Altered folate receptor 2 expression in uraemic patients on haemodialysis: implications for folate resistance

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Keywords: folates, folate receptors, heterogeneous nuclear ribonucleoprotein-E1, homocysteine, haemodialysis

ABSTRACT

Background. Folate therapy reduces, but does not normalize homocysteine (Hcy) levels, frequently elevated in chronic kidney disease (CKD). The mechanisms of this folate resistance are unknown. Cellular acquisition of folate is mediated by folate receptors (FRs), whose expression is also modulated by folate status, through an Hcy-dependent regulation mechanism involving heterogeneous nuclear ribonucleoprotein-E1 (hnRNP-E1). Our objective was to evaluate whether an alteration of the FR2 (the form present in nucleated blood cells) expression is present in CKD patients on haemodialysis (HD), and its susceptibility to folate treatment.

Methods. A population of chronic uraemic patients on HD was enrolled, along with a control group, and studies on FR2 receptor expression and related items were performed in plasma and mononuclear cells from peripheral blood. A subgroup of patients was treated with methyltetrahydrofolate for 1 month.

Results. In HD, there was a significant reduction in FR2 protein expression compared with controls, not correlated with Hcy concentrations, while its mRNA levels were significantly increased. After folate treatment, there was a significant mRNA decrease, in the absence of significant changes in receptor protein expression. hnRNP-E1 gene and protein expression levels increased pre-treatment, while decreased post-treatment.

Conclusions. In HD, FR2 expression is altered in peripheral mononuclear cells, since its levels are decreased and are not responsive to variations in Hcy concentration, while the intracellular machinery (receptor mRNA and hnRNP-E1), possibly triggering its regulation, is conserved. These findings provide insight into the mechanisms of folate resistance in uraemia.

INTRODUCTION

Chronic kidney disease (CKD) and especially end-stage renal disease (ESRD) patients, a steadily growing population in advanced countries, have a mortality rate for cardiovascular (CV) disease several fold higher than the general population. This excess mortality is still unexplained, hence the interest for risk factors as hyperhomocysteinaemia (HHcy). Homocysteine (Hcy) is a risk factor for CV disease and is almost always elevated in this patient population [1, 2].

Folate treatment reduces, but does not normalize, the Hcy levels in this population, even when administered at high doses, perhaps explaining the negative results of intervention trials aiming at CV risk reduction [3, 4]. A state of folate resistance therefore exists in uraemia, where its mechanisms are not yet known [5, 6].

Folate transport into cells is predominantly mediated by cell surface receptors, the folate receptors (FRs). Four FR isoforms are described: alpha (or FR1), beta, gamma (or FR3) and gamma1. For isoform alpha at least, FR expression increases or decreases in response to folate repletion or depletion, through a mechanism regulated by the Hcy levels. In fact, Hcy intracellular levels increase during folate deficiency and vice versa. Hcy stimulates the interaction between heterogeneous nuclear ribonucleoprotein-E1 (hnRNP-E1, Q15365; NM_006196.3) and a FR mRNA cis-
element leading to increased FR biosynthesis [7–10]. It has been recently shown that Hcy, through hnRNP-E1 homocysteinylation, unmasks an RNA-binding pocket in hnRNP-E1, a post-translational modification preparatory to FR up-regulation [11].

To summarize, under normal conditions, the low folate levels lead to high circulating levels of Hcy, thus resulting in an increase in receptor expression to capture all circulating folate. In contrast, the high levels of folate lead to low Hcy levels, with a decrease in receptor expression. It is unknown whether in ESRD, where plasma Hcy is increased and folate levels vary, an alteration of FR expression is present, thus explaining folate resistance. In addition, the effects of folate treatment on the FR expression are unknown. FR beta (β), also known as FR2 (P14207; NM_000803.4), is a cell surface glycoprotein expressed in haematopoietic cells (neutrophils, monocytes and activated macrophages) [12–16] and is therefore available for testing in an in vivo environment. FR1 and FR2 have a high degree of similarity, and it is conceivable that both forms are regulated by the same mechanism [16–18].

Considering blood cells [the only readily available tissue in haemodialysis (HD) patients], FR1 is present on the red cell lineage [19], where, in mature red cells, is a vestigial remnant [20]; on peripheral mononuclear cells (PBMs), which are important for CV risk and atherosclerosis [21], the FR2 and FR3 isoforms are present. While FR1 and FR2 possess a glycoprophosphatidylinositol (GPI) anchor, FR3 lacks this moiety and its secretory protein. Therefore, in the selected cells, FR2 is the only functionally involved isoform expressed on the cell surface.

We studied, in a population of chronic HD patients, (i) FR2 receptor expression levels on mononuclear cells from peripheral blood, (ii) FOLR2 gene (encoding for the FR2 protein) expression levels, (iii) plasma Hcy levels, (iv) circulating levels of various B vitamins such as folate, vitamin B12 and holotranscobalamin (HolOTC; active B12), (v) methylenetetrahydrofolate reductase (MTHFR) C677T and A1298C gene polymorphisms, (vi) hnRNP-E1 gene and protein expression levels. In a subgroup of patients, the same studies were performed before and after treatment with methylenetetrahydrofolate (MTHF), the active circulating form, as the methyl donor, in Hcy metabolism.

### Materials and Methods

#### Patients

A control group of 21 healthy subjects and a group of 41 ESRD patients on HD were recruited. All patients were in stable clinical conditions, treated with erythropoietin and other drugs commonly utilized in this population for anaemia, hypertension and secondary hyperparathyroidism and recruited according to the following inclusion criteria:

(i) Chronic three times a week HD for at least 3 months.
(ii) No clinical evidence of diabetes, lupus erythematosus, viral hepatitis, cancer.
(iii) No folate (any form) treatment for at least 2 months.

All previous transplant recipients were excluded.

A subgroup of 11 patients was treated with 15 mg MTHF (Prefolic, Zambon, Italy) intravenously after each session for 13 consecutive dialysis sessions.

All patients gave their informed consent. Procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000. The study was approved by our institution’s ethics committee (protocol number 374/2007 of the Second University of Naples). The trial was registered in a public trial register, the European Clinical Trials Database (http://eudract.emea.eu.int/), number: 2007-000451-34.

Blood samples were drawn under fasting conditions before the dialysis session in the patient group and in the controls and collected in Vacutainer tubes (containing ethylenediaminetetraacetic acid (EDTA) 1 mg/mL). After withdrawal, an aliquot was centrifuged at 4°C for 15 min at 1700 × g to obtain plasma, while the buffy coat was utilized for DNA extraction. An aliquot was immediately processed for biochemical analysis, flow cytometry and for the extraction of protein and RNA. A complete blood count was also performed.

#### Immunoassays for the determination of Hcy, folatemia and vitamin B12

Total Hcy was quantitated, on plasma samples, by fluorescence polarization immunoassay (FPIA-IMX®; Abbott Diagnostics). Folate-binding assay, an ionocapture assay, was used for the quantitative determination of folate in plasma and in human red blood cells (RBGs; AxSYM®; Abbott Diagnostics). Total plasma vitamin B12 and active B12 (HolOTC) were carried out using microparticle capture enzyme immunoassay (AxSYM®; Abbott Diagnostics).

#### Preparation of whole cell protein extracts

Mononuclear cells were isolated on Histopaque-1077 gradient, washed twice with 1% ice-cold phosphate buffer saline (PBS) and lysed in radioImmunoprecipitation assay buffer after protease and phosphatase inhibitors. After centrifugation (4°C, 10 min, 10 000× g), protein concentration was determined according to Bradford [22]. Samples were stored at −20°C in preparation for western blot analysis.

#### Western blot analysis

Fifty microgram of proteins were separated on sodium dodecyl sulphate, 12–15% (as appropriate) polyacrylamide gels and transferred to polyvinyl difluoride membrane (Millipore, USA). After being blocked with 5% non-fat dry milk, membranes were incubated overnight at 4°C with each of the following primary antibodies against: FOLR2 and PCBP1 (ABCAM), anti-actin Ab-5 (BD), as appropriate. Secondary antibodies were: goat anti-rabbit and goat anti-mouse (R&D Systems), as appropriate. After incubation with secondary antibodies conjugated with horseradish peroxidase for 1 h at room temperature, immunocomplex visualization was obtained by chemiluminescence, utilizing the ECL-Plus kit (GE Healthcare). Signal intensity was quantified with the ChemiDoc™ (Bio-Rad) with the Bio-Rad Quantity One® software.

Alteration of FOLR2 expression in haemodialysis
Cytoluciferometric analysis

Flow cytometric analyses were performed with a FACS-Calibur instrument (FACSCalibur, BD Biosciences, Milan, Italy).

For the cytoluciferometric assay, whole blood was withdrawn in EDTA (1 mg/mL blood). Cells were labelled by fluorescent antibodies. Two hundred microlitres of whole blood was added to parallel tubes containing 20 µL of Ab FOLR2 for 30 min in the dark as the primary antibody or the blanks containing only 10 µL of fluorescein isothiocyanate (FITC) goat anti-rabbit Ig secondary Ab. Ten microlitres of FITC goat anti-rabbit Ig Ab, as the secondary antibody, was then added to all tubes and kept for 30 min in the dark. Effective elimination of interfering erythrocytes, while preserving the leukocytes, was achieved by hypotonical lysis, by means of FACS Lysing Solution (Becton Dickinson). Four millilitres of 1× lysing buffer was added, the tubes were then vortexed and incubated at room temperature for 15 min. Cells were then washed twice in 1 mL of 1× PBS each. The analyses were performed with a FACS-Calibur instrument (Becton Dickinson) equipped with the Cell Quest Pro and ModFit LT version 3 softwares (Verity). For each sample, 2 × 10⁴ events were acquired. Blanks prepared as above, were analysed and subtracted as appropriate, for each set of samples.

Relevant forward scatter (FSC) and side scatter (SSC) parameters of leucocyte subpopulations were detected. Cell identification was further refined using anti-CD45 PerCP (BD Biosciences) pan-leucocyte fluorescent marker and anti-CD11c-PE (BD Biosciences). For FR2 evaluation, FOLR2 (ABCAM) and FITC goat anti-rabbit immunoglobulins (BD Pharmingen™) were used as the primary and secondary antibodies, respectively. Each experiment was performed in duplicate.

RNA extraction from whole blood

Mononuclear cells were isolated from whole blood withdrawn in EDTA by isopnicic centrifugation by stratification of a blood layer on an equal volume of Histopaque®-1077 solution (Sigma-Aldrich). Mononuclear cells were recovered at the gradient interface, gently removed and washed twice with 1% PBS and lysed with 1 mL of TRIZOL Reagent (Invitrogen). RNA was then extracted by chloroform–isoopropanol. The resulting pellet was washed in 70% ethanol, and suspended in H₂O diethylpyrocarbonate (DEPC). RNA concentration was measured by means of NanoDrop (ThermoScientific) and analysed by electrophoresis on 2% agarose gel in Tris base, Acetic acid and EDTA buffer 1×, to verify its integrity. RNA samples were stored at −80°C.

Reverse transcription

cDNA was synthesized from 1 µg of RNA. For reverse transcription, the SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen™, Carlsbad, CA, USA) was used. The reaction mixture contained: 1 µg RNA, 4 µL VILO reaction mix, 2 µL SuperScript enzyme mix, brought to 20 µL final volume by DEPC water. Reaction was carried out in Veriti™ 96-Well Thermal Cycler (Applied Biosystems) under the following conditions: 25°C for 10 min, 42°C for 60 min, 85°C for 5 min, 4°C infinitely. cDNA samples were stored at −20°C.

Quantitative real-time PCR gene expression analysis (qPCR)

qPCR experiments were performed for 35 cycles, using an iQ real-time polymerase chain reaction detection System (Bio-Rad, Bio-Rad Laboratories S.r.l., Segrate, Milan, Italy) in a total volume of 25 µL reaction mixture, containing 1 µL cDNA, 12.5 µL iQ SYBR Green Supermix (Bio-Rad) and 1 µL of each primer.

Primers were: GAPDH sense: 5′-TTGGATATCGTTGGAAGGACTCATG-3′; GAPDH antisense: 5′-CAGTATGAGGCAGGGATGTATGTC-3′; FOLR2 sense: 5′-AATCGGCAACAGAGGATGG-3′; FOLR2 antisense: 5′-TAGTTGCTGACCTTGATGAG-3′; hnRNP-E1 sense: 5′-GGACAAACACACATTCTC-3′; hnRNP-E1 antisense: 5′-CACAATCTGCCCCAATAAGC-3′.

Primer pairs were designed using Beacon Designer™ (PREMIER Biosoft). Sequence data, for each gene of interest, were desummed from the NCBI ‘Nucleotide’ database (http://www.ncbi.nlm.nih.gov/sites/entrez?db=nucleotide&cmd=search&term=); hnRNP-E1 Accession: NM_006196.3; FOLR2 Accession: NM_000803.4. SYBR® Green PCR that were specific to our target gene and efficiently amplify the target region. Amplification conditions were the following: 95°C for 15 min, followed by 35 cycles of 94°C for 15 s, 52°C (54°C for hnRNP-E1) for 30 s and 72°C for 30 s.

All our real-time PCR experiments were performed by using a housekeeping gene as an internal control (glyceraldehyde 3-phosphate dehydrogenase, GAPDH), to normalize the results of the transcript levels of the inducible genes of interest (GOI), namely: FOLR2 in Figures 4 and 6; HnRNP-E1 in Figures 4, 6 and 7C), using the formula:

\[ \Delta C = C_{\text{GOI}} - C_{\text{GAPDH}} \]

where 2^−ΔΔCt indicates the relative abundance of each GOI transcripts was then calculated by comparison with an adequate baseline control condition (labelled as ‘Controls’ in Figures 4, 6 and 7C), using the formula: ΔΔCt = ΔCtGOI treatment − ΔCtGOI control. The relative expression was then determined as 2^−ΔΔCt, where 2^−ΔΔCt >1 reflects increased expression of the target GOI, compared with the control, while 2^−ΔΔCt <1 reflects decreased expression with respect to baseline condition. A ΔΔCt of 0 indicates a ratio of 1 between the sample and the control (2^−ΔΔCt = 2^0 = 1) and represents the baseline (a threshold at which there is no difference in the expression of a certain target gene between the sample and the control). Notably, in all these figures, this number 1
value is indicated by a dotted line. In addition, we should underscore that all real-time PCR experiments were performed according to the minimum information for publication of quantitative real-time PCR experiments (MIQE) guidelines [24], as already applied in previous studies also by us [25].

**Extraction of genomic DNA from whole blood**

Genomic DNA extraction was performed from whole blood, buffy coat, by magnetic beads technology, using the kit ‘GeneCatcher™’ (gDNA Blood Kits; Invitrogen). DNA concentration was measured by means of NanoDrop UV/Vis micro-spectrophotometry (ND-1000; NanoDrop Technologies, ThermoScientific).

**Analysis of the major MTHFR polymorphisms**

Patient genotypization for MTHFR C677T and A1298C gene polymorphisms was accomplished by the analysis of the appropriate restriction fragments length polymorphisms as described previously [26–28].

**Statistical analysis**

Sample sizes were estimated to be adequate and able to detect differences with an 80% statistical power. The formula reported by Dawson-Saunders and Trapp was utilized to estimate sample size, employing the mean and standard deviation of data pertaining to the first pilot experiments [29].

Data are presented as mean ± standard error (SE). All calculations were performed using the software package GraphPad Prism, Version 5.0 for Windows (GraphPad Software, San Diego, CA, USA). A P-value <0.05 is considered for statistical significance. All experiments were done in triplicate unless stated otherwise. An unpaired or paired t-test was performed, as appropriate, to evaluate the P values between patients and controls, or patients after and before treatment, respectively. Both Pearson’s correlation, two-tailed and linear regression were performed to assess correlations. Grubb’s method was used to assess outliers.

**RESULTS**

**Patient basic data and co-morbidities**

Patient basal characteristics (means ± SE) were: age 55 ± 5.60 years in controls and 63.3 ± 2.14 years in patients; body weight 66 ± 3.7 kg in controls and 68 ± 4.4 kg in patients. Among the causes of ESRD (patient specific primary pathologies), those above 1% were: nephroangiosclerosis 30%, miscellaneous glomerulonephritides 15%, reflux nephropathy and pyelonephritis 10%, polycystic kidney disease 5%.

Patients were reported not to have any residual renal function. In addition, no vitamin B12 supplements were administered at any time and there were no vegetarians among the groups.

**Evaluation of Hcy, folate, vitamin B12 and related haematological parameters, and MTHFR polymorphisms**

In HD patients, Hcy levels were significantly higher than in controls, with a 5-fold average increase (Table 1).

Plasma folate was significantly lower in patients than in healthy controls, but this did not occur at the level of red blood cell folate reserve (Table 1).

Plasma vitamin B12 concentration showed a significant increase of vitamin B12 in uremic patients with respect to controls (Table 1). HoloTC was several times higher in CKD HD patients compared with control. However, since holoTC appears not to be predictive of B12 deficiency as is normal [30], this could also explain the slightly higher mean cell volume (MCV) in our HD patients.

Patients showed a significant decrease in haemoglobin (HGB) concentration values compared with controls, a

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<th>Table 1. Plasma Hcy, Folate, vitamin B12, HGB, MCV and RDW in the study populations (ESRD patients and healthy controls)</th>
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<td><strong>Controls</strong></td>
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<tr>
<td>Hcy</td>
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<tr>
<td>Plasma folate</td>
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<td>RBC folate</td>
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<td>Vitamin B12</td>
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<td>MCV</td>
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<td>HGB</td>
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<td>RDW</td>
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Total Hcy is expressed in µmol/L. Plasma folate and RBC folate are expressed in ng/mL. Vitamin B12 and active B12 (HoloTC) are expressed as pg/mL and pmol/L, respectively. MCV is expressed as fl, HGB as g/dL and RDW as CV(%). All values are expressed as the mean ± SE. The confidence interval is reported in parentheses (CI). *P < 0.05, **P ≤ 0.01 and ***P < 0.001, respectively.
reflection of the well-known uraemic anaemia. The significant increase in MCV probably reflects the folate depletion present in these patients, selected according to criteria including the absence of folate supplementation (Table 1). The significantly higher red blood cell distribution width (RDW) in the patients is consistent with the anisocytosis commonly detected in ESRD.

FR2 expression is reduced in peripheral leucocytes from HD patients

The results of western blot analysis to detect the presence of FR2 in mononuclear cell protein extracts from peripheral blood of healthy controls and HD patients are shown in Figure 1. FR2 protein expression was significantly reduced, in patients with respect to controls (Figure 1A and B), although its pattern of migration on sodium dodecyl sulfate polyacrylamide gel electrophoresis was not different from that of controls.

Hereafter, a flow cytometric analysis was performed to evaluate the extent of expression of mature FR2 on cellular surface (percentage of FR2-positive cells) in peripheral leucocytes (Figure 2A–C). Results from cytofluorimetric analyses showed that peripheral leucocytes from patients exhibited FR2 levels significantly lower than in healthy controls (Figure 2D; confidence interval [CI] of controls = 5.518/13.54, patients = 3.590/5.475). This difference in the expression of FR2 was detected in both granulocytes (Figure 2E; CI of controls = 1.639/6.234, patients = 0.9852/2.345) and mononuclear cells (Figure 2F; CI of controls = 3.672/7.511, patients = 2.317/3.419).

All correlations between FR2 and other parameters were tested with the appropriate statistical tools. A significant negative correlation between Hcy and plasma folate was found (Figure 3A) in the patient population, confirming a previous finding [31] and indicating that, with increasing plasma folate concentration, Hcy levels decrease. This is mirrored by findings in red blood cell folate (Figure 3B). Other correlations did not reach statistical significance (not shown).

FOLR2 Transcriptional levels are elevated in HD

Under conditions of low folate levels and high Hcy, FR2 receptor is expected to be up-regulated. A transcriptional increase of FR (FOLR2) mRNA was indeed detectable in cell culture models under low folate/high Hcy conditions (about 2.5-fold) [10].

To assess this aspect, we measured the gene expression levels of FOLR2 by qPCR on mRNA of mononuclear cells from peripheral blood. FOLR2 expression levels were significantly higher in patients compared with control (Figure 4).

These levels were, in the patient group, well above the baseline, thus reflecting a significant positive transcriptional response in vivo, in uraemic patients, in the presence of low plasma folate and HHcy.

MTHFR polymorphisms

The influence of the most common MTHFR polymorphisms, C677T and A1298C, was evaluated. The mutated genotypes are expected to determine, particularly in the homozygous, an increase in plasma Hcy levels. Therefore, it is possible that these polymorphisms could affect FR2 expression, either through the mentioned Hcy level increase or as the consequence of the direct involvement of this enzyme (the product of MTHFR gene) in the folate cycle.

Aside from the known increase in Hcy levels in the TT and CC [32, 33] homozygous individuals, no difference was found relative to the FR2 protein expression among the CC, CT and TT genotypes, and among the AA, AC and CC genotypes (data not shown).

Effect of MTHF treatment on HD patients

To evaluate the effects of folate on the parameters listed in Table 1 and on FR2 expression, a selected group of patients was treated with intravenously MTHF for 1 month. If regulation is conserved, one should expect that FR2 levels would decrease after folate therapy.

After treatment, Hcy levels decreased significantly (Table 2), although not to normative levels, consistently with previous reports [3]. Plasma (Table 2) and red blood cell folate (Table 2) levels were significantly increased post-treatment. In consideration of the role of cobalamin coenzymes in folate-dependent Hcy remethylation, we also evaluated vitamin B12 and HoloTC, whose levels were unchanged after treatment (Table 2). The haematological parameters did not indicate, as expected, a substantial improvement after MTFR therapy (Table 2).

Despite the decrease in circulating Hcy and the increase in folate after treatment, flow cytometric analysis demonstrated that FR2 levels did not change after folate treatment.
(Figure 5A–C) in whole leucocytes, as well as in white blood cell subpopulations.

Furthermore, MTHF treatment induced a decrease in FOLR2 gene transcript levels (Figure 6), and FOLR2 expression, post-treatment, was even lower than the baseline, indicating a conserved responsiveness to down-regulation at the transcriptional level upon folate therapy, in the face of a lack of decrease of FR2 protein levels.

**hnRNP-E1 is decreased post-treatment**

Since Hcy, accumulating during folate deficiency, stimulates the interaction between hnRNP-E1 and an 18-base FR-α mRNA cis-element that leads to increased FR biosynthesis and up-regulation of FR at cell surface, hnRNP-E1 is a plausible candidate sensor of folate deficiency [11]. In response to HHcy, hnRP-E1 levels would also be expected to increase, as shown in a folate deficiency murine model, demonstrating a
parallel up-regulation of FR and hnRNP-E1 [8]. We therefore evaluated hnRNP-E1 expression both at gene and protein levels, upon folate therapy, in uraemic patients. Our data demonstrated, according to western blot analysis, that hnRNP-E1 protein was up-regulated, in the folate untreated patient population compared with healthy controls. Conversely, hnRNP-E1 expression levels were significantly reduced, in the patients, after MTHF treatment, compared with the same subjects before treatment (Figure 7A and B). Furthermore, qPCR analysis demonstrated a significant increase of hnRNP-E1 gene expression levels of all patients compared with control levels (Figure 7C), whereas in the folate-treated group hnRNP-E1 levels decreased significantly post-treatment (Figure 7D).

**DISCUSSION**

In this study, the mechanisms of folate resistance in uraemia (as expressed by the lack of Hcy normalization upon treatment in this population) [3, 31] have been explored: FR2 levels are decreased and its expression is not responsive to variations in Hcy concentration, while FR2 mRNA levels do change in response to Hcy variations and/or folate administration and are accompanied by consistent modifications of hnRNP-E1 at mRNA and protein levels.

Higher Hcy levels have been postulated to be a CV risk factor, while many aspects need to be clarified relative to the adequacy of folate therapy as a Hcy-lowering means [34, 35].

In ESRD, Hcy levels are in the moderate–intermediate range (20–50 μM), if patients are untreated with respect to B vitamin supplementation [1–4, 31, 35–38]. In this population, Hcy-lowering therapy does not consistently lead to a reduction in CV outcomes [1, 3, 4, 36], similar to that found in the general population. However, if in the general population, we are dealing with Hcy levels within or near the upper normal range, uraemic patients do not reach normal levels and remain relatively hyperhomocysteinaemic (levels >20 μM) despite treatment [3]. This lack of normalization present in most treated patients occurs independently of the form of folate used, and it persists even if folate is utilized at very high dosage [37]. This finding is consistent with a state of relative resistance to folate, the basis of which we have attempted to analyse this study.

For this study, we utilized carefully selected HD patients and measured the levels of FR2 and its gene expression in their circulating mononuclear cells, a readily available tissue we have been using also in previous studies [38].

PBMs are regarded with increasing importance in the mechanism of vascular damage in patients with CKD [21]. In addition, HHcy is important in eliciting inflammatory monocyte differentiation in atherosclerosis animal models [39].

**Table 2. Effect of MTHF treatment on plasma Hcy, Folate, vitamin B12, HGB, MCV and RDW**

<table>
<thead>
<tr>
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<th>Patients pre-treatment</th>
<th>Patients post-treatment</th>
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<tr>
<td>Hcy</td>
<td>55.75 ± 9.096 (35.38/76.02)</td>
<td>29.90 ± 2.835** (23.58/36.22)</td>
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<tr>
<td>Plasma folate</td>
<td>3.555 ± 0.5387 (2.354/4.755)</td>
<td>2167 ± 521.1** (988.2/3346.0)</td>
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<tr>
<td>RBC folate</td>
<td>354.9 ± 46.01 (252.4/457.4)</td>
<td>2780 ± 942.2* (648.7/4911)</td>
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<tr>
<td>Vitamin B12</td>
<td>1685 ± 719.3 (82.32/3288.0)</td>
<td>1067 ± 459.6 (43.01/2091)</td>
</tr>
<tr>
<td>HoloTC</td>
<td>567.3 ± 189.3 (145.4/899.1)</td>
<td>1070 ± 529.1 (108.8/2249)</td>
</tr>
<tr>
<td>MCV</td>
<td>97.13 ± 1.725 (93.28/101.0)</td>
<td>99.72 ± 1.992** (95.28/104.2)</td>
</tr>
<tr>
<td>HGB</td>
<td>11.59 ± 0.2581 (11.02/12.17)</td>
<td>12.46 ± 0.3040 (11.79/13.14)</td>
</tr>
<tr>
<td>RDW</td>
<td>15.36 ± 0.3093 (14.67/16.05)</td>
<td>15.40 ± 0.3446 (14.63/16.17)</td>
</tr>
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</table>

Values are expressed as the mean ± SE and, in parentheses, the confidence interval (CI). Plasma Hcy (μmol/L); plasma folate and red blood cell folate (ng/mL). Plasma vitamin B12 (pg/mL) and HoloTC (pmol/L). Red cell parameters: HGB (g/dL); MCV (fL); RDW (CV%). *P < 0.05, **P ≤ 0.01 and ***P < 0.001, respectively.
Since in PBMs, FR2 is expressed and is able to bind folate, although with a lower affinity with respect to FR1, PBMs can be regarded as a readily available, suitable model to test our hypothesis.

Findings relative to Hcy and folate status display macrocytic anaemia, intermediate HHcy and plasma folate depletion (red blood cell folate levels were comparable with normal), which characterize the typical pattern of folate resistance described in uraemia [31]. Particularly, HHcy, almost constantly present in uraemia, persists as a key feature of this folate resistant state in the face of a preserved intracellular folate reserve and a mild haematological response to folate administration.

In the serum, vitamin B₁₂ (cobalamin) is bound to two proteins, transcobalamin (TC) and haptocorrin. The vitamin B₁₂–TC complex is called HoloTC. HoloTC levels were also significantly higher in patients than in controls (Table 1), thus ruling out the hypothesis that, in these patients, a vitamin B₁₂ deficiency was present. The mechanisms responsible for high cobalamin levels are not yet known, but one possible explanation may lie in a failure of renal uptake of HoloTC-bound cobalamin, whose receptors are abundant in the kidney [40]. This may also contribute to explain the relative inability of holoTC to predict B₁₂ deficiency in HD patients compared with that assessed in what is considered as normal [30].

Under normal conditions, the expression of FR is up-regulated in the presence of high Hcy levels, according to a mechanism which is modulated in part at the transcriptional level [10], but particularly at the translational level, given the increased interaction of the FR mRNA with the hnRNP-E1 translational modulator protein [11]. We found that FR2 expression is significantly lower in HD patients, both considering the FR2 protein expression as revealed by both western blot and by cytofluorimetric analysis, in the face of high Hcy circulating levels. In these patients, however, FR (FOLR2) mRNA is increased, in PBMs, compared with controls, as expected on the basis of previous studies on cell models [10]. We can therefore conclude that in ESRD circulating mononuclear cells are exposed to a high Hcy microenvironment and consistently respond by transcriptionally increasing FOLR2 mRNA (Figure 8). Nevertheless, this is not followed by a consistent increment of translational efficiency of this mRNA, despite the fact that, in ESRD, high protein homocysteinylation has been detected [32], which is the molecular mechanism by which high Hcy increases the binding of hnRNP-E1 to FR mRNA [10, 11]. The presence of a defective processing of FR2 precursor protein can be ruled out, since the results of immunoblot analysis and surface receptor cytofluorimetric analysis were highly consistent (compare Figures 1 and 2), and no intracellular accumulation of precursor proteins could be proven at the immunoblot analysis. We can therefore infer that, despite the presence of high Hcy in uraemia, this cannot exert its positively modulating action as proposed by the homocysteinylation of the hnRNP-E1 sensor mechanistic model (Figure 8).

This interpretation of a disrupted Hcy sensor mechanism in ESRD is even further underscored by the results of folate therapy, which only partially corrects HHcy and haematological parameters, particularly MCV (in accordance with the folate resistance model in uraemia), but is able to decrease FOLR2 mRNA, although its corresponding FR2 protein levels are not consistently modified.

It has been also reported that FR expression is susceptible to modulation by DNA methylation-dependent mechanisms [41]. Since DNA hypomethylation has been reported to occur in folate-depleted uraemic patients [38], we have to take into account these mechanisms to complete the understanding of the mechanisms responsible for the FR2 expression in ESRD.

**FIGURE 5:** Cytofluorimetric analysis of FR2-positive leucocytes in uraemic patients after MTHF treatment. Cytofluorimetric analysis was performed on patients before (black bar) and after treatment (cross-hatched bar). FR2 is expressed as the percentage of positive cells in whole leucocytes (A), granulocytes (B) and mononuclear cells (C). All data are presented as the mean ± SE.

**FIGURE 6:** FOLR2 gene expression levels after MTHF treatment. White bar, control; black bar, patients before treatment; cross-hatched bar, patients after treatment. $2^{-\Delta\Delta Ct}$ and baseline (indicated by a dotted line) are defined in the ‘Materials and methods’ section. *P < 0.05 compared with control; **P < 0.001, compared with patients pre-treatment. All data were presented as the mean ± SE.
account the possibility that DNA methylation status may influence, in these patients, FR expression levels.

Since in ESRD the FOLR2 gene is transcriptionally up-regulated, in circulating mononuclear cells, compared with normal, in the absence of a corresponding increase in the relevant protein levels, it is plausible that a defect in the post-translational regulation of this gene expression may be present. For example, a defective regulation of hnRNP-E1 can be hypothesized to occur in ESRD, since this is an important sensor of folate status, which is also responsive to high Hcy, a metabolite whose circulating levels are directly influenced by methylenetetrahydrofolate bioavailability [10, 11]. Reduced folate availability would result in Hcy build-up and increased FR translation, through the homocysteinylation of hnRNP-E1 and unmasking of the mRNA cis-element binding site [11]. In response to HHcy, in fact, hnRNP-E1 levels increase [8]. On the other hand, hnRNP-E1 levels decrease, after folate repletion, in response to lowered Hcy levels, presumably contributing to FR down-regulation [8].

hnRNP-E1 concentration levels were explored by us, both at protein and gene levels. Its levels in the uraemic state are increased with respect to control and, after folate treatment, are decreased, a finding compatible with an adequate pattern of response of this folate sensor.

It has been shown that hnRNP-E1 is also sensitive to Hcy levels, which may induce, in the presence of HHcy, the formation of homocysteinylated hnRNP-E1 stable adducts [11]. Increased plasma protein homocysteinylation has been detected in ESRD patients [42], and albumin N-homocysteinylation, at levels comparable with those present in the uraemic milieu, has been found to stimulate monocyte adhesion to an endothelial monolayer and the release of inflammatory chemokines and cytokines [25]. We can therefore speculate that the presence of a uraemic toxin(s), over 90 molecules including low-molecular weight organic substances and peptides [43], could be able to variously interfere with the FR2 protein expression, through a number of mechanisms such as molecular mimicking, enzyme inhibition or ligand competition. In consideration of the protein homocysteinylation increase in uraemia [42], and since homocysteinylated hnRNP-E1 modulates FR expression, an educated guess can be made that a switch mechanism, continuously turned on, could affect the normal sensitivity of this sensor, an analogy of what is observed for the calcium-sensing receptor in uraemia.

We trust that although our results do not claim to be exhaustive of all possible mechanisms underlying folate resistance in uraemia and relevant aspects, they can be illustrative of derangements of folate-dependent mechanisms and therefore contribute to the understanding of what actually occurs in many tissues and cells in vivo in CKD. At the present time, however, we believe that the information provided in the present paper sheds some light on folate resistance mechanism in CKD: a previously rather opaque box.

In conclusion, our studies have uncovered that at the basis of the well-known phenomenon of folate resistance in uraemia is a state of FR unresponsiveness, which, at least in our ex vivo model, i.e. circulating mononuclear cells obtained from ESRD

**FIGURE 7:** hnRNP-E1 expression evaluated at gene and protein levels. (A) Western blot analysis of hnRNP-E1 in controls, patients pre- and post-folate treatment. (B) Densitometry analysis of the quantitative difference in expression of hnRNP-E1. hnRNP-E1 protein before treatment with MTHF, compared with controls (*P < 0.05). Post- compared with pre-treatment levels (**P < 0.001). (C) qPCR analysis. **P ≤ 0.01 compared with control levels. In the folate-treated group (D), ***P < 0.001 compared with pre-treatment levels. 2^−ΔΔCt is defined in ‘Materials and Methods’. Fold change is relevant to baseline expression (indicated by dotted line; see ‘Materials and methods’). All data are presented as the mean ± SE.
patients on HD, is present in the face of a normal intracellular machinery, responding appropriately to folate deficiency and repletion. Our results further support the notion that folate supplementation is not an adequate and sound Hcy-lowering measure, also because of the now molecularly documented existence of a state of folate resistance in these patients.

CONFLICT OF INTEREST STATEMENT

None declared.

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Received for publication: 29.7.2012; Accepted in revised form: 28.9.2012