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A role for homocysteine increase in haemolysis of megaloblastic anaemias due to vitamin B_{12} and folate deficiency: results from an in vitro experience

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Abstract

Megaloblastic anaemias (MA) are frequently associated with haemolysis. The pathogenesis of these finding is not clear, but it is thought to depend on the greater destruction of abnormal and fragile megaloblastic erythrocytes. Vitamin B_{12} and folate deficiencies are the commonest cause of MA; these deficiencies may simultaneously induce a significant alteration in homocysteine metabolism leading to hyperhomocysteinemia. Blood cells have enzymes involved in homocysteine metabolism. Considering the possible effects of hyperhomocysteinemia in erythrocyte toxicity (due to oxidative damage and/or to interaction with sulfhydryl residues of structural and enzymatic proteins), the aim of our study was to evaluate (1) the homocysteine blood cells production in patients with MA due to vitamin B_{12} and folate deficiency and (2) the possible role and mechanism of hyperhomocysteinemia in MA haemolysis. After incubation at 37 °C, blood samples from MA patients showed higher and significant levels of Hcy, LDH, lipid peroxidation parameters (MDA), and ghost protein-bound Hcy than controls. Haemolysis (%) was higher in MA patients than controls and was significantly correlated with Hcy accumulation in the medium, lipid peroxidation indices and ghost protein-bound Hcy. No significant (or significantly lower) alterations through time in considered parameters were observed in the corresponding samples incubated at 4° C or in samples incubated with methionine-free medium (lower Hcy production). Our data, deriving from an in vitro experience, suggest a possible role of Hcy accumulation due to vitamin B_{12} and folate deficiencies in haemolysis associated to MA due to vitamin deficiency.

Keywords: Disulfide compound; Haemolysis; Homocysteine; Megaloblastic anaemia; Peroxidation

1. Introduction

Homocysteine (Hcy) is a sulfur amino acid deriving from the metabolic conversion of methionine, mainly in liver and kidney, through different enzymatic pathways dependent on vitamins as cofactors [1–3]. High level of plasma homocysteine (hyperhomocysteinemia, HHcy) may result both from several different genetic alterations in enzymes involved in its metabolism (inherited forms) [2,4–8] and from deficiencies of their cofactors, namely betaine, folates, pyridoxine, or cobalamine (vit. B_{12}) (acquired forms) [9,10] (Fig. 1). The importance of low availability of cobalamine and folates as determinants of HHcy has been well documented by many different epidemiological studies [11,12] and high level of plasma homocysteine has been advocated as a possible marker, even at subclinical level, of cobalamine and folate deficiency [13–16]. HHcy has been reported to negatively influence normal cell function in many different tissues, ranging from vascular endothelial and smooth muscle cells to blood cells [17,18]. These effects, in turn, have been advocated in explaining the association of HHcy with vascular diseases (thrombosis of arterial and venous districts and atherosclerosis) [17,19–21]. Many hypotheses have been proposed to explain the

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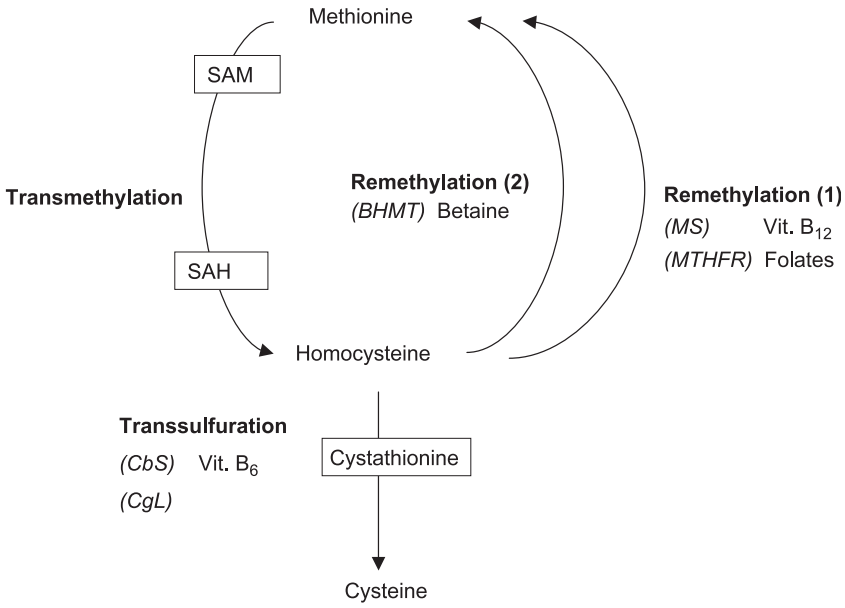

Fig. 1. Schematic representation of homocysteine metabolism. SAM=*S*-adenosyl-methionine; SAH=*S*-adenosyl-homocysteine; CbS=cystathionine-beta-Synthase; CgL=cystathionine-gamma-Lyase; MS=methione synthase; MTHFR=methylene-tetra-hydrofolate-reductase; BHMT=betaine-homocysteine-methyl-transferase; vitamin cofactors for each enzyme are indicated.

pathological mechanism by which HHcy promotes atherosclerosis, venous thrombosis and cell toxicity in general [22]. Due its peculiar chemical structure, homocysteine is a very reactive thiol: different reports have shown that Hcy, with respect to other thiols (especially cysteine), has a preferential interaction with -SH groups of proteins, being able also to displace other thiols from protein binding sites to form homocysteine-protein disulfides [23–25]. Moreover, it has been demonstrated that during Hcy oxidation in vitro to homocystine and to other mixed disulfides, free radical oxygen species, such as H_2O_2 and Superoxide Anion (O_2.), well-known promoters of molecular peroxidation, are generated [26–29].

Megaloblastic anaemias (MA) are a heterogeneous group of disorders sharing common morphologic characteristics: erythrocytes are larger and have higher nuclear-to-cytoplasmic ratios compared to normoblastic cells. Neutrophils can be hypersegmented, and megakaryocytes are abnormal. On the molecular level, in megaloblastic cells the maturation of nuclei is delayed, while cytoplasm development is normal. The molecular basis for megaloblastosis is failure in the synthesis and assembly of DNA. The most common causes of megaloblastosis are cobalamine and folate deficiencies, in turn due to many different possible situations [30]. Cobalamine and folate metabolisms are intricately related, and abnormalities in these pathways are believed to lead to the attenuated production of DNA. A hallmark of megaloblastic anaemia is ineffective erythropoiesis, as evidenced by erythroid hyperplasia in the bone marrow, decreased peripheral reticulocyte count, and elevation in plasma lactate dehydrogenase (LDH) and indirect bilirubin levels. The pathogenesis of these findings is not completely cleared, but it is thought to depend on the intramedullary destruction of fragile and abnormal megaloblastic erythroid precursors [30–32].

It is well known that oxidative stress causes important modifications in cellular macromolecules (in membrane and in cytoplasm) leading to cell damage; damage to cytoskeleton or membrane protein thiols by the formation of high molecular aggregates was proposed to be related to the haemolytic process and mixed disulfide formation between protein thiols and other sulfhydryl compounds has been demonstrated in red blood cells under oxidative stress [33]. Moreover, interference with the functional capability of membrane sulfhydryl groups, either by their oxidation or by their blockade, results in premature red cell destruction in vivo [34,35].

There are different data about the presence in blood cells of enzymes involved in the conversion of methionine into homocysteine [36–39]; Malinow et al. in their elegant studies showed that synthesis of homocysteine occurs in erythrocytes (RBC), whereas leucocytes (WBC) both synthesize and transulfurate homocysteine [40].

Considering the possible effects of Hcy in red blood cell toxicity (interaction with sulfhydryl groups of protein membrane and cytoskeleton and induction of peroxidation), we undertook this study in order to evaluate (1) the homocysteine blood cell production in patients with megaloblastic anaemias due to vitamin B_{12} and folate deficiency, and (2) the possible role of homocysteine excess in the haemolysis commonly observed in these diseases.

2. Materials and methods

Blood samples were obtained from 14 healthy volunteers (Control Group, CG; age 38±6, 6 f) and 16 subjects with documented megaloblastic anaemia (Megaloblastic Anemia Group, MAG; age 52±8, 7 f). MAG subjects were recruited as having: (1) red blood mean corpuscolar volume greater than 100 fl (measured by a Coulter Haematology Analyser; STKS Coulter Electronics, Miami, USA); (2) haemoglobin levels ≤11.0 g/dl; (3) peripheral blood smears showing hypersegmentation of neutrophils (defined as the presence of 5% or more five-lobed neutrophils, or one or more six-lobed neutrophils as reviewed by a haematologist); (4) significant vitamin B_{12} or folate deficiency (or both). Serum vitamin B_{12} was measured as serum cobalamine by ELISA commercial kit (Abbott Lab, IL, USA); red blood cell folates were measured as previous described [41]. The normal range of serum cobalamine was 148 to 445 pmol/l and that of RBC folate was 360 to 1400 nmol/l. In MAG group subjects, six had frank (levels under reference values) serum B_{12} deficiency, five frank folate deficiency and five both folate and B_{12} frank deficiency; moreover, the patients with B_{12} deficiency had serum folates levels within the low quartile of reference values and, similarly, folate deficient subjects had serum cobalamine levels within the low quartile of cobalamin reference values. All subjects gave their informed consent to participate to the study. Blood cell variables were determined on automatic blood cell counter. Haptoglobin and LDH values were measured by standard methods.

2.1. Samples collection and study design

Fasting venous blood samples were drawn into 20-ml Vacutainer® tubes containing Na_2-EDTA as an anti-coagulant. After centrifugation at 3000×*g* at room temperature for 10 min, the plasma supernatant was aspired and used for homocysteine (HPLC) and vitamin B_{12} determinations. Blood cell fractions were separated by density gradient and differential centrifugation; "WBC fraction" was obtained by single-density gradient centrifugation with Ficoll-Paque (Pharmacia Biotech, USA) at 500×*g* for 30 min at room temperature; the WBC band was then harvested from the interface and washed three times with phosphate-buffered saline (PBS) before reconstitution. RBC fraction was prepared by removing the RBCs pelletted through the Ficoll-Plaque gradient and by three times washing with PBS. The washed RBCs were then suspended in RPMI-1640 Medium Modified (free of L-methionine and other sulfur containing amino acid; Sigma-Aldrich, St. Louis, MO, USA) containing 3% BSA, 10 mM glucose with a hematocrit value of 30%. Each sample was added with its corresponding WBCs, to obtain a WBCs:RBCs ratio 1:500. Methionine concentration was adjusted to 50 μmol/l by addition in the medium of L-methionine (Sigma-Aldrich).

All samples were prepared in duplex and incubated under slight mixing at 4 °C and 37 °C. Four samples from both groups were also incubated at 37 °C in a free-methionine medium. Samples from supernatant and RBC were taken at the beginning of the study (time 0) and after 1, 4, 8 and 24 h of incubation for different assays.

2.2. Biochemical assays

Cysteine (Cys) and homocysteine (Hcy) in supernatant and within cytoplasm erythrocytes were measured by HPLC with fluorimetric detection [42].The assay has a sensitivity of 0.1 μmol/l for Hcy and 10 μmol/l for Cys and an intra- and inter-CV of 4.1% and 4.6%, respectively. Methionine was measured in supernatant of methionine-enriched medium at time 0, 8 and 24 h by HPLC method with fluorimetric detection [43]. The assay has a sensitivity of 3.5 μmol/l and an intra- and inter-CV of 4.0% and 3.0%, respectively.

In order to assess erythrocyte thiols content, each time-collected sample of RBCs was washed twice with PBS and haemolysed with hypotonic 5 mM Tris–HCl buffer (pH 7.4); the haemolysed (300 μl) was then assessed for Hcy and Cys by HPLC. In order to assess thiol content in membrane proteins, the ghost membranes were prepared by centrifugation at 10 000×*g* for 10 min. The resultant pellets were washed with the same buffer until no red color remained. The final pellets were resuspended with an equal volume of 3% sodium dodecyl-phosphate solution and incubated at 37 °C for 20 min; the solubilized ghosts were then incubated at 60 °C for 30 min in the absence or presence of dithiothreitol (DTT, Sigma Chemicals, St. Louis, MO, USA) 30 mM. Ghost protein content was assessed by the method of Lowry (Lowry); ghost protein-bound thiol content (Hcy, Cys) (thiol-protein mixed disulfide amount) was assessed by HPLC as the difference between the amount of thiols in the ghost medium after DTT treatment and the amount present before DTT (DTT, due its high reductive potential, induces the reduction of disulfide bounds, leading to release in solution of thiols bound to protein cysteine residuals).

Haemolysis measurement was assessed by the measurement in the cell-free supernatant of lactate dehydrogenase activity (LDH) and by photometrical (545 nm) detection of the haemoglobin amount [44].

As a marker of lipid peroxidation, we measured the thiobarbituric acid-reactive substances [malondialdehyde (MDA)] in the supernatant, according to the method of Fukunaga et al. [45]. Although the level of MDA is thought to be nonspecific due to the large amount of artefacts it may produce, it is very commonly used as an indicator of lipid peroxide levels; moreover, the HPLC method reduces the rate of artefacts, making the results more reliable.

2.3. Statistical analysis

Conventional statistic methods were used for calculation of means and S.D. When the distribution of the various

parameters within the population studied shows a significant departure from normality at the Shapiro–Wilk *W* test, data were log-converted before statistical analysis. Therefore, basal data of continuous variables were compared using the unpaired Student *t* test and effects of incubation in considered groups were analysed by two-factorial repeated measures ANOVA (group *x* time) with time as the repeated measure. The repeated measures were over three to five time periods (Basal–1–4–8–24 h, excluding measurements where no detectable measurement was noticed) for supernatant and cytoplasm parameters. When significant interaction was noted in the ANOVAs (Wilks' Lambda test), post-hoc pairwise comparisons using Tukey's test were used to compare each group over each time interval and groups within each time period. The Pearson *r* coefficient was used to assess the correlation between continuous variables. In all statistical evaluations a $P<0.05$ was taken as significant. Statistical analysis was performed by SPSS v.11.0 commercial package (SPSS, Chicago, IL).

3. Results

All data are reported as mean±standard deviation (S.D.). Clinical data of studied groups were reported in Table 1. Table 2 shows haemolysis increase rate through time of incubation in two considered groups during incubation at 37 °C compared with Hcy levels in the medium and in RBC cytoplasm; trends of lipoperoxidation (MDA levels in the supernatant) and Hcy-protein bound in RBC ghosts are also reported. In Table 3 are reported the same data from the corresponding samples from the two groups through incubation time at 4 °C. Considering the results from incubation at 37 °C samples, Hcy shows a progressive and significant increase in medium over time in both MAG and CG groups ($F=23.3$, $P<0.01$ and $F=15.6$, $P<0.01$, MAG and CG, respectively), a similar significant increase trend over time was observed with regard to RBC cytoplasm homocysteine content in both groups ($F=7.29$, $P<0.05$ and $F=6.48$, $P=0.048$, MAG and CG, respectively). Hcy levels both in the supernatant and in RBC cytoplasm were always significantly higher in MAG group than in CG group in each corresponding time assessment (cf. legend of Table 2). Similar significant trends over time were observed within both groups with regards to haemolysis ($F=8.71$, $P<0.01$ and $F=6.52$, $P<0.05$, MAG and CG, respectively), LDH ($F=9.02$, $P<0.01$ and $F=6.88$, $P<0.05$, MAG and CG, respectively) and MDA ($F=9.15$, $P<0.01$ and $F=7.12$, $P<0.05$, MAG and CG, respectively) increase in the supernatant and RBC ghost protein-bound Hcy increase ($F=13.2$ and $F=8.43$, MAG and CG, respectively $P<0.01$ for both); even for these parameters the values were always significantly higher in MAG group than in CG group in each corresponding time assessment (cf. legend of Table 2). With regards to results from samples incubated at 4 °C (Table 3), no significant increase over time was observed in Hcy levels both in RBC cytoplasm and in the supernatant in both groups, even if Hcy in cytoplasm and in supernatant was significantly higher in MAG group than in CG group in each corresponding time assessment (legend Table 2). Hcy levels in supernatant and RBC cytoplasm were always significantly higher at 1, 4, 8 and 24 h in samples at 37 °C with respect to correspondent samples at 4 °C ($P<0.01$ for all samples).

With regards to haemolysis parameters (LDH levels in supernatant and haemolysis% measurement), a significant increasing trend over time ($P<0.05$) was observed in samples at 4 °C within each group, whereas no significant difference was observed between groups. Significant and higher haemolysis parameters were observed in both groups comparing data from samples incubated at 37 °C with those incubated at 4 °C ($P<0.01$ for all assessment at 1, 4, 8 and 24 h). MDA levels in the supernatant showed a not significant increase trend over time in both groups with no significant difference between groups. Similarly, no significant increase over time was noticed in both groups for ghost protein-bound Hcy, whereas significant difference between groups was noticed for every time measurement. LDH, haemolysis%, MDA and ghost-protein bound Hcy from samples obtained at 37 °C incubation were always higher ($P<0.01$ for all; 1–4–8–24 h time determinations) vs. the corresponding samples at 4 °C incubation. No significant differences in all considered parameters were observed comparing different MAG subgroups (i.e., subjects with folate deficiency, cobalamine deficiencies or with both deficiencies; data not reported).

Table 1
Clinical and biological characteristics of studied subjects

	CG (n=14)	MAG (n=16)	P
Age (years)	38±6	52±8	<0.01
Sex (M/F)	8/6	9/7	ns*
Plasma homocysteine (μmol/l)	8.8±1.4	26.8±4.6	<0.01°
Plasma vit. B_{12} (mmol/l)	890±281	125±62	<0.01°
RBC folates (mmol/l)	921±324	310±90	<0.01°
RBC count ($n\times10^6$/mm^3)	4.7±0.5	3.0±0.3	<0.01°
RBC MCV (fl)	88.2±2.3	112±2.6	<0.01°
Haemoglobin (g/dl)	14.2±1.2	9.6±1.3	<0.01°
WBC count ($n\times10^3$/mm^3)	7.4±1.1 (70%)	6.9±1.3 (68%)	0.056°
Serum LDH (U/l)	180±45	280±60	<0.05°
Plasma haptoglobin (mg/dl)	122±25	54±31	<0.01°

RBC=red blood cell; WBC=white blood cell (in brackets percentage is referred to PMN); MCV=mean corpuscolar volume; LDH=lactate dehydrogenase. Results are expressed as mean±S.D. *p* value of unpaired Student's *t*-test and *chi-square test. ns, not significant. °Comparison after age adjustment. Vitamins reference range: RBC folate 360–1400 nmol/l; B_{12}: 148–445 pmol/l; LDH reference range: 100–240 U/l. Haptoglobin reference range: 30–140 mg/dl.

Table 2
Trend over time of considered parameters in samples from controls (CG, n=14) and patients with megaloblastic anemia (MAG, n=16) at 37 °C incubation

		Basal	1 h	4 h	8 h	24 h
Supernatant Hcy (μmol/l)	MAG group	n.d.	3.9 ± 0.6^{A}	$7.5\pm0.9^{1\ A}$	$9.6\pm1.1^{1\ A}$	$13.8\pm1.2^{1\ A}$
	HG group	n.d.	2.4 ± 0.5	4.8 ± 0.8	6.9 ± 1.2	8.1 ± 1.3
RBC Hcy (μmol/l)	MAG group	2.5 ± 0.5^{B}	$2.8\pm0.6^{2\ B}$	$3.1\pm0.5^{2\ A}$	$3.4\pm0.6^{2\ A}$	$3.6\pm0.7^{1\ A}$
	HG group	2.1 ± 0.2	2.3 ± 0.5^{0}	2.5 ± 0.6^{1}	2.6 ± 0.6^{1}	2.8 ± 0.5^{1}
Supernatant LDH (UI/l)	MAG group	n.d.	15 ± 0.8^{A}	$28\pm2.2^{1\ A}$	$46\pm5.3^{1\ A}$	$67\pm8.2^{1\ A}$
	HG group	n.d.	8 ± 1.1	12 ± 3.2^{NS}	22 ± 6.1^{1}	42 ± 9.1^{1}
Haemolysis (%)	MAG group	n.d.	5.3 ± 0.5^{A}	$8.2\pm0.7^{2\ A}$	$10.1\pm1^{1\ A}$	$15.2\pm0.8^{1\ A}$
	HG group	n.d.	1.1 ± 0.1	3.1 ± 1.1^{0}	4.1 ± 0.5^{2}	6.1 ± 0.7^{2}
Protein-bound Hcy	MAG group	0.21 ± 0.04^{A}	$0.45\pm0.11^{1\ A}$	$0.61\pm 0.12^{1\ A}$	$0.83\pm0.18^{1\ A}$	$1.12\pm0.21^{1\ A}$
(μmol/g protein)	HG group	0.12 ± 0.03	0.18 ± 0.07^{NS}	0.21 ± 0.08^{2}	0.41 ± 0.11^{1}	0.61 ± 0.18^{1}
Supernatant MDA	MAG group	n.d.	0.20 ± 0.06^{B}	$0.26\pm0.08^{2\ B}$	$0.36\pm0.10^{1\ B}$	$0.48\pm0.12^{1\ A}$
(nmol/ml)	HG group	n.d.	0.11 ± 0.04	0.15 ± 0.06^{0}	0.18 ± 0.08^{2}	0.25 ± 0.11^{1}

Intragroup difference (with respect to first detectable assessment): 0=ns; 1=P<0.01; 2=P<0.05. Intergroup difference: A=P<0.01; B=P<0.05; NS=not significant.

Table 4 reports data observed in samples (four MAG patients and four CG subjects) incubated at 37 °C in a methionine-free medium. Supernatant, RBC and ghost protein-bound Hcy increased significantly over time in both groups; this increase was higher for MAG patients than CG subjects; in both groups Hcy levels reached through time were significantly lower (up to 4–6 time lower at 24 h) than those observed in methionine-enriched samples (P<0.01 for all time measurement comparisons). No significant difference in haemolysis parameters between groups through time was observed.

A significant correlation between supernatant Hcy levels and MDA levels (r=0.468, P<0.05), LDH levels (r=0.395, P<0.05) and haemolysis% (r=0.332, P<0.05) was observed in samples from 37 °C incubation but not in those from 4 °C incubation; similarly, a significant or nearly significant correlation was found between ghost protein-bound Hcy levels and haemolysis (r=0.210, P=0.056) or LDH levels (r=0.341, P<0.05) in 37 °C samples, whereas no significant correlation was observed regarding these parameters in the samples from 4 °C incubation.

Fig. 2 shows cysteine levels in supernatant through time in incubation samples at 37 and 4 °C. Both groups showed a significant increase in cysteine supernatant levels (F=7.48, P<0.05 and F=7.05, P<0.05) at 37 °C; MAG group had always higher cysteine supernatant levels than controls over time with respect to CG group at 37 °C (cf. Fig. 2 legend). Fig. 3 shows methionine levels in supernatant through time in incubation samples at 37 and 4 °C. A significant decrease in methionine supernatant levels over time was observed in samples at 37 °C (F=9.48, P<0.01 and F=7.46, P<0.05, MAG and CG samples, respectively). No significant alteration in supernatant methionine levels was observed over time in samples at 4 °C from both groups (cf. legend of Fig. 3).

4. Discussion

Oxidative stress may cause important modification of cellular macromolecules at different levels, leading to cell damage: membrane lipids and membrane and cytoskeletal proteins are thought to be important target of oxidative

Table 3
Trend over time of considered parameters in samples from controls (CG, n=14) and patients with megaloblastic anemia (MAG, n=16) at 4 °C incubation

		Basal	1 h	4 h	8 h	24 h
Supernatant Hcy (μmol/l)	MAG group	n.d.	0.21 ± 0.04^{B}	$0.22\pm0.03^{0\ B}$	$0.23\pm0.04^{0\ B}$	$0.23\pm0.05^{0\ B}$
	HG group	n.d.	0.15 ± 0.02	0.17 ± 0.02^{0}	0.18 ± 0.03^{0}	0.18 ± 0.04^{0}
RBC Hcy (μmol/l)	MAG group	$2.5\pm0.5^{0\ B}$	$2.4\pm0.6^{0\ B}$	$2.6\pm0.4^{0\ B}$	$2.4\pm0.6^{0\ NS}$	$2.6\pm0.6^{0\ NS}$
	HG group	2.1 ± 0.2	2.0 ± 0.3^{0}	2.2 ± 0.4^{0}	2.2 ± 0.5^{0}	2.3 ± 0.5^{0}
Supernatant LDH (UI/l)	MAG group	n.d.	4 ± 0.5^{NS}	$8\pm1.2^{1\ NS}$	$15\pm3.2^{1\ NS}$	$22\pm3.6^{1\ NS}$
	HG group	n.d.	3 ± 0.6	7 ± 1.3^{2}	12 ± 4.1^{2}	18 ± 2.8^{1}
Haemolysis (%)	MAG group	n.d.	1.2 ± 0.3^{NS}	$1.3\pm0.4^{0\ NS}$	$2.7\pm1.0^{0\ NS}$	$4.1\pm0.5^{2\ NS}$
	HG group	n.d.	1.1 ± 0.2	1.1 ± 0.3^{0}	2.5 ± 0.8^{0}	3.7 ± 0.7^{2}
Protein-bound Hcy (μmol/g protein)	MAG group	0.21 ± 0.04^{A}	$0.23\pm0.05^{0\ B}$	$0.24\pm0.06^{0\ B}$	$0.24\pm0.07^{0\ A}$	$0.25\pm0.07^{0\ B}$
	HG group	0.12 ± 0.03	0.14 ± 0.04^{0}	0.15 ± 0.05^{0}	0.15 ± 0.06^{0}	0.16 ± 0.06^{0}
Supernatant MDA (nmol/ml)	MAG group	n.d	n.d.	0.12 ± 0.02^{NS}	$0.15\pm0.03^{0\ NS}$	$0.16\pm0.06^{0\ NS}$
	HG group	n.d	n.d.	0.11 ± 0.03	0.13 ± 0.04^{0}	0.13 ± 0.05^{0}

Intragroup difference (with respect to first detectable measurement): 0=ns; 1=P<0.01; 2=P<0.05. Intergroup difference: A=P<0.01; B=P<0.05; NS=not significant.

Table 4
Trend over time of considered parameters in samples from controls (CG, n=4) and patients with megaloblastic anemia (MAG, n=4) after incubation in a methionine-free medium

		Basal	1 h	4 h	8 h	24 h
Supernatant Hcy (μmol/l)	MAG group	n.d.	1.8 ± 0.04^{B}	$2.4\pm0.04^{2\ B}$	$2.7\pm0.05^{2\ B}$	$2.9\pm0.05^{1\ B}$
	HG group	n.d.	1.4 ± 0.03	1.8 ± 0.05^{2}	2.2 ± 0.05^{2}	2.4 ± 0.06^{1}
RBC Hcy (μmol/l)	MAG group	$2.3\pm0.5^{\ B}$	$2.5\pm0.6^{0\ B}$	$2.7\pm0.4^{2\ B}$	$2.8\pm0.6^{2\ B}$	$2.8\pm0.6^{2\ B}$
	HG group	1.9 ± 0.2	2.1 ± 0.3^{0}	2.3 ± 0.4^{0}	2.5 ± 0.4^{2}	2.4 ± 0.5^{2}
Supernatant LDH (UI/l)	MAG group	n.d.	5 ± 0.6^{NS}	$9\pm1.6^{1\ NS}$	$16\pm3.5^{1\ NS}$	$25\pm3.7^{1\ NS}$
	HG group	n.d.	4 ± 0.7	8 ± 1.2^{1}	11 ± 4.1^{1}	21 ± 2.8^{1}
Haemolysis (%)	MAG group	n.d.	1.4 ± 0.4^{NS}	$1.7\pm0.3^{0\ NS}$	$2.8\pm1.0^{0\ NS}$	$5.8\pm0.4^{1\ NS}$
	HG group	n.d.	1.3 ± 0.1	1.5 ± 0.4^{0}	2.4 ± 0.8^{0}	4.5 ± 0.7^{1}
Protein-bound Hcy (μmol/g protein)	MAG group	$0.21\pm0.04^{\ A}$	$0.25\pm0.05^{0\ B}$	$0.28\pm0.06^{0\ B}$	$0.31\pm0.07^{2\ B}$	$0.34\pm0.07^{2\ B}$
	HG group	0.12 ± 0.03	0.17 ± 0.04^{0}	0.20 ± 0.05^{0}	0.24 ± 0.06^{2}	0.27 ± 0.06^{2}
Supernatant MDA (nmol/ml)	MAG group	n.d	n.d.	n.d.	0.13 ± 0.03^{NS}	$0.16\pm0.06^{0\ NS}$
	HG group	n.d	n.d.	n.d.	0.12 ± 0.04	0.15 ± 0.05^{0}

Intragroup difference (with respect to first detectable measurement): 0=ns; 1=P<0.01; 2=P<0.05. Intergroup difference: A=P<0.01; B=P<0.05; NS=not significant.

damage in RBC. Progressive peroxidation of membrane lipids has been advocated as a possible mechanism of haemolysis in the presence of many different experimental and clinical conditions [46–49]. A similar role in haemolytic process has been advocated for modifications concerning RBC membrane-related and cytoskeletal proteins. Particularly, protein thiol groups (cysteine residues) represent one of the sites most likely to be modified: interference with the functional capability of membrane sulfhydryl groups, either by their oxidation or by their blockade, results in premature RBC destruction in vivo; moreover it has been demonstrated that high concentrations of sulfhydryl-active agents (including molecules able to form mixed disulfide linkages) in vitro interfere with the transport and metabolism of glucose and the membrane permeability to cations, thus leading to osmotic swelling and haemolysis [34,35]. Homocysteine, with respect to other circulating thiols, has been attributed some peculiar characteristics that make this compound of interest as a potential "haemolytic toxin": (1) pro-oxidant effect due to induction of free-radicals during the autoxidation to homocystine or during the oxidation in the formation of different mixed disulfide compounds [22,50,51]; (2) greater disposition (about 100 times greater than cysteine) to form disulfide bounds than other thiols (hence, higher disposition to bind to cysteine lateral residues of proteins) [23,24].

Different authors demonstrated the presence in blood cells of enzymes involved in the conversion of methionine into homocysteine [36,38,39]; Malinow et al. in their elegant studies showed that synthesis of homocysteine occurs in erythrocytes, whereas leucocytes both synthesize and transulfurate homocysteine. Elevation of homocysteine in red blood suspensions would represent the effect of a continue conversion of methionine to homocysteine, and its

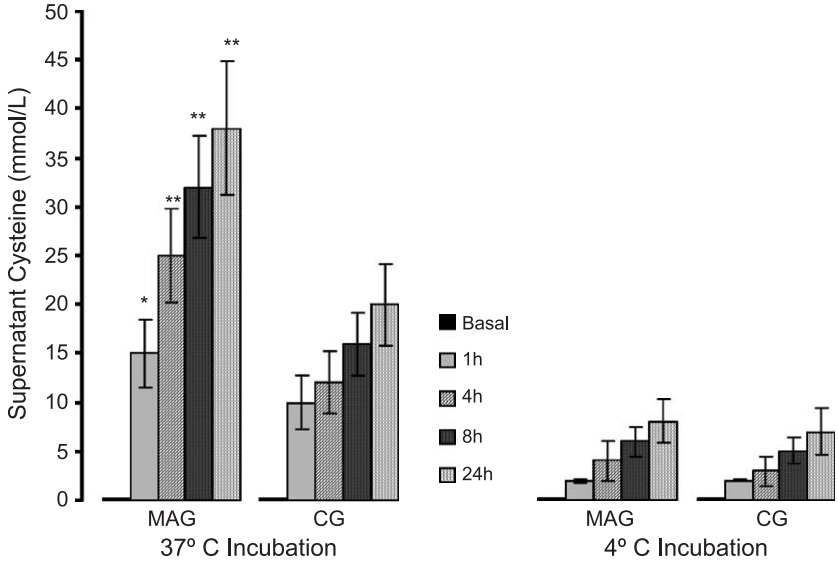

Fig. 2. Trend over time of supernatant cysteine in samples from controls (CG) and patients with megaloblastic anemia (MAG) (results from samples at 4 and 37 °C incubation are presented). *P<0.05 and **P<0.01, MAG vs. CG samples at 37 °C. No difference was found in intragroup comparison at 4 °C incubation at any correspondent time assessment. MAG and CG values at 37 °C always showed significant higher (P<0.01 for all assessments) supernatant cysteine levels with respect to their correspondent time assessment at 4 °C.

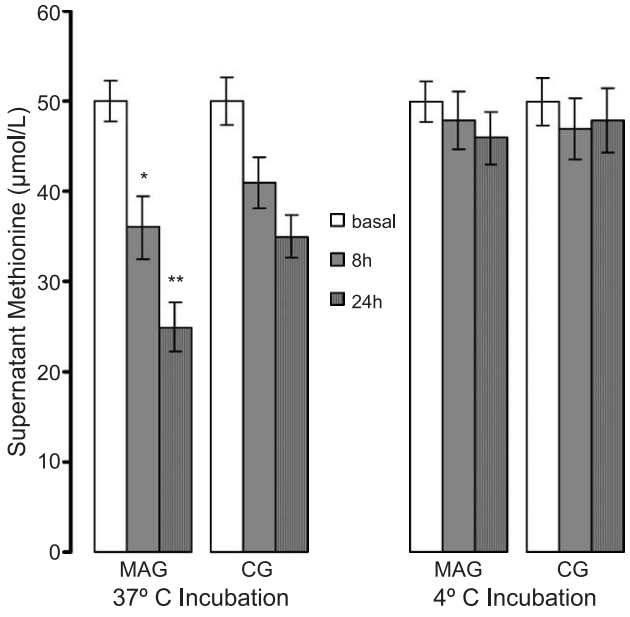

Fig. 3. Trend over time of supernatant methionine in samples from controls (CG) and patients with megaloblastic anemia (MAG) (results from basal, 8- and 24-h samples at 4 and 37 °C incubation are presented) *P<0.05 and **P<0.01, MAG vs. CG samples at 37 °C. No difference was found in intragroup comparison at 4 °C incubation. MAG and CG values at 37 °C showed significant lower (P<0.01) supernatant methionine levels with respect to their correspondent time (8 and 24 h) assessment at 4 °C.

prompt export into plasma [40]. No conclusive data are available on the presence within mature red blood cells of remethylation activities, even if Bagley and Selhub [52] showed the role of MTHFR variants in influencing the kind of folates stored within red blood cells and, more recently, the presence within RBC of soluble cytochrome b_5 (an important cofactor of methionine synthase, a key enzyme involved in remethylation of homocysteine to methionine) has been demonstrated [53]. WBCs seem to be able of Hcy remethylation by methionine synthase (MS) pathway (vit. B_{12}- and folate-dependent; this pathway is thought to responsible for about 27–30% of Hcy consumed), whereas Hcy remethylation by betaine-homocysteine methyl transferase (BHMT) (about 27–30% of Hcy consumed) seems to occur only in liver and kidney [54].

To our knowledge, no data are available concerning the homocysteine production by blood cells in megaloblastic anaemias due to vitamin B_{12} and folate deficiencies (both conditions known to lead to hyperhomocysteinemia) and if this production may have a role in increased haemolytic process observed in these diseases.

Our data (Table 1) confirm the well-known link between vitamin B_{12} and folate status and plasma homocysteine: MAG group patients have significantly higher levels of plasma homocysteine than CG; this is likely the consequence of the impairment of homocysteine metabolism due the severe lack of vitamin cofactors, essential for Hcy remethylation by MS pathway. Moreover, our data suggest that blood cells from MA patients produce in vitro significantly greater amounts of Hcy than controls. The metabolic activity seems to be relevant in this production (i.e., it is not a consequence of cellular leakage due to haemolysis), as may argued by the scanty effect in Hcy production in samples at 4 °C (Table 3). This observation is in agreement with previous data about the continuous production (and export) of Hcy by metabolic active RBCs in suspension as an effect of continuous transmethylation of methionine [40,55,56]. In our model, part of this Hcy is likely to be metabolized by WBCs, whose regulatory effect on whole-blood Hcy production has been previously demonstrated [55]; the higher Hcy levels in supernatant from MAG patients with respect to CG subjects may be a consequence of the impairment of MS remethylation pathway in WBCs of MAG group, consequent to deficient vitamin status: in these cells only (or in prevalence) the transsulfuration is likely to be functioning (and partially compensating for remethylation impairment); transsulfuration has been demonstrated able to increase significantly in response to conditions leading to HHcy [54]. These observations may explain the higher cysteine levels through time found in the supernatant of MAG group with respect to CG group (at 37 °C) (Fig. 2). Similarly, the greater supernatant methionine decrease over time in MAG group compared to CG group (Fig. 3) may be explained by new methionine synthesis (remethylation) which is possible only by blood cells of CG group.

With regards to the possible effect of relative increase of Hcy production on RBC damage, our data (cf. results about differences and correlations about Hcy increase, MDA production, ghost protein-bound protein and haemolysis in metabolic active MAG samples with respect CG) seem to confirm the possible haemolytic effect of homocysteine excess in vitro, especially in presence of polymorphonuclear cells [18]. Goth and Vitai [57] recently confirmed the possibility of Hcy to induce RBC membrane peroxidation in vivo, especially in the presence of decreased cellular antioxidant capacity, and it has been observed that folates or vitamin B_{12} deficiency is associated to a reduced availability of glutathione, an important factor in antioxidant defence of RBC [58,59].

With concerns to the possible toxic effect by methionine per se [60], it is to be considered that the methionine levels we used in our experimental model were adjusted at 50 µmol/l, which is a concentration near at the higher level of the physiological plasma range for adults (15–45 µmol/l). Moreover, in our study, methionine levels reduced during incubation at 37 °C (i.e., during increase in peroxidation and haemolysis parameters), whereas they remained almost unchanged during incubation at 4 °C (Fig. 3). In this last situation, even though RBC remained exposed to relative high levels of methionine through time, no significant signs of haemolysis were noticed. Moreover, in samples at 37 °C,

methionine levels in supernatant were always significantly higher over time in CG group (Fig. 3), where RBC damage parameters were significantly lower then those observed in NAG group samples.

On the other hand, a low increase in Hcy levels, as that observed in samples incubated in a methionine-free medium (Table 4), seems to induce scanty effects on RBC damage, supporting a dose-dependent effect in Hcy-induced damage. This scanty increase in Hcy supernatant even in the absence of methionine, the homocysteine natural precursor, may be explained by metabolism of methionine and *S*-adenosylmethionine present in cell cytoplasm at time 0, according to previous observation by other authors in similar conditions [55,56,61].

Nevertheless, before drawing definitive clinical conclusions, some issues have to be considered. The first issue regards data from human models: so far, no data about clinical evidence of haemolysis have been reported in patients with homocystinuria (homocysteine plasma levels >200 μmol/l) not due to B_{12} or folate deficiencies, or to inherited metabolic defects influencing B_{12} or folates cellular utilization (as in CbL diseases) [10,62]. It is to be considered that our study was conducted in vitro and under conditions far enough from physiological: for example, our model lacks plasma antioxidants and proteins (both able to modulate Hcy toxicity). Moreover, in vivo, the Hcy transsulfuration by other tissues (mainly the liver and the kidney) may be important in limiting Hcy accumulation as well [3,63]. On the other hand, our model (blood cells only) somehow reflects a condition quite similar to that present in the blood marrow, where haemolysis in megaloblastic anaemias is thought to take place.

Similarly, though our data would suggest a relation between ghost protein-bound Hcy and RBC damage, nevertheless it is to be reminded that different authors have demonstrated that a cytoplasm increase in Hcy may influence the cytoskeletal proteins, and hence favour haemolytic processes, by altering the processes of protein methylation; a Hcy accumulation in fact progressively blocks the transmethylation of methionine, this inducing a reduction in cell *S*-adenosyl-methionine (SAMe) availability, a key substrate for methylation reactions needed to maintain the integrity of RBC structural proteins through RBC life-time [64–67]. Both mechanisms (direct, by functional alteration due to mixed-disulfide formation with protein-ghost cysteine residues; indirect, by SAMe synthesis blockade) may be relevant in Hcy-induced damage to RBC cytoskeletal or membrane proteins.

In conclusion, our data from an in vitro experience suggest a higher production of Hcy by blood cells in the presence of vit. B_{12} and folate deficiency leading to megaloblastic anaemias. The same data suggest a possible effect of Hcy accumulation in inducing erythrocyte damage and favouring haemolysis by mechanisms involving both lipid peroxidation and membrane/cytoskeletal protein derangement [57,68–70].

Nevertheless, more data are needed in order to evaluate and confirm a similar effect in vivo of Hcy accumulation on erythrocyte survival in case of megaloblastic anaemias due to cobalamine and folate deficiency.

References

[1] M.R. Malinow, Hyperhomocyst(e)inemia. A common and easily reversible risk factor for occlusive atherosclerosis, Circulation 81 (1990) 2004–2006.
[2] L.H. Mudd SH, Disorders in transsulfuration, in: J.B. Wingarden, W.J. Stanbury, D.S. Fredrickson, J.L. Goldstein, M.S. Brown (Eds.), Metabolic Bases of Inherited Diseases, McGraw-Hill Book, New York, NJ, 1995, pp. 1279–1327.
[3] J. Selhub, Homocysteine metabolism, Annu. Rev. Nutr. 19 (1999) 217–246.
[4] A.M. Engbersen, D.G. Franken, G.H. Boers, E.M. Stevens, F.J. Trijbels, H.J. Blom, Thermolabile 5,10-methylenetetrahydrofolate reductase as a cause of mild hyperhomocysteinemia, Am. J. Hum. Genet. 56 (1995) 142–150.
[5] B. Christensen, P. Frosst, S. Lussier-Cacan, J. Selhub, P. Goyette, D.S. Rosenblatt, J. Genest Jr., R. Rozen, Correlation of a common mutation in the methylenetetrahydrofolate reductase gene with plasma homocysteine in patients with premature coronary artery disease, Arterioscler. Thromb. Vasc. Biol. 17 (1997) 569–573.
[6] H.J. Blom, Genetic determinants of hyperhomocysteinaemia: the roles of cystathionine beta-synthase and 5,10-methylenetetrahydrofolate reductase, Eur. J. Pediatr. 159 (Suppl. 3) (2000) S208–S212.
[7] M.Y. Tsai, M. Bignell, F. Yang, B.G. Welge, K.J. Graham, N.Q. Hanson, Polygenic influence on plasma homocysteine: association of two prevalent mutations, the 844ins68 of cystathionine beta-synthase and A(2756)G of methionine synthase, with lowered plasma homocysteine levels, Atherosclerosis 149 (2000) 131–137.
[8] M.Y. Tsai, B.G. Welge, N.Q. Hanson, M.K. Bignell, J. Vessey, K. Schwichtenberg, F. Yang, F.E. Bullemer, R. Rasmussen, K.J. Graham, Genetic causes of mild hyperhomocysteinemia in patients with premature occlusive coronary artery diseases, Atherosclerosis 143 (1999) 163–170.
[9] E. Joosten, A. van den Berg, R. Riezler, H.J. Naurath, J. Lindenbaum, S.P. Stabler, R.H. Allen, Metabolic evidence that deficiencies of vitamin B-12 (cobalamin), folate, and vitamin B-6 occur commonly in elderly people, Am. J. Clin. Nutr. 58 (1993) 468–476.
[10] J.B. Mason, J.W. Miller, The effects of vitamins B12, B6, and folate on blood homocysteine levels, Ann. N.Y. Acad. Sci. 669 (1992) 197–203 (discussion 203-194).
[11] D.E. Wilcken, B. Wilcken, B vitamins and homocysteine in cardiovascular disease and aging, Ann. N.Y. Acad. Sci. 854 (1998) 361–370.
[12] A. Lobo, A. Naso, K. Arheart, W.D. Kruger, T. Abou-Ghazala, F. Alsous, M. Nahlawi, A. Gupta, A. Moustapha, F. van Lente, D.W. Jacobsen, K. Robinson, Reduction of homocysteine levels in coronary artery disease by low-dose folic acid combined with vitamins B6 and B12, Am. J. Cardiol. 83 (1999) 821–825.
[13] D.G. Savage, J. Lindenbaum, S.P. Stabler, R.H. Allen, Sensitivity of serum methylmalonic acid and total homocysteine determinations for diagnosing cobalamin and folate deficiencies, Am. J. Med. 96 (1994) 239–246.
[14] M.A. Vilaseca, D. Moyano, R. Artuch, I. Ferrer, M. Pineda, E. Cardo, J. Campistol, C. Pavia, J.A. Camacho, Selective screening for hyperhomocysteinemia in pediatric patients, Clin. Chem. 44 (1998) 662–664.
[15] M. Carrasco, A. Remacha, A. Sureda, P. Sarda, R. Martino, J. Sierra, Acute megaloblastic anemia: homocysteine levels are useful for diagnosis and follow-up, Haematologica 84 (1999) 767–768.

[16] G.G. Klee, Cobalamin and folate evaluation: measurement of methylmalonic acid and homocysteine vs. vitamin B(12) and folate, Clin. Chem. 46 (2000) 1277–1283.
[17] M. Cattaneo, Hyperhomocysteinemia, atherosclerosis and thrombosis, Thromb. Haemost. 81 (1999) 165–176.
[18] R. Olinescu, F.A. Kummerow, B. Handler, L. Fleischer, The hemolytic activity of homocysteine is increased by the activated polymorphonuclear leukocytes, Biochem. Biophys. Res. Commun. 226 (1996) 912–916.
[19] R. Clarke, L. Daly, K. Robinson, E. Naughten, S. Cahalane, B. Fowler, I. Graham, Hyperhomocysteinemia: an independent risk factor for vascular disease, N. Engl. J. Med. 324 (1991) 1149–1155.
[20] M. den Heijer, F.R. Rosendaal, H.J. Blom, W.B. Gerrits, G.M. Bos, Hyperhomocysteinemia and venous thrombosis: a meta-analysis, Thromb. Haemost. 80 (1998) 874–877.
[21] I.M. Graham, L.E. Daly, H.M. Refsum, K. Robinson, L.E. Brattstrom, P.M. Ueland, R.J. Palma-Reis, G.H. Boers, R.G. Sheahan, B. Israelsson, C.S. Uiterwaal, R. Meleady, D. McMaster, P. Verhoef, J. Witteman, P. Rubba, H. Bellet, J.C. Wautrecht, H.W. de Valk, A.C. Sales Luis, F.M. Parrot-Rouland, K.S. Tan, I. Higgins, D. Garcon, G. Andria, et al., Plasma homocysteine as a risk factor for vascular disease. The European Concerted Action Project, Jama 277 (1997) 1775–1781.
[22] J.S. Stamler, A. Slivka, Biological chemistry of thiols in the vasculature and in vascular-related disease, Nutr. Rev. 54 (1996) 1–30.
[23] M.A. Mansoor, A.B. Guttormsen, T. Fiskerstrand, H. Refsum, P.M. Ueland, A.M. Svardal, Redox status and protein binding of plasma aminothiols during the transient hyperhomocysteinemia that follows homocysteine administration, Clin. Chem. 39 (1993) 980–985.
[24] L.A. Smolin, N.J. Benevenga, The use of cyst(e)ine in the removal of protein-bound homocysteine, Am. J. Clin. Nutr. 39 (1984) 730–737.
[25] V.C. Wiley, N.P. Dudman, D.E. Wilcken, Interrelations between plasma free and protein-bound homocysteine and cysteine in homocystinuria, Metabolism 37 (1988) 191–195.
[26] G. Starkebaum, J.M. Harlan, Endothelial cell injury due to copper-catalyzed hydrogen peroxide generation from homocysteine, J. Clin. Invest. 77 (1986) 1370–1376.
[27] R.T. Wall, J.M. Harlan, L.A. Harker, G.E. Striker, Homocysteine-induced endothelial cell injury in vitro: a model for the study of vascular injury, Thromb. Res. 18 (1980) 113–121.
[28] D. Lang, M.B. Kredan, S.J. Moat, S.A. Hussain, C.A. Powell, M.F. Bellamy, H.J. Powers, M.J. Lewis, Homocysteine-induced inhibition of endothelium-dependent relaxation in rabbit aorta: role for superoxide anions, Arterioscler. Thromb. Vasc. Biol. 20 (2000) 422–427.
[29] J.W. Heinecke, M. Kawamura, L. Suzuki, A. Chait, Oxidation of low density lipoprotein by thiols: superoxide-dependent and -independent mechanisms, J. Lipid Res. 34 (1993) 2051–2061.
[30] B. Babior, The megaloblastic anemias, in: E. Beutler, M.A. Lichtman, B.S. Coller, K. TJ (Eds.), Williams Hematology, McGraw-Hill, New York, NY, 1995, pp. 471–489.
[31] R. Carmel, Megaloblastic anemias, Curr. Opin. Hematol. 1 (1994) 107–112.
[32] S.N. Wickramasinghe, The wide spectrum and unresolved issues of megaloblastic anemia, Semin. Hematol. 36 (1999) 3–18.
[33] P. Caprari, A. Bozzi, W. Malorni, A. Bottini, F. Iosi, M.T. Santini, A.M. Salvati, Junctional sites of erythrocyte skeletal proteins are specific targets of *tert*-butylhydroperoxide oxidative damage, Chem.-Biol. Interact. 94 (1995) 243–258.
[34] Y. Ohmiya, K. Nakai, Relationship between inhibition of membrane SH groups and hemolysis induced by SH inhibitors, Jpn. J. Pharmacol. 27 (1977) 596–599.
[35] E. Baumann, G. Stoya, A. Volkner, W. Richter, C. Lemke, W. Linss, Hemolysis of human erythrocytes with saponin affects the membrane structure, Acta Histochem. 102 (2000) 21–35.
[36] K.L. Oden, S. Clarke, *S*-Adenosyl-L-methionine synthetase from human erythrocytes: role in the regulation of cellular *S*-adenosylmethionine levels, Biochemistry 22 (1983) 2978–2986.
[37] C. Gomes Trolin, B. Regland, L. Oreland, Decreased methionine adenosyltransferase activity in erythrocytes of patients with dementia disorders, Eur. Neuropsychopharmacol. 5 (1995) 107–114.
[38] M.S. Hershfield, N.M. Kredich, D.R. Ownby, H. Ownby, R. Buckley, In vivo inactivation of erythrocyte *S*-adenosylhomocysteine hydrolase by 2′-deoxyadenosine in adenosine deaminase-deficient patients, J. Clin. Invest. 63 (1979) 807–811.
[39] J.E. Kaminska, I.H. Fox, Decreased *S*-adenosylhomocysteine hydrolase in inborn errors of purine metabolism, J. Lab. Clin. Med. 96 (1980) 141–147.
[40] M.R. Malinow, M.K. Axthelm, M.J. Meredith, N.A. MacDonald, B.M. Upson, Synthesis and transsulfuration of homocysteine in blood, J. Lab. Clin. Med. 123 (1994) 421–429.
[41] S.F. Choumenkovitch, P.F. Jacques, M.R. Nadeau, P.W. Wilson, I.H. Rosenberg, J. Selhub, Folic acid fortification increases red blood cell folate concentrations in the Framingham study, J. Nutr. 131 (2001) 3277–3280.
[42] P.E. Cornwell, S.L. Morgan, W.H. Vaughn, Modification of a high-performance liquid chromatographic method for assay of homocysteine in human plasma, J. Chromatogr. 617 (1993) 136–139.
[43] K. Ou, M.R. Wilkins, J.X. Yan, A.A. Gooley, Y. Fung, D. Sheumack, K.L. Williams, Improved high-performance liquid chromatography of amino acids derivatised with 9-fluorenylmethyl chloroformate, J. Chromatogr., A 723 (1996) 219–225.
[44] Y. Sato, S. Kamo, T. Takahashi, Y. Suzuki, Mechanism of free radical-induced hemolysis of human erythrocytes: hemolysis by water-soluble radical initiator, Biochemistry 34 (1995) 8940–8949.
[45] K. Fukunaga, T. Suzuki, K. Takama, Highly sensitive high-performance liquid chromatography for the measurement of malondialdehyde in biological samples, J. Chromatogr. 621 (1993) 77–81.
[46] U.J. Dumaswala, L. Zhuo, D.W. Jacobsen, S.K. Jain, K.A. Sukalski, Protein and lipid oxidation of banked human erythrocytes: role of glutathione, Free Radic. Biol. Med. 27 (1999) 1041–1049.
[47] E. Niki, Y. Yamamoto, M. Takahashi, K. Yamamoto, E. Komuro, M. Miki, H. Yasuda, M. Mino, Free radical-mediated damage of blood and its inhibition by antioxidants, J. Nutr. Sci. Vitaminol. (Tokyo) 34 (1988) 507–512.
[48] E. Niki, S. Minamisawa, M. Oikawa, E. Komuro, Membrane damage from lipid oxidation induced by free radicals and cigarette smoke, Ann. N.Y. Acad. Sci. 686 (1993) 29–37 (discussion 37-28).
[49] G. Facorro, F. Aguirre, L. Florentin, M. Diaz, T. De Paoli, J.E. Ihlo, A.A. Hager, J.C. Sanchez Avalos, H.A. Farach, C.P. Poole Jr., Oxidative stress and membrane fluidity in erythrocytes from patients with hemolytic uremic syndrome, Acta Physiol. Pharmacol. Ther. Latinoam. 47 (1997) 137–146.
[50] J. Loscalzo, The oxidant stress of hyperhomocyst(e)inemia, J. Clin. Invest. 98 (1996) 5–7.
[51] G.N. Welch, G.R. Upchurch Jr., J. Loscalzo, Homocysteine, oxidative stress, and vascular disease, Hosp. Pract. (Off. Ed) 32 (1997) 88–92.
[52] P.J. Bagley, J. Selhub, A common mutation in the methylenetetrahydrofolate reductase gene is associated with an accumulation of formylated tetrahydrofolates in red blood cells, Proc. Natl. Acad. Sci. U. S. A. 95 (1998) 13217–13220.
[53] Z. Chen, R. Banerjee, Purification of soluble cytochrome *b*5 as a component of the reductive activation of porcine methionine synthase, J. Biol. Chem. 273 (1998) 26248–26255.
[54] J.D. Finkelstein, J.J. Martin, Methionine metabolism in mammals. Distribution of homocysteine between competing pathways, J. Biol. Chem. 259 (1984) 9508–9513.
[55] A. Andersson, A. Isaksson, B. Hultberg, Homocysteine export from erythrocytes and its implication for plasma sampling, Clin. Chem. 38 (1992) 1311–1315.
[56] A. Andersson, A. Lindgren, B. Hultberg, Effect of thiol oxidation and thiol export from erythrocytes on determination of redox status of homocysteine and other thiols in plasma from healthy subjects and patients with cerebral infarction, Clin. Chem. 41 (1995) 361–366.

[57] L. Goth, M. Vitai, The effects of hydrogen peroxide promoted by homocysteine and inherited catalase deficiency on human hypocatalasemic patients, Free Radic. Biol. Med. 35 (2003) 882–888.
[58] P.I. Ho, D. Ashline, S. Dhitavat, D. Ortiz, S.C. Collins, T.B. Shea, E. Rogers, Folate deprivation induces neurodegeneration: roles of oxidative stress and increased homocysteine, Neurobiol. Dis. 14 (2003) 32–42.
[59] U.J. Dumaswala, L. Zhuo, S. Mahajan, P.N. Nair, H.G. Shertzer, P. Dibello, D.W. Jacobsen, Glutathione protects chemokine-scavenging and antioxidative defense functions in human RBCs, Am. J. Physiol., Cell Physiol. 280 (2001) C867–C873.
[60] A.M. Troen, E. Lutgens, D.E. Smith, I.H. Rosenberg, J. Selhub, The atherogenic effect of excess methionine intake, Proc. Natl. Acad. Sci. U. S. A. 100 (2003) 15089–15094.
[61] A. Schaefer, F. Piquard, P. Haberey, The effects of changes in plasma amino acid concentrations on erythrocyte amino acid content, Clin. Biochem. 23 (1990) 237–240.
[62] C. Steen, D.S. Rosenblatt, H. Scheying, H.C. Braeuer, A. Kohlschutter, Cobalamin E (cblE) disease: a severe neurological disorder with megaloblastic anaemia, homocystinuria and low serum methionine, J. Inherit. Metab. Dis. 20 (1997) 705–706.
[63] J.D. House, M.E. Brosnan, J.T. Brosnan, Characterization of homocysteine metabolism in the rat kidney, Biochem. J. 328 (Pt. 1) (1997) 287–292.
[64] P.N. McFadden, S. Clarke, Methylation at D-aspartyl residues in erythrocytes: possible step in the repair of aged membrane proteins, Proc. Natl. Acad. Sci. U. S. A. 79 (1982) 2460–2464.
[65] J.R. Barber, S. Clarke, Inhibition of protein carboxyl methylation by *S*-adenosyl-L-homocysteine in intact erythrocytes, Physiological consequences, J. Biol. Chem. 259 (1984) 7115–7122.
[66] A.F. Perna, D. Ingrosso, E. Satta, M. Romano, A. Cimmino, P. Galletti, V. Zappia, N.G. De Santo, Metabolic consequences of hyperhomocysteinemia in uremia, Am. J. Kidney Dis. 38 (2001) S85–S90.
[67] D. Ingrosso, S. D'Angelo, E. di Carlo, A.F. Perna, V. Zappia, P. Galletti, Increased methyl esterification of altered aspartyl residues in erythrocyte membrane proteins in response to oxidative stress, Eur. J. Biochem. 267 (2000) 4397–4405.
[68] C.G. Zou, N.S. Agar, G.L. Jones, Oxidative insult to human red blood cells induced by free radical initiator AAPH and its inhibition by a commercial antioxidant mixture, Life Sci. 69 (2001) 75–86.
[69] B. Bayes, M.C. Pastor, J. Bonal, J. Junca, R. Romero, Homocysteine and lipid peroxidation in haemodialysis: role of folinic acid and vitamin E, Nephrol. Dial. Transplant. 16 (2001) 2172–2175.
[70] S.K. Lee, B.M. Lee, Oxidation of erythrocyte protein and lipid, and hemolysis in rabbit red blood cells treated with benzo[*a*]pyrene or adriamycin, J. Toxicol. Environ. Health 51 (1997) 557–569.