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ORIGINAL ARTICLE

Homocysteinylation score of high-molecular weight plasma proteins

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Abstract Ultrafiltration rates (filterability) of protein-bound homocysteine (Hcy), unlike glutathione (Glt), are significantly decreased in patients with cardiovascular disorders, end-stage renal disease, and prothrombotic conditions. Reduced filterability of Hcy through 300,000 MWCO PES membranes in these groups of patients was observed, regardless of hyperhomocysteinemia (HHcy) degree. Filterability of Hcy, but not of glutathione, was impaired in plasma preparations. It is possible that the patients with impaired filterability of Hcy in mixed disulfide state are characterized by increased ability of partial Hcy retention by proteins and complexes with higher than albumin molecular weight. These findings led us to conclude that evaluation of protein-bound Hcy filterability may provide more complete diagnostic data, to interpret clinical significance of HHcy. The proposed Hcy filterability test may be performed as a simple laboratory procedure, in addition to conventional tests for total Hcy.

Keywords Homocysteine · Glutathione · Centrifugal ultrafiltration

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Introduction

Hyperhomocysteinemia (HHcy) is defined as elevated (>12 μmol/L) blood plasma levels of the homocysteine species (Hcy). The term "total homocysteine" (tHcy) refers to Hcy values in plasma preparations after mixed disulfides reduction. It is possible to assume that *S*- and *N*-homocysteinylation of proteins may explain some principal toxic effects of high tHcy levels. (Glushchenko and Jacobsen 2007; Ueland et al. 1996; Fridman et al. 2011; Jakubowski and Głowacki 2011; Głowacki et al. 2011). Protein binding, as a ratio of free-to-mixed disulfides, was assayed at different proportions for various plasma aminothiols, being approximately 0.2, 0.6, and 5 for Hcy, cysteine, and glutathione (Glt), respectively (Mansoor et al. 1992, 1994). Lower Hcy ratio is due to the very high Hcy pK_a value ($\sim$10.0), therefore, causing a more strong disulfide bond of its mixed disulfides. It was estimated that Hcy plasma protein binding capacity reached 4.88 ± 0.51 and 4.74 ± 0.68 μmol/g for healthy men and women, respectively (Togawa et al. 2000). Plasma albumin, due to its Cys-34 residue, serves as the main Hcy transporter under normal condition (Sengupta et al. 2001). With increasing age, an elevation of tHcy and other aminothiols bound to blood proteins was shown (Giustarini et al. 2006; Rossi et al. 2009). Homocysteinylation of other proteins including fibronectin, transthyretin, and metallothionein may present a mechanistic explanation for endotoxicity of elevated tHcy (Glushchenko and Jacobsen 2007; Hubmacher et al. 2011; Silla et al. 2013). Among the large proteins, alpha-2-macroglobulin (A2M, 720 kDa) is characterized by substantial capacity of Hcy binding (Catanescu et al. 2007). It was also suggested that apolipoprotein B (Mr >300 kDa) homocysteinylation can cause a receptor-mediated endocytosis of Hcy by the cells of vascular lining

(Zinellu et al. 2006). Hypothetically, this homocysteinylation may play a quite significant role in the vascular wall remodeling in the course of atherogenesis.

According to our assumption, detection of elevated Hcy binding to high-molecular weight proteins (>300 kDa) and its reduced binding to middle-sized proteins, e.g., albumin, may be of additional diagnostic and prognostic significance. Hcy redistribution between the plasma proteins can be detected by the same analytic procedures, as tHcy assay. The procedure includes comparisons between test results obtained before and after filtration of plasma preparations through molecular filters. The aim of the present study was to evaluate the levels of tHcy in fresh and thawed plasma samples and their ultrafiltrates.

Materials and methods

Chemicals

DL-homocysteine, L-cysteine, Glt oxidized form, ethylenediaminetetraacetic acid (EDTA), 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), dithiothreitol (DTT), and 5-sulfosalicylic acid were purchased from Sigma (St. Louis, MO, USA). HPLC-grade acetonitrile was purchased from Cryochrom (St. Petersburg, Russia). Human serum albumin was obtained from Serva chemicals (Heidelberg, Germany). Potassium dihydrogen phosphate, O-phosphoric acid, and all other chemicals used in this study were of analytical grade.

Plasma samples

Our study included healthy blood donors, patients with cardiovascular diseases (CVD), and subjects with end-stage renal disease treated at the First State I.Pavlov Medical University Hospital from 2009 to 2013. After obtaining the approval of the University Ethics Committee, the study has been performed in accordance with the standards of Helsinki Declaration and its later amendments. Peripheral blood samples were collected after overnight fast (for CVD) by venipuncture into cooled tubes with EDTA. The tubes were placed on ice and processed within 20 min. After centrifugation at 3000 rpm at 4 °C for 15 min, the clear plasma supernatant was collected and stored at −80 °C until analysis (for 1–4 weeks). In some cases, the plasma samples were split into two aliquots. One of them was ultrafiltered and analyzed within subsequent 2 h and the other was treated in a similar way after 12–15 h of freezing at −80 °C and subsequent thawing. In all cases, there was informed consent for anonymous use of the resultant data mining.

Table 1 Plasma samples characterization

	1	2	3	4	5
Clinical state	CVD patients with documented folate deficiency, freeze samples	CVD patients with activated intravascular coagulation, freeze samples	Donors, freeze samples	Donors, fresh samples	CVD high-cholesterolemic patients with folate deficiency, freeze samples	Hemodialysis patients with end-stage renal disease, fresh and freeze samples
N	20	17	22	9	8	23
Male/Female	10/10	7/10	8/14	3/6	2/6	11/12
Age, years	55–80	35–67	19–58	27–58	>55	55–73
tHcy, μmol/L M ± SD (Me)	19.1 ± 3.2 (22.9)	12.0 ± 6.5 (10.8)	7.4 ± 2.5 (6.9)	7.2 ± 2.3 (6.9)	15.6–18.5	31.8 ± 14.4 (28.9)
Comment	Methylmalonic acid: >0.6 μmol/L Low folate level: 10.3 ± 7.7 ng/mL; B_{12}: normal. Without vitamin supplementation	D-dimer level >600 μg/L. Without vitamin supplementation	Without vitamin supplementation	Used for experiments with fresh and freeze samples	Total cholesterol 5.28–7.33 mmol/L; C-reactive protein: 1.65–9.57 mg/L	Dialysis-dependence >3 years; With supplementation of B6 and folic acid. Samples taken before hemodialysis procedure; creatinine: 0.993 ± 0.288 mM. Used for experiments with fresh and freeze samples

CVD cardiovascular diseases, *tHcy* total homocysteine

Five groups of samples have been included in this study (Table 1). Plasma samples of the first group were obtained from CVD patients with mildly elevated tHcy level (19.1 ± 3.2 μmol/L), mild folic acid deficiency and methylmalonic acidemia, whereas B12 contents were within a reference interval of 133–675 pmol/L. The second group included CVD patients with tHcy levels approaching the upper reference limit (12.0 ± 6.5 μmol/L), along with elevated D-dimer amounts (>600 μg/L). Blood samples of the third group were taken from healthy donors (19–58 years old), being characterized by normal tHcy levels ranging from 5 to 11.7 μmol/L. Nine samples from this group were ultrafiltered and analyzed without freezing, as described above. The fourth group consisted of CVD patients characterized by HHcy and hypercholesterolemia. A series of 23 nonfasting blood samples obtained before hemodialysis from patients with end-stage renal disease and markedly elevated tHcy were designated as group 5 (Table 1). Routine biochemical data and clinical characteristics of the patients were gathered retrospectively.

Centrifugal ultrafiltration

Low-molecular-mass ultrafiltrates were obtained using Vivaspin 500 (Sartorius, Germany) 100,000 MWCO PES (~100 kDa cutoff) and 300,000 MWCO PES (~300 kDa cutoff) centrifugal devices. These membranes were used according to the manufacturer's specifications. Centrifugal ultrafiltration was carried out after 5-fold dilution of plasma with 9 g/L NaCl solution. The samples of diluted plasma were centrifuged at 6,000×*g* in a fixed-angle rotor for 60 min at 23–25 °C, and then tHcy was measured in diluted plasma and its ultrafiltrate. Protein-bound Hcy was evaluated using ultrafiltration coefficient which was designated as *F* factor and calculated as: *F* = tHcy in ultrafiltrate/tHcy in plasma. Factor *F* for Glt was evaluated with the same calculations.

Hcy and other aminothiols assay

tHcy was determined by HPLC method as described previously with some modifications (Zhloba and Blashko 2004). Briefly, 100 μL of sample was mixed with 50 μL of water or calibrator, incubated with 25 μL of DTT (10 mmol/L, dissolved in 1 mmol/L EDTA) for 10 min at 60 °C to reduce the disulfides and release protein-bound Hcy. Then, it was mixed with 100 μL of 10 mmol/L DTNB dissolved in the 100 mmol/L potassium phosphate buffer, pH 8.0 and approximately 5 min later deproteinization was achieved by the addition of 150 μL of sulfosalicylic acid (100.0 g/L) containing 0.2 mmol/L EDTA. Precipitated proteins were removed by centrifugation at 8000×*g* for 5 min, and the supernatant was filtered through 0.2 μm pore-sized filter.

Agilent 1100 HPLC system (Agilent Technologies, Germany) with a degasser, a quarterly pump with low-pressure gradient flow control valve, a thermostat for the column, an autosampler, a VWD detector with a 14 μL cell and chemstation Leochem RUS verA08.03 were used. A reversed-phase Zorbax Eclipse XDB-C8 (150 × 4.6 mm i.d. 5 μm) column was placed in the thermostat at 30 °C. A mobile phase of 9 % acetonitrile and 91 % of 0.1 M pH 3.78 potassium phosphate buffer with the flow rate of 0.8 mL/min was used for isocratic elution of aminothiol derivatives which lasted for 4.7 min. Then, with the flow rate of 1.6 mL/min the buffer was substituted in the linear gradient mode for water by 6.0 min, with the increase of acetonitrile amount up to 35 %. From the 6.1 min up to the 7.0 min, isocratic elution was occurred with 80 % acetonitrile and 20 % of water and then by the 11.0 min a return to the initial amounts of the mobile phase components was achieved with the linear gradient mode. The aminothiol-derivative peaks were detected at 330 nm.

Supporting measurements

A2M was measured in plasma immunoturbidimetrically using Beckman Coulter, Inc. assay kit. D-dimer concentration was determined quantitatively with Smart D-dimer test reagent kit (Eurolyser Diagnostica GmbH, Austria). Plasma folic acid and vitamin B12 measurements as levels of the two important cofactors of Hcy metabolism were performed by chemiluminescent immunoassay with an Access 2 system and reagent kits (Beckman Coulter, Fullerton, CA, USA). Plasma methylmalonic acid concentration was determined by HPLC method (Babidge and Babidge 1994) with fluorimetric detection (Agilent 1100, Agilent Technologies, Germany). Plasma creatinine, albumin levels and electrophoresis of plasma preparations were performed by means of standard equipment for clinical laboratories.

Statistical analysis

The statistical analysis was performed by methods of nonparametric statistics using SPSS 16.0 for Windows software package (SPSS Inc., Chicago, IL, USA). Between-group differences were assessed by Mann–Whitney *U* test, whereas Wilcoxon matched pairs test was used for dependent variables. Correlation analysis was performed using the Spearman correlation coefficient. A value of $p < 0.05$ was considered statistically significant.

Results

The aim of the initial experiments was to determine the type of filter which does not transmit to ultrafiltrate the A2M molecule or its dissociated subunits. Eight samples from

CVD patients of group 4 with moderate HHcy were used. After ultrafiltration through Vivaspin 100,000 MWCO PES from 8 to 37 % tHcy (Me = 21 %) and from 7 to 33 % protein (Me = 21 %) were found in ultrafiltrates. After ultrafiltration of the same plasma specimen through filters, 300,000 MWCO PES ultrafiltrates contained 20–39 % tHcy (Me = 28 %) and 37–56 % protein. In this regard, in all subsequent experiments, 300,000 MWCO PES filters were used. To assess the reproducibility of Hcy filterability, seven membrane devices Vivaspin 300,000 MWCO PES were taken in experiments with one plasma preparation with tHcy content of 55 μmol/L. At seven parallel experiments on the average, 14.2 μmol/L tHcy was found in ultrafiltrates. The coefficient of variation was 7.75 %.

The subsequent experiments allowed us to proceed with evaluation of filterability for Hcy contained in preparations of plasma samples from subjects with different CVD, including the intravascular coagulation/fibrinolysis syndrome. The *F* Factor values for Hcy were estimated in the sample groups 1, 2, and 4, with respect to the reference range of donors (Table 2).

Filterability of Hcy in plasma of patients with CVD was significantly reduced, as compared with reference group 3 ($p < 0.05$). In particular, reduced filterability of Hcy was more pronounced when CVD was accompanied by a prothrombotic state (group 2), where the average value of the *F* factor comprised only a half of that for normal subjects (0.29 ± 0.032 vs. 0.60 ± 0.189, respectively). Such low *F* values characterize a possible retention of Hcy by plasma proteins with a molecular weight of >300 kDa. It is essential to note that none of the studied groups revealed significant correlations between tHcy concentrations in plasma and ultrafiltrates.

In the reference group 3, the *F* value at the lower limit of interquartile range (25 percentile) was 0.44. This borderline level was used to determine the bottom reference value characterizing filterability of Hcy bound to plasma proteins. More clearly, the relationship between the plasma tHcy concentrations and its filterability is shown in Fig. 1. The lines, being parallel to *X* and *Y* axes, correspond to the reference limits of 0.44 for filterability and 12.0 μmol/L for plasma tHcy levels, respectively. When considering these data, an attention should be drawn to larger scatter of variables in the samples from group 1, rather than in groups 2 and 4. We can conclude that the *F* values in patients with CVD are not always significantly lower than in healthy donors. Eight samples from group 1 had $F \geq 0.44$, but were characterized by plasma tHcy ≥12 μmol/L. On the contrary, tHcy levels were not increased in 14 samples from group 2 (4.0–11.7 μmol/L), but their filterability was impaired.

The above-mentioned data were obtained with samples stored at −80°. Meanwhile, sensitivity of *F* factor to the sample freezing/thawing remained unclear. In the following series of experiments we compared the variables, including factor *F* for Hcy and Glt, having been assayed in fresh plasma samples and after storage of the specimens in frozen state. For this purpose, 23 plasma samples from the patients with end-stage renal disease treated with hemodialysis for several years (group 5) and nine plasma samples taken from healthy donors (group 3) were used. The results are shown in Table 3.

As seen from Table 3, the group of patients was characterized by moderate HHcy. In any case the level of tHcy was normal (range 15.2–59.0 μmol/L). Storage of samples at low temperature was accompanied by a small decrease of detectable amounts of tHcy and Glt. This tendency was observed in both groups, but reached statistical significance for tHcy in patients group. In absolute value, the reduction in detectable tHcy content was about 0.8 μmol/L, which is less than 3 %. Also, slight but significant reduction in factor *F* values for Hcy and Glt was observed after freezing of patients' plasma. The *F* values for Hcy, in contrast to those for Glt, were significantly lower in samples of hemodialysis patients compared to reference group. Only in two cases *F* was above reference limit of 0.44 (for freeze samples).

A moderate HHcy was found in the samples of group 5. In our earlier work (Zhloba and Blashko 2004) performed with a large clinical sample, we have shown that, in patients under chronic hemodialysis, the HHcy level may reach 45.5 ± 41.2 μmol/L. A lower level of HHcy of group 5 patients in present study is clearly due to vitamin therapy (Table 1). In spite of this treatment, the tHcy concentrations in the samples from group 5 are significantly higher than in

Table 2 Ultrafiltration rates of homocysteine across Vivaspin 300,000 MWCO PES membranes for species from freeze plasma

Sample Group	Ultrafiltration factor *F* (M ± SD)	Median (25–75 % percentile)
1. CVD patients with folate deficiency ($N = 20$)	0.38 ± 0.052	0.27* (0.18–0.62)
2. CVD patients with prothrombotic state ($N = 17$)	0.29 ± 0.032	0.27* (0.18–0.36)
3. Donors, reference group ($N = 22$)	0.60 ± 0.189	0.62 (0.44–0.79)
4. CVD high-cholesterolemic patients ($N = 8$)	0.28 ± 0.061	0.27* (0.23–0.32)

CVD cardiovascular diseases, *F* ultrafiltration coefficient which is calculated as:

F = tHcy in ultrafiltrate/tHcy in plasma

* $p < 0.05$ compared to reference group 3

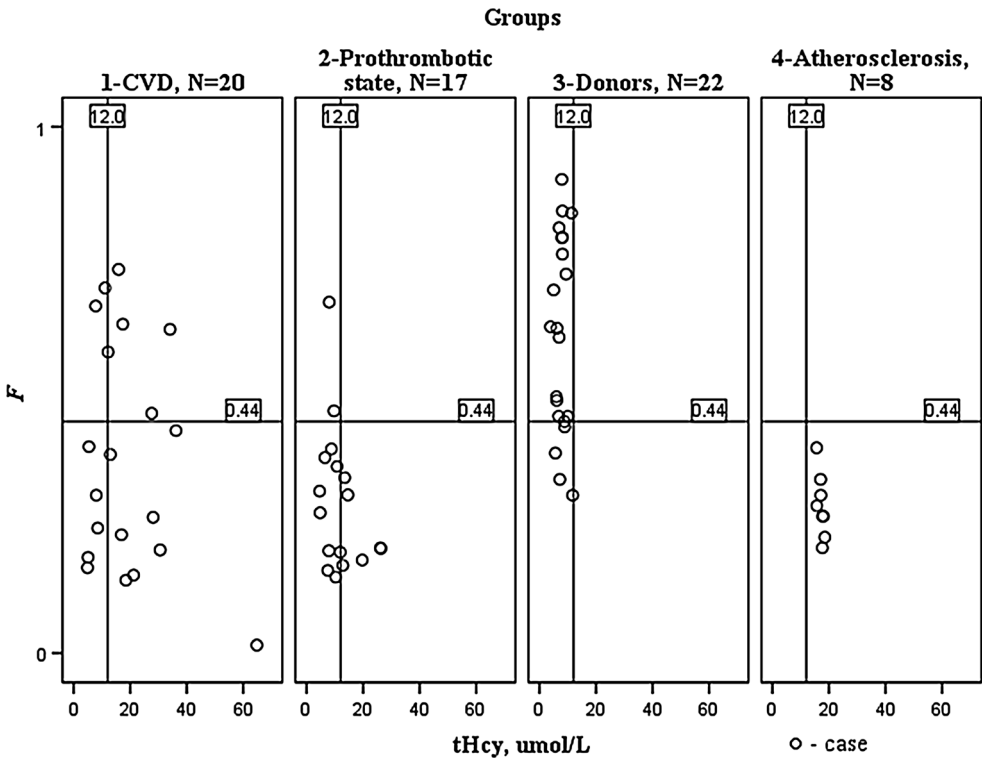

Fig. 1 Dot plots of tHcy and *F* in the groups of patients and donors. Lines parallel to the axes *X* and *Y* are the boundaries of the reference ranges of *F* (0.44) and o tHcy (12 μmol/L), respectively; *CVD* cardiovascular diseases, *tHcy* total homocysteine, *F* ultrafiltration coefficient which is calculated as: *F* = tHcy in ultrafiltrate/tHcy in plasma

Table 3 Effect of freezing on ultrafiltration rates of homocysteine and glutathione across Vivaspin 300,000 MWCO PES membranes

Variables	Fresh sample		After freezing	
	Hemodialysis patients (group 5), $N = 23$	Donors (from group 3), $N = 9$	Hemodialysis patients (group 5), $N = 23$	Donors (group 3), $N = 22$[a]
tHcy, μmol/L	28.9[#] (20.3–36.3)	6.9 (6.0–8.1)	28.1*,[#] (18.9–33.6)	6.9 (6.1–8.8)
Ultrafiltration factor *F* for Hcy	0.40[#] (0.35–0.52)	0.81 (0.77–0.84)	0.36*,[#] (0.33–0.43)	0.62 (0.44–0.79)
Glt, μmol/L	8.3[#] (7.3–9.9)	6.0 (4.7–7.4)	7.5[#] (7.2–9.1)	5.5 (4.4–7.3)
Ultrafiltration factor *F* for Glt	0.82 (0.75–0.92)	0.83 (0.75–0.88)	0.74* (0.70–0.82)	0.81 (0.75–0.86)

tHcy total homocysteine, *Glt* glutathione, data are presented as median (25–75 % percentile)

F ultrafiltration coefficient which is calculated as: *F* = tHcy in ultrafiltrate/tHcy in plasma and similarly for Glt

* $p < 0.05$ compared to fresh sample (matched pairs test)

[#] $p < 0.05$ between-group difference

[a] $N = 9$ for matched-pairs comparison

other experimental groups. Interestingly, despite high concentration of tHcy, its filterability *F* in group 5 was significantly better (0.36 for freeze samples) than in the groups 2 and 4 (0.27 for the both cases, see Table 2) and were not significantly different from that in group 1 ($p = 0.004$; 0.001, and 0.436, resp.). All this shows once again that there is no simple inverse relationship between the Hcy concentrations and its filterability.

To compare filtration efficiency for various plasma proteins, the preparations from six randomly selected samples were analyzed using standard electrophoretic procedure. It was found that, on average, ultrafiltrates contained 76 % of albumin, 70 % of alpha-1-globulins, but less than 10 % of alpha-2- and beta globulins from the initial plasma levels (see Online Resource 1 for the example of a case report). According to the immunoturbidimetric data, A2M was retained in the non-filterable fraction and was not detected in the filtrates. The effect of freezing on filtration efficiency of proteins across 300,000 MWCO PES membranes was insignificant, according to the data of electrophoretic fractionation.

Discussion

The purpose for *F* factor determination is to obtain more information about the degree of Hcy binding by the high-

molecular weight plasma proteins. We did not observe any correlations between tHcy and filterability factor F in the groups 1, 2, 3, and 5, with respective R(p) values: −0.1 (0.6), −0.2 (0.3), 0.04 (0.9), −0.2 (0.3). Our study shows that the F factor was significantly decreased in cases of atherogenic processes (groups 4 and 5). In group 4 ($N = 8$), a decreased filterability of Hcy was accompanied by HHcy in all cases, whereas a significant reduction of F factor below the reference range (<0.44) was found in 21 cases from the group 5 ($N = 23$). This tendency was observed in group 1 of samples taken from patients with various CVD ($N = 20$), for which the F factor was decreased in 14 cases, being accompanied by low Hcy rates (<12 μmol/L) in six of these cases (Fig. 1). Since a significant reduction of Hcy filterability was observed in groups 4 and 5 marked by the progression of atherosclerosis, it may be assumed that the F factor indicates a presence of characteristic bound Hcy fraction. A mechanism for decreased filterability of protein-bound Hcy remains unclear. Our findings allow a conclusion that Hcy bonding in mixed disulfide with other non-albumin proteins may cause some changes in filterability of Hcy. Apparently, a variety of proteins may be involved in the transport of Hcy in plasma (Silla et al. 2013). It should be noted that there is a rather short list of candidates in plasma proteome with a molecular mass equal to or exceeding 300 kDa and thiolate-anion expressing capability, or having a large number of sites for thiol-disulfide exchange. In particular, such proteins may be complement component C3 (190 kDa, 5–15). Also, immunoglobulins with a total concentration of light and heavy chains about 500 μmol/L may have unpaired thiols in some of the variable regions. A2M (720 kDa) is characterized by rapid disulfide bond formation with Hcy (Catanescu et al. 2007) with a capacity in plasma of about 20 μmol/L after proteinase-induced activation of A2M. It was also shown that apoB of low density lipoproteins (550 kDa) can bind Hcy (Luoma et al. 1994; Schulz et al. 2003); therefore, internalization of the activated A2M as well as LDL carrying Hcy occurred at the expense of LDL receptors (Zinellu et al. 2006, 2009, 2010). Thereby, proteins remaining in the retentate chamber contain potential carriers of Hcy; however, our data do not indicate specifically on any of them and make it necessary to study this in the future.

Decreased filterability of Hcy only, but normal passage of Glt, was revealed for the samples of group 5, as compared both in fresh samples and those after freezing/thawing. Most likely, this is due to higher ability of Hcy to form mixed disulfides with plasma proteins, since Hcy forms strong disulfide bonds, causing displacement of other aminothiols by a thiol-disulfide exchange (Sengupta et al. 2001; Glushchenko and Jacobsen 2007; Zinellu et al. 2006, 2010; Rossi et al. 2009).

Concerning a decrease of Hcy in plasma samples after storage in frozen state, one may suggest a conversion of aminothiols into other forms, including N-derivatives by protein homocysteinylation (Jakubowski and Głowacki 2011).

Perhaps, those patients who maintain high F levels may be characterized by the less "dangerous" HHcy, whereas patients without HHcy and with low values of F can be described by an increased ability of Hcy retention in their tissues, including vascular cell lining, e.g., by endocytosis with various proteins. Hence, the present results lead us to a conclusion that the Hcy filterability assays allow us to estimate its retention by various plasma proteins, including their modified forms and complexes with higher molecular mass than the albumin monomers. This approach may offer more complete diagnostic information useful for evaluation of clinical significance of HHcy. The proposed testing of Hcy filterability may be performed as a simple laboratory procedure, in addition to conventional tests for total Hcy. We suggest that the poor filterability of Hcy may be indicative for unfavorable transfer of Hcy to alternative targets, thus, likely, causing adverse biological effects. Therefore, some extensive clinical trials with a large number of observations would be necessary, to explain possible significance of the F factor, as a marker of altered Hcy transport forms in blood, and to reveal a potential role of this test as a predictor of adverse outcomes in CVD.

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