B12 (47.1%), reaching an FA/B12 group average in the normal range, corresponds to [39] who also achieved normal Hcy values in a folic acid therapy upon additional supplementation with vitamin B12. However, another group was recently able to normalize Hcy levels with only folic acid supplementation for 6 months in dialysis patients [40].

Inhibitory effects of Hcy, through S-adenosylhomocysteine, on DNA methyltransferases have been reported [23,41–42], as well as the restoration of DNA–cytosine–methylation levels by folic acid supplementation [23,43–44]. However, methylation levels were not altered during the course of our study, possibly because our patients had only slightly, although significantly, reduced DNA–cytosine–methylation levels at the beginning of the study. We detected 5.72 ± 0.37% methylated cytosine bases (mean of all 27 patients), while we found 7.1 ± 0.43% as an average of eight healthy controls (P < 0.05; Wilcoxon two-sample test; individual data of eight healthy controls not shown, data gained in parallel but separate from this study). Furthermore, some of the patients were taking oral multivitamins containing 1 mg folic acid, so this may influence the response to the higher (15 mg, i.v.) dose folic acid. Also, an observation by Stenvinkel et al. [12] is interesting, who reported an elevated cytosine-methylation level in haemodialysis and pre-dialysis patients with inflammation (mean CRP >20) compared to healthy individuals, although hyperhomocysteinemia was linked anyway to DNA hypomethylation. This suggests that multiple elements can play a role in DNA methylation patterns, and that we have only begun to unravel the complex interplay between the various elements.

It has been proposed that some of the adverse effects associated with hyperhomocysteinemia may be due to the generation of reactive oxygen species [e.g. 45–46]. However, Hcy treatment did not elicit an oxidative stress response in HUVEC [47] and LLC-PK1 cells [48]. Thus, the generation of oxidative stress by Hcy may be cell-type-specific. This idea is supported by a publication stating that Hcy can both inhibit and promote LDL oxidation, depending on the experimental conditions [49]. In our study, lymphocytes of dialysis patients without additional folic acid and vitamin B12 supplementation (CO group) showed 132 ± 74 εdAdo per 10^9 DNA bases (with εdAdo representing an oxidative stress-induced DNA alteration mediated by lipid peroxidation), while cells of FA/B12 group patients exhibited slightly (not significant) reduced εdAdo levels (96 ± 35 εdAdo/10^9) compared to the CO group or to the FA group (161 ± 27 εdAdo/10^9). In eight healthy controls, we measured an average of 93 ± 41 εdAdo/10^9. Considering that micronuclei were lowered in both supplementation groups, and a possible alteration of oxidative stress levels was only observed in the FA/B12 group, the role of oxidative stress in the reduction of genomic damage in the patients is not clear. Haemodialysis patients are known to exhibit elevated amounts of reactive oxygen species in their peripheral blood, which are thought to be caused not only by Hcy but also by chronic inflammation as well as the dialysis treatment itself. Most recently Alvaraes Delfino et al. [40], who lowered Hcy in dialysis patients by folic acid supplementation, found an increased antioxidant capacity level in dialysis patients after a 6-month supplementation with folic acid, but no decrease in lipid peroxidation was measured as plasma hydroperoxide. Au-Young et al. [50] recently detected that folic acid inhibits Hcy-induced NADPH oxidase-dependent superoxide anion production and nuclear factor kappaB activation in macrophages independent of its Hcy-lowering ability. Higashi-Okai et al. [51] found in an in vitro study pro-oxidant and antioxidant effects of folic acid on lipid peroxidation, while vitamin B12 showed only antioxidant effects.

In conclusion, folic acid, and more efficiently the combination of folic acid and vitamin B12, led to a reduction in micronucleus frequency and Hcy level. While the protective effects do not seem to be mediated by alterations in DNA-cytosine–methylation, it cannot be excluded that antioxidant properties of vitamin B12 might further support the beneficial effects of the Hcy-lowering treatment.

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Conflict of interest statement. None declared.

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