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Review

# The disturbance of hemostasis induced by hyperhomocysteinemia; the role of antioxidants

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**Elevated concentration of homocysteine (Hcy) in human tissues, definied as hyperhomocysteinemia has been correlated with some diseases, such as cardiovascular, neurodegenerative, and kidney disorders. Homocysteine occurs in human blood plasma in several forms, including the most reactive one, the homocysteine thiolactone (HTL) — a cyclic thioester, which represents up to 0.29% of total plasma Hcy. In the article, the effects of hyperhomocysteinemia on the complex process of hemostasis, which regulates the flowing properties of blood, are described. Possible interactions of homocysteine and its different derivatives, including homocysteine thiolactone, with the major components of hemostasis such as endothelial cells, blood platelets, plasmatic fibrinogen and plasminogen, are also discussed. Modifications of hemostatic proteins (N-homocysteinylation or S-homocysteinylation) induced by Hcy or its thiolactone seem to be the main cause of homocysteine biotoxicity in hemostatic abnormalities. It is suggested that Hcy and HTL may also act as oxidants, but various polyphenolic antioxidants are able to inhibit the oxidative damage induced by Hcy or HTL. We also discuss the role of phenolic antioxidants in hyperhomocysteinemia -induced changes in hemostasis.**

**Key words**: hyperhomocysteinemia, homocysteine, homocysteine thiolactone, hemostasis, blood platelets, fibrinogen, plasminogen, antioxidant

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## INTRODUCTION

L-Homocysteine (Hcy) is an endogenous amino acid, containing a free thiol group, which in healthy cells is involved in methionine and cysteine synthesis/resynthesis. Indirectly, Hcy participates in methyl, folate, and cellular thiol metabolism (D'Angelo & Selhub, 1997). Approximately 80% of total plasma Hcy is protein-bound, and only a small amount exists as a free reduced Hcy (about 0.1 μM). The majority of the unbound fraction of Hcy is oxidized, and forms dimers (homocystine) or mixed disulphides consisting of cysteine and Hcy (Table 1) (Mansoor *et al.,* 1992; Ramakrishnan *et al.*, 2006).

Over the years, several theories concerning the toxicity of Hcy have been elaborated, but despite the efforts, none fully explains the toxicity of this compound. However, two main pathways of Hcy biotoxicity are discussed: 1) Hcy-dependent oxidative stress and 2) Hcy-induced protein structure modifications, named homocysteinylation. In the first case, oxidative stress is generated during oxidation of the free thiol group of Hcy, when Hcy binds *via* a disulphide bridge with plasma proteins — mainly albumin, or with other low-molecular plasma thiols or with a second Hcy molecule. Oxidation of Hcy may induce the subsequent oxidation of proteins, lipids and nucleic acids (Zou & Banerjee, 2005). Accumulation of oxidized biomolecules alters the biological functions of many cellular pathways. In the second case, two main types of homocysteinylation exist: S-homocysteinylation and N-homocysteinylation; both of them can be considered as posttranslational protein modifications. S-homocysteinylation occurs when Hcy reacts, by its free thiol group, with another free thiol derived from a cysteine residue in a protein molecule. These changes can alter the thiol-dependent redox status of proteins (Sengupta *et al.,* 2001; Jakubowski 2004). N-homocysteinylation takes place after acylation of the free ε-amino lysine groups of proteins by the most reactive form of Hcy — its cyclic thioester (Hcy thiolactone — HTL), representing up to 0.29% of total plasma Hcy (Jakubowski, 1999 and 2003; Perla-Kajan *et al.,* 2007). The chemical structure of Hcy and its thiolactone is presented in Fig. 1. In human blood, N-homocysteinylated (N-Hcy-protein) and S-homocysteinylated proteins (S-Hcy-protein) such as N-Hcy-hemoglobin, N-(Hcy-S-S-Cys)-albumin, and S-Hcy-albumin were described (Jakubowski, 2002; 2005; 2006; Chwatko & Jakubowski, 2005a; 2005b; Perla-Kajan *et al.,* 2007). Moreover, also other pathways of Hcy biotoxicity may exist, i.e. apoptosis and excitotoxicity mediated through glutamate receptors.

Kang *et al.* (1992) classified several types of hyperhomocysteinemia, in relation to the total plasma Hcy concentration. They defined hyperhomocysteinemia as severe, for concentrations higher than 100 μM, intermediate for concentrations between 31 and 100 μM, and moderate for concentrations of 16–30 μM, and a reference total plasma Hcy range as 5 to 15 μM (mean, 10 μM). Lentz & Haynes (2004) defined hyperhomocysteinemia as severe (>100 μM), moderate (30–100 μM) and mild (10–30 μM). They defined the physiological range

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**Abbreviations**: AARS, aminoacyl-tRNA synthetase; ADP, adenosine diphosphate; CBS, cystathionine β-synthase; CSE, cystathionine γ-lyase; Cys, cysteine; FN, fibronectin; GFR, glomerular filtration rate; Hcy, homocysteine; HTL, homocysteine thiolactone; HTLase, homocysteine thiolactonase; Lp(a), lipoprotein (a); Met, methionin; Met, methionine; MetRS- methionyl-t-RNA synthetase; MS, methionine synthase; MTHFR, methylenetetrahydrofolate reductase; ·NO, nitric oxide; $O_2^{\cdot-}$, superoxide anion; PAF, platelet aggregating factor; PAI-1, plasminogen activator inhibitor type-1; PAI-2, plasminogen activator inhibitor type-2; PDI, protein disulphide isomerase; PON, paraoxonase 1; RNS, reactive nitrogen species; ROS, reactive oxygen species; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; SAM synthetase, methionine adenosyltransferase; t-PA, tissue plasminogen activator; TXA2, thromboxane A2; u-PA, urokinase plasminogen activator.

**Table 1. Different forms of homocysteine present in human blood.**
*Total level of homocysteine — the term "total homocysteine" describes the pool of homocysteine released by reduction of all disulphide bonds in the sample (Perla-Kajan *et al.,* 2007; Zimny, 2008; Manolescu *et al*., 2010, modified).

| The form of Hcy | The concentration in human blood |
|---|---|
| Homocysteine thiolactone (HTL) | 0–35 nM |
| Protein N-linked homocysteine: N-Hcy-hemoglobin, N-(Hcy-S-S-Cys)-albumin | about 15.5 μM: 12.7 μM, 2.8 μM |
| Protein S-linked homocysteine — S-Hcy-albumin | about 7.3 μM* |
| Homocystine (Hcy-S-S-Hcy) and combined with cysteine to from mixed disulphides (Hcy-S-S-Cys) | about 2 μM* |
| Free reduced Hcy | about 0.1 μM* |

of total plasma Hcy as the 2.5th to 97.5th percentile interval for the analyzed group that corresponds to a lower limit of 5 μM, while the upper limit varies considerably between laboratories and different populations. As in the case of other biochemical parameters, a reference interval for Hcy which will take into account the influence of different non-modifiable and modifiable factors, should be established. The relationship between homocysteine and risk appears to hold for total plasma concentrations of homocysteine between 10 and 30 μM.

The elevated plasma level of Hcy may be a result of the genetic defects of enzymes involved in its metabolism, such as cystathionine-β-synthase (CBS), methionine synthase (MS) and methylenetetrahydrofolate reductase (MTHFR) (Brattstörm *et al*., 1998). As early as in the 1960s it was noted that the risk of atherosclerosis is markedly increased in patients with homocystinuria, an inherited disease resulting from homozygous CBS deficiency and characterized by episodes of thromboembolism, mental retardation, lens dislocation, hepatic steatosis, and osteoporosis. Homocystinuria is associated with abnormal, very high concentrations of plasma homocysteine and methionine. Patients with homocystinuria have very severe hyperhomocysteinemia, with plasma homocysteine concentration reaching even 400 μM, and represent a very small proportion of the population (approximately 1 in 200,000 individuals). In patients with residual CBS activity (between 0% and 2% of normal), who represent approximately half of the cases of the homozygous homocystinuria, pyridoxine treatment can decrease hyperhomocysteinemia. Heterozygous lack of CBS, CBS mutations and polymorphism of the methylenetetrahydrofolate reductase gene are considered to be the most probable causes of hyperhomocysteinemia (Franken *et al*., 1994). In CBS, Ile278Thr and Gly307Ser are the most common mutations. If they occur homozygously, these mutations may lead to a significant elevation of plasma homocysteine level and to an increased risk of atherosclerosis of carotid arteries (Klerk *et al*., 2002). Thirty-three point mutations of the CBS gene, which is located on chromosome 21, have been identified. Among these mutations, the G919A transition, frequently encountered in the Irish population, resulting in the substitution of guanine by adenine in position 919, is observed in subjects who are refractory to vitamin $B_6$ treatment. The T833C mutation, frequent in the German population, is observed in vitamin $B_6$ responders. People who are heterozygous for CBS deficiency have normal plasma homocysteine levels in 30% to 50% of cases (Lentz *et al*, 2000; Durand *et al*, 2001).

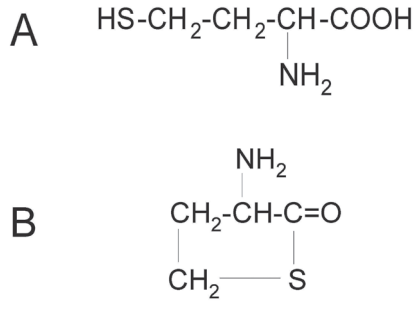


**Figure 1. The chemical structure of homocysteine (A) and its thiolactone (B).**

Methylenetetrahydrofolate reductase gene polymorphism involves substitution of cytosine by thymine in position 677 (C677→T) and leads to the synthesis of a thermolabile enzyme form with decreased activity. Prevalence of this mutation is race dependent. In Asian and Caucasian populations (including Poland) there are 50% C7T heterozygotes and 10–13% T/T homozygotes, while in the Afro-American population this polymorphism is rare (Brattstörm *et al*., 1998; Beltowski 2005). T/T homozygotes have, on average, Hcy levels higher by 2.5 μM than C/C homozygotes. However, this difference has been observed in the case of folic acid and riboflavin deficiency in the diet (Bednarek-Tupikowska *et al*., 2004). It has also been observed that A→C transversion in position 1298 of the MTHFR gene results in the substitution of glutamate with alanine in the enzyme, which leads to its decreased activity (Chango *et al*., 2000). Nine thermostable mutations of the MTHFR gene, located on chromosome 1, inherited as an autosomal recessive trait, could be responsible for its reduced activity. The two main mutations are the C599T and the G482A transitions. Hyperhomocysteinemia is only observed in the homozygous state. No vitamin treatment has been effective in these patients, although their hyperhomocysteinemia is less severe than in CBS deficient homozygotes.

Acquired hyperhomocysteinemia may result from various factors, such as lifestyle, diet, various diseases, medications, drugs, sex and age. Unbalanced diet, leading to significant deficiency of vitamins $B_2$, $B_6$, $B_{12}$ and folic acid may cause an increase in plasma Hcy concentration. Additionally, alcohol, tobacco smoking and coffee also lead to increased plasma Hcy level. The difference observed in homocysteine level between men and women could be explained by body size or estrogen, the dependence of homocysteine concentration on age could result from the decline of the glomerular filtration rate (GFR) with increasing age. Also alimentary tract disorders connected with impaired absorption of vitamins $B_2$, $B_6$, $B_{12}$ and folic acid may play an important role. In some countries, e.g. in the U.S.A., supplementation of food with folic acid is a common occurrence. In Poland, however, the food is not supplemented with folic acid and therefore the levels of total plasma homocysteine should

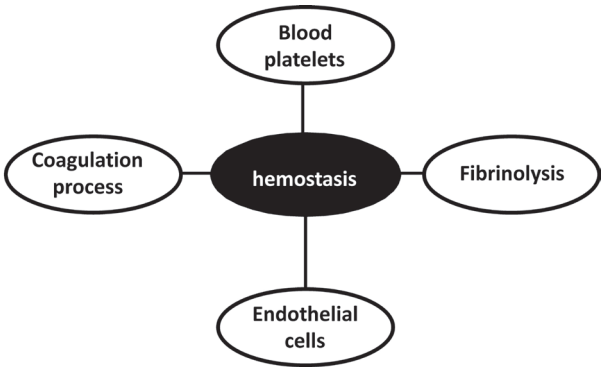


**Figure 2. Different elements of hemostasis.**

more closely depend on the dietary and genetic variation (Suwala *et al*., 2009).

Elevated plasma Hcy (>15 μM; Hcy) is associated with an increased risk of cardiovascular diseases (Harpel *et al.,* 1992; 1996; Boysen *et al.,* 2003; Li *et al.,* 2003; Karolczak & Olas, 2009; Bloom *et al*., 2011; Cacciapuoti, 2011), such as thrombosis (Harpel *et al*., 1992; 1996; Matteo *et al.,* 2010; Kamat *et al*., 2010) and thrombosis-related diseases, such as stroke (Boysen *et al.,* 2003; Li *et al*., 2003; Suwala *et al*., 2009). Every increase of 2.5 μM in plasma Hcy may be associated with an increase of stroke risk of about 20% (Clarke *et al*., 2002). Suwala *et al.* (2009) analyzed the occurrence of ischemic brain stroke in the population of Northern Poland with regard to risk factors of the disease. With the use of a modified HPLC method (described by Suwala *et al*., 2008), those authors demonstrated that hyperhomocysteinemia might be a risk factor of ischemic brain stroke, independent of other, conventional risk factors of this disease. Moreover, total plasma Hcy level above 20 μM are associated with a nine-fold increase of the myocardial infarction and stroke risk, in comparison to the concentrations below 9 μM (Nygard *et al*., 1997). The increase of Hcy concentration has been also found in other human pathologies, including neurodegenerative diseases (Ravaglia *et al.,* 2005). However there are many different opinions on the biotoxicity of Hcy on the hemostasis process.

This review describes the effect of hyperhomocysteinemia on different components of hemostasis (blood platelets, endothelial cells, plasmatic fibrinogen and plasminogen (Fig. 2)) and hemostatic abnormalities (Fig. 3). The normal hemostatic mechanisms prevent the hemorrhage and thrombosis by complex interactions between the blood vessel wall, blood platelets, coagulation factors and fibrinolytic proteins. The vascular endothelium maintains the balance between prevention and stimulation of platelet activation, coagulation and fibrinolysis and between vasoconstriction and vasodilation. In the present article, we also discuss the results of various experiments, performed to investigate the beneficial effects of phenolic antioxidants on changes in hemostasis induced by hyperhomocysteinemia.

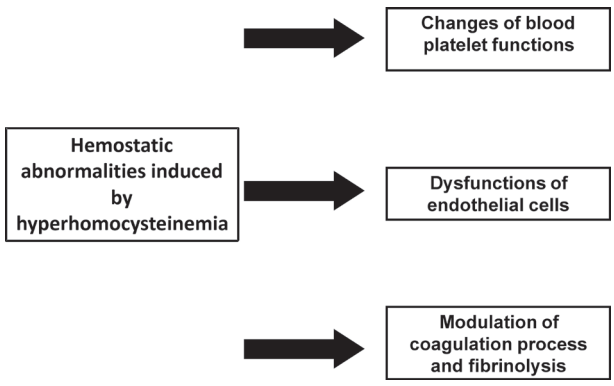


**Figure 3. Hemostatic abnormalities induced by hyperhomocysteinemia.**

## PRODUCTION AND METABOLISM OF HOMOCYSTEINE AND ITS THIOLACTONE

Hcy is a homologue of the amino acid cysteine (Cys), differing by an additional methyl group. Hcy is formed as a result of the transformation of methionine (Met) to Cys in all types of animal cells, including human. In the reaction catalyzed by methionine adenosyltransferase (SAM synthetase) adenosine is transferred from ATP to Met to form S-adenosylmethionine (SAM). SAM is the major donor of methyl groups for various methylation reactions, therefore, is called "active methyl" or "active methionine". When a methyl group ($-CH_3$) is transferred by SAM synthetase to individual acceptors, SAM is converted to S-adenosylhomocysteine (SAH). Then, SAH is hydrolyzed by a specific SAH hydrolase to adenosine and Hcy. Hcy is transported outside the cell and its concentration can be determined in plasma.

In human cells Hcy can be metabolised *via* two B-vitamins-dependent pathways: remethylation and transsulfuration. In the first pathway, Hcy may be remethylated to Met by MS, which uses 5-methyltetrahydrofolate and vitamin $B_{12}$ as cofactors. The second pathway — transsulfuration — leads to cysteine. This pathway of Hcy metabolism is catalyzed by CBS and CSE, with pyridoxal-5 phosphate as a cofactor (active form of vitamin $B_6$) (Fig. 4) (Perla-Kajan *et al.,* 2007).

The synthesis of homocysteine thiolactone is associated with the activation of the amino acid by aminoacyl-tRNA synthetase (AARS). Hcy may also undergo erroneous activation, e.g. by methionyl-t-RNA synthetase (MetRS) (Jakubowski, 1990). In the first step of conversion of–Hcy to HTL, MetRS misactivates Hcy giving rise to homocysteinyl-adenylate. In the next phase, the homocysteine side chain thiol group reacts with the activated carboxyl group and HTL is produced. The level of HTL synthesis in cultured cells depends on Hcy and Met levels. HTL may be hydrolyzed nonenzymatically,

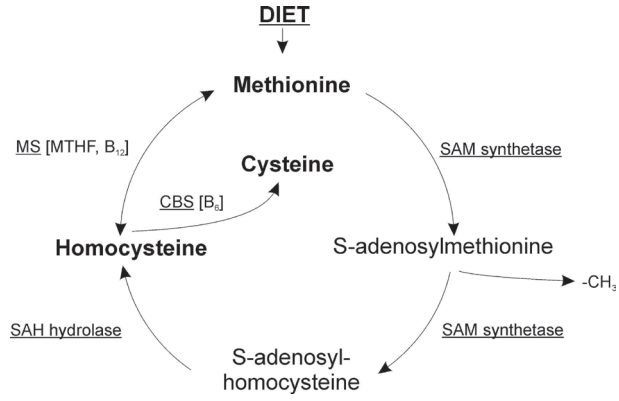


**Figure 4. Metabolism of homocysteine.**
Abbreviations: CBS, cystathionine β-synthase; CSE, cystathionine γ-lyase; MS, methionine synthase; 5-MTHF, 5-methyltetrahydrofolate.

but it is mostly metabolized by plasma HTL hydrolase, also known as homocysteine thiolactonase (HTLase) or paraoxonase (PON 1) (Jakubowski, 2000; Perla-Kaján & Jakubowski, 2010; Jakubowski & Głowacki, 2011).

## HYPERHOMOCYSTEINEMIA AND CHANGES IN FIBRINOLYSIS AND COAGULATION PROCESS

The fibrinolytic system comprises a proenzyme, plasminogen, which can be converted to the active enzyme, plasmin, by plasminogen activators. The main plasminogen activator in the intravascular fibrinolysis is tissue plasminogen activator (t-PA); another plasminogen activator — urokinase plasminogen activator (u-PA) is mostly involved in the extracellular proteolysis (Lijnen, 2002). Both u-PA and t-PA are serine proteinases that act directly on the peptide bond Arg560-Val561 of the plasminogen molecule, but the plasminogen activation process induced by t-PA occurs on the fibrin clot surface. The fibrinolytic activity of blood is regulated by specific inhibitors; the inhibition of fibrinolysis takes place at the level of plasminogen activation (by PA-inhibitors: plasminogen activator inhibitor type-1, -2; PAI-1 or PAI-2) or at the level of plasmin activity (mainly by $\alpha_2$-antiplasmin) (Booth, 2000).

Hyperhomocysteinemia disturbs hemostasis and shifts the hemostatic mechanisms in favor of thrombosis. The recent reports indicate that the prothrombotic state observed in hyperhomocysteinemia may arise not only due to endothelium dysfunction or blood platelet and coagulation activation, but also due to impaired fibrinolysis (Kołodziejczyk *et al.*, 2010). It has been shown, that Hcy-modified fibrinogen is more resistant to the fibrinolytic action (Undas *et al.,* 2006). The studies of Freyburger *et al.* (1997) demonstrated that the increased level of total Hcy, induced by oral methionine load, may diminish the fibrinolytic activity of the euglobulin plasma fraction. Also the enzymes of the fibrinolytic system may be a potential target for homocysteine action (Colucci *et al.*, 2008). Moreover, other clinical studies carried by Speidl *et al.* (2007) on cardiovascular disease patients after their first myocardial infarction, who also had hyperhomocysteinemia showed a reduced t-PA activity independently from cardiovascular risk factors and medical treatment. Authors suggest that homocysteine-lowering therapies may increase fibrinolytic activity and prevent atherothrombotic events in patients with cardiovascular diseases after the first myocardial infarction.

The other possible mechanism of the prothrombotic effect of homocysteine may be the direct blockade of the t-PA binding domain of annexin II, its major endothelial cell receptor (Hajjar *et al.,* 1998). t-PA attaches to endothelial cells *via* the calcium-regulated phospholipid-binding protein annexin II and this interaction is inhibited by Hcy complexing with Cys9 within annexin II putative hexapeptide binding domain (LCKLSL). It has been estimated that Hcy at the concentration of about 11 μM is able to inhibit t-PA binding to annexin II by as much as 50%; this extent of the loss of fibrinolytic potential seems to be an important factor in the etiology of vascular disorders.

In further studies, Dassah and coworkers (2009) incubated annexin II in a purified protein system with homocysteine. Annexin II was modified by Hcy. This treatment blocked the ability of t-PA to bind to annexin II. Finally, incubation of cultured ECs with 35S-Hcy led to metabolic labeling of annexin II that was sensitive to protein reduction, suggesting a disulfide-mediated association between Cys8 and Hcy. These data revealed a binding domain for t-PA in the N-terminal tail of annexin II, and showed its susceptibility to modification by Hcy, an agent highly associated with atherothrombotic vascular disease.

The impairment of fibrinolytic efficacy may be also a result of the enhanced level of lipoprotein (a) (Lp(a)), which is one of the common risk factors of atherosclerosis. Since Lp(a) contains apoprotein (a), with a sequence highly homologous to plasminogen, it can act as a competitive inhibitor of plasminogen. Lp(a) may bind to cellular plasminogen receptors and to fibrin(ogen) and competes for the binding of plasminogen to these molecules (Xue *et al.*, 1999). According to the study of Harpel *et al.* (1992) Hcy promotes the attachment of lipoprotein (a) to fibrin, decreasing the possibility of plasminogen binding and activation. Nardulli *et al.* (2005) suggest the potential mechanism for the homocysteine-enhanced antifibrinolytic action of lipoprotein(a) in human plasma. According to these scientists, Hcy reduces the apo(a)/apoB disulphide bond and causes the appearance of free apo(a) with high affinity for fibrin, which is able to strongly inhibit plasminogen binding. Furthermore, it seems very likely, that one of the prothrombotic mechanisms of homocysteine action is the modification of the fibrinogen structure (Acevedo *et al.,* 2002). Fibrinogen is a key protein in blood coagulation. The most significant biological role of fibrinogen is related to its ability to form the scaffold of a blood clot and thereby prevent the loss of blood after injury. It is of greatest consequence for the maintenance of health that the capacity of the soluble fibrinogen to be converted into insoluble fibrin is meticulously regulated, and that stable fibrin is formed only when and where it is needed. Fibrinogen plays also a role in the physiological and pathological processes related to wound healing, tumor growth, and metastasis as well as defense mechanisms. In order to fulfill its numerous functions, fibrinogen interacts in highly specific ways with a large number of other proteins as well as low molecular weight cellular components, primarily in the blood stream or blood vessel walls. Human fibrinogen is subject to modification at a number of different sites both during and after biosynthesis. These modifications include oxidation, nitration, non-enzymatic glycation and homocysteinylation. Other studies have shown, that Hcy- and HTL-modified fibrinogen is more resistant to the fibrinolytic action (Undas *et al.,* 2006; Malinowska *et al.,* 2011). Homocysteine thiolactone-mediated changes in the fibrinogen molecule may increase the resistance of the fibrin clot to degradation. Studies by Sauls *et al.* (Sauls *et al.,* 2003 and 2006) with the use of mass spectrometric analysis of Hcy fibrinogen showed homocysteinylation of twelve lysine residues. Moreover, several of them were localized close to t-PA and plasminogen binding sites. The authors suggested that the Hcy-induced modification of lysine might occur *in vivo*. Lysine residues in the fibrinogen molecule are crucial for the interaction with fibrinolytic enzymes, thus modification of these amino acids may lead to increased resistance of clots to lysis and contribute to the thrombotic tendency occurring in hyperhomocysteinemia. HTL can react with primary amines such as those in lysine, asparagine, arginine, and glutamine. Homocysteinylation introduces a new free sulfhydryl group into the protein, as well as alters the size of the modified amino acid. Under physiological conditions the reaction with the epsilon

amino group of lysine is favored (Jakubowski, 1999; Hop & Bakhitiar, 2002; Głowacki & Jakubowski, 2004; Sikora *et al*., 2010; Marczak *et al*., 2011). Limited homocysteinylation induces the features of the homocysteinemia-associated dysfibrinogenemia including the formation of thin, tightly packed fibrin fibers and increased resistance to fibrinolysis. Fibrin formed from N- homocysteinylated fibrinogen also has a decreased ability to support activation of plasminogen by t-PA (Sauls *et al.,* 2006).

Another hemostatic protein, susceptible for homocysteinylation is factor V (Undas *et al.,* 1996). Homocysteine rapidly incorporates into its structure *in vitro* due the modification of cysteine(s) and formation of heterologous disulphide bonds. The homocysteine treatment of factor V protects α-thrombin-derived factor Va from inactivation by supporting fibrinolysis.

## THE EFFECT OF HYPERHOMOCYSTEINEMIA ON BLOOD PLATELET FUNCTIONS

Blood platelets are multiresponding cells, both with respect to the number of agonists and number of responses. They can be activated by different compounds including coagulation factors (thrombin), hormones (epinephrine, vasopressin, low-molecular-weight substances (serotonin, adenosine diphosphate (ADP)), lipid derivatives (platelet aggregating factor (PAF), thromboxane $A_2$ ($TXA_2$)), and other protein substances (collagen or immune complexes). The responses of platelets to agonists, known as platelet activation, include mainly adhesion (to foreign surfaces such as collagen or glass), shape change, aggregation and secretion of active compounds from three different types of storage granules (dense granules, α-granules and lysosomes), shedding of microvesicles, development of platelet procoagulant activity and retraction of fibrin clots (Wu, 1996; Levy-toledano, 1999; Ryningen & Holmsen, 1999). Blood platelets are involved in the hemostatic process. In hemostasis, platelet plug formation represents the primary response to vascular injury, with the coagulation cascade and fibrin formation. Normal primary hemostasis requires three critical events: platelet adhesion, secretion and aggregation. If a blood vessel is injured, platelets are exposed to subendothelial collagen. This initial interaction is mediated through the collagen receptor — integrin $\alpha_2\beta_1$, and by another platelet receptor — the glycoprotein complex GPIb/IX/V. Adherent platelets then change their shape from discs to spheres with pseudopods and release their granule contents (degranulation process). Few seconds later, more platelets are deposited on the collagen fibrils, and they start to stick to each other (platelet aggregation) (Wu, 1996; Levy-toledano, 1999; Ryningen & Holmsen, 1999). Increased platelet activation with hyperaggregability is one of the risk factors in the pathogenesis of cardiovascular diseases.

Recently, it has been shown that homocysteine may modulate blood platelet functions (Rajkumar *et al.,* 1999; Alexandru *et al.,* 2007; Signorello *et al.,* 2007; Olas *et al*., 2008; 2009). Some studies demonstrated that homocysteine promotes arachidonic acid release, the formation of thromboxane $A_2$ (Signorello *et al*., 2002) and protein tyrosine phosphorylation in blood platelets (Leoncini *et al*., 2006; 2007). Results of Undas *et al.* (2007) demonstrated that the elevated total Hcy level is associated with increased platelet activation at the site of microvascular injury. McDonald *et al.* (1964) observed increased platelet adhesion in homocysteinuric patients. In an animal model of hyperhomocysteinemia (induced by a diet poor in folic acid), the aggregation of platelets stimulated by ADP or thrombin was higher than in control animals (Durand *et al*., 1996). Blood platelets obtained from patients with peripheral occlusive arterial disease and hyperhomocysteinemia, are more reactive and sensitive to agonists, but also far less sensitive to inhibitors (Riba *et al*., 2004). In diabetic patients, a high level of plasma Hcy is associated with more potent aggregation of blood platelets (Rajkumar *et al.,* 1999). However, a direct action of Hcy and its derivatives on blood platelets is rather controversial. Some studies have reported that homocysteine alone does not induce platelet aggregation, but increases platelet aggregation induced by a strong platelet agonist — thrombin (Olas *et al*., 2008). Furthermore, these results suggest that this increase may be partly dependent on integrin $\alpha_{IIb}\beta_3$ activation on the platelet surface (Olas *et al*., 2008). It was also observed that the most reactive form of Hcy - HTL did not induce platelet aggregation on its own, but like Hcy, increased platelet aggregation induced by thrombin (Olas *et al.,* 2008). In these studies authors used washed blood platelets (Olas *et al*., 2008). Other results showed that not only the reduced form of Hcy, but also HTL, may augment blood platelet activation; induced by other physiological agonists (ADP and collagen) and measured by flow cytometry or turbidimetry (Olas *et al.,* 2009). These results (Olas *et al.,* 2008; 2009) suggest that changes in the state of thiol groups in the platelet proteins, induced by Hcy or HTL, may potentiate aggregation stimulated by ADP or collagen. The tested concentrations of Hcy (10–100 μM) and HTL (0.1–1 μM) used in studies of Olas *et al.* (2008 and 2009) corresponded to the levels found in plasma under hyperhomocysteinemia. Essex and Li (2003) observed the same process; when the effects of different low-molecular-weight thiols on platelet aggregation were tested. They found that not only glutathione at concentrations normally found in the blood, but also cysteine, cysteinylglycine and homocysteine potentiated platelet aggregation.

Recently, it has been suggested that N-homocysteinylated or S-homocysteinylated hemostatic proteins induced by Hcy or its thiolactone seem to be the main cause of the biotoxicity of homocysteine in cardiovascular diseases. The possibility that Hcy or HTL reacts with carbonyl groups present in human blood platelet proteins (Olas *et al*., 2009) and in human plasma proteins (Olas *et al.,* 2010), also exists. In this context, the reaction of Hcy and HTL with carbonyl groups, for which the term C-homocysteinylation has been proposed, gains new significance. C-homocysteinylation induced by homocysteine or its thiolactone, may block protein degradation;; it may be also one of the pathomechanisms of some age-related diseases associated with hyperhomocysteinemia, such as Alzheimer disease, Parkinson disease and atherosclerosis.

Thiol homeostasis determines critical aspects of cell functions. Oxidation of thiol groups and reduction of the disulphide bonds in proteins is a dynamic, reversible process that occurs under physiological conditions in cells (Inayama *et al.,* 2002; Martin *et al.,* 2001). The concentration of thiol groups in proteins is much greater than that of glutathione; protein thiols are present as free thiols, disulphides, and mixed disulphides when conjugated with glutathione, cysteine, and γ–glutamylcysteine (Inayama *et al.,* 2002). There is evidence that the metabolism of platelet protein thiols changed after incubation of platelets with Hcy and its thiolactone (Olas *et al.,* 2009). It is suggested that Hcy may act as a platelet agonist,

because it may act as an antagonist at the glycine site of the NMDA receptor (in the presence of normal or low glycine levels), or as an agonist at the glutamate site of this receptor (when glycine levels are increased) (Franconi *et al.,* 1998; Morrell *et al.,* 2008). Hcy, like other platelet agonists probably causes changes in protein disulphide isomerase (PDI) activity in platelets. Upon activation, platelets release PDI into the medium. This enzyme has been shown to be on the external surface of the platelet plasma membrane (Essex & Li, 2003; Essex, 2004); it catalyses the formation as well as isomerisation of disulphide bonds. Essex and Li (2003) showed that PDI mediates platelet aggregation and secretion, and activates integrin $\alpha_{IIb}\beta_3$; these findings suggest that this receptor is a target of PDI. PDI also denitrosates S-nitrosothiols, releasing nitric oxide ($^{\bullet}NO$) that, *via* the guanylate cyclase/G-kinase route, attenuates platelet activation. Moreover, S-nitrosothiols are denitrosated at the same PDI-active site that catalyses the disulphide bond formation between integrins and their ligands, thereby attenuating irreversible aggregation.

The decrease in free $-NH_2$ protein groups after 2–30 minutes of incubation of platelets with Hcy or HTL is a very important result of studies on blood platelets (Olas *et al.*, 2009). At all the remaining times of incubation an increase in free amino groups was noted; this finding was inconsistent with the expected effect of N-homocysteinylation, which should decrease the number of free ε-amino groups. This may suggest that changes in the secondary protein structure induced by both Hcy and HTL are responsible for the exposure of new amino groups.

Blood platelets, in analogy to other circulating blood cells, generate reactive oxygen/nitrogen species (ROS/RNS) that may act as second messengers and regulate platelet functions. Accumulating evidence suggests a role of ROS/RNS in platelet activation. On the other hand, an increased production of ROS/RNS causes oxidative stress, and thus, contributes to the development of various diseases, including vascular complications, inflammation, cancer and psychiatric illnesses (Olas & Wachowicz, 2007). The results of our studies suggest that the reduced formation of nitric oxide ($^{\bullet}NO$) in blood platelets treated with Hcy in a reduced form, or with HTL, may lead to an increase in platelet aggregation, because $^{\bullet}NO$ is a powerful aggregation inhibitor (Olas *et al.,* 2008). It is known that homocysteine inhibits the synthesis of $^{\bullet}NO$ in platelets and reduces $^{\bullet}NO$ bioavailability (Upchurch *et al.,* 1997; Mutus *et al.,* 2001; Undas *et al.*, 2007); these effects of Hcy contribute to platelet hyperactivity and reduce tyrosine nitration in platelet proteins (Olas *et al.* 2008). The changes in protein tyrosine nitration may play an important role, since they may modulate tyrosine phosphorylation — a very important process for signal transduction in platelets. According to the results obtained by Olas *et al.* (2008), homocysteine and its thiolactone may influence the formation of the superoxide anion ($O_2^{\bullet-}$) in blood platelets, as was estimated by the method of cytochrome c reduction. The described study showed that not only Hcy, but also HTL induces increased production of $O_2^{\bullet-}$ both in resting and thrombin-activated platelets. Moreover, other results obtained by these authors (Olas *et al.*, 2010) indicate that Hcy and its thiolactone may promote *in vitro* apoptotic events in human platelets.

## THE EFFECT OF HOMOCYSTEINE AND ITS THIOLACTONE ON ENDOTHELIAL CELLS

At the cellular level the pathological role of homocysteine seems to be associated with alterations in endothelial cells, which play an important role in hemostasis. Endothelial cells are very sensitive even to a mild increase in Hcy concentration. This susceptibility may be explained by the fact; that human endothelial cells do not express the active form of cystathionine β-synthase, and consequently are not able to initiate homocysteine catabolism by the transsulfuration pathway (Jacobsen, 1998). The elevated level of Hcy may modulate functions of the vascular endothelium, changing the character of its surface from anticoagulant to procoagulant (Jacobsen, 1998). The anticoagulative properties of vascular endothelium are based on heparin-like glycosaminoglycan-antithrombin III interactions. Results of Rodgers and Kane (1986) demonstrated a significant increase in the coagulation factor V activity, after exposure of endothelium to Hcy. Coagulation factor V in Hcy-modified endothelium is cleaved into fragments different than those obtained after factor V cleavage by thrombin or coagulation factor Xa (Rodgers & Kane, 1986). Moreover, in hyperhomocysteinemia the prothrombotic tendency may be related to impaired inactivation of S-homocysteinylated coagulation factor Va by the activated protein C (Undas *et al.*, 2001). It has been demonstrated that not only Hcy, but also HTL may modulate the properties and functions of endothelial cells. Results of Raposo *et al.* (2004) indicate that both compounds, Hcy and its thiolactone, inhibit the activity of lysyl oxidase (an enzyme involved in extracellular matrix maturation) in vascular endothelial cells. The report of Jakubowski *et al.* (2000) showed that protein N-homocysteinylation occurred in endothelial cells and that it depended on the concentration of Hcy. Modification of endothelial cell proteins may cause different pathophysiological consequences, such as modulation of the hemostasis system, that may contribute to the development of cardiovascular diseases. Detailed description of the effect of Hcy on endothelial cells has been provided by Karolczak and Olas (2009).

## HOMOCYSTEINE — FIBRONECTIN INTERACTION AND ITS CONSEQUENCES

Human fibronectin (FN) is a multifunctional glycoprotein encoded by the FN gene on chromosome 2q34. This glycoprotein plays key roles in cell adhesion and migration, embryogenesis, differentiation, hemostasis, thrombosis, wound healing, as well as in tissue remodeling (Romberger, 1997). The FN subunit contains two fibrin binding sites. The site close to the amino terminus serves as a transglutamination site for activated factor XIII which crosslinks FN to various other proteins including fibrin, fibrinogen, and FN. The interaction with fibrin starts at the N-terminal site of FN due to its higher affinity, followed by binding to the C-terminal site, which strengthens the fibrin-FN interaction. Interaction of FN with fibrin, mediated by factor XIII transglutaminase, is thought to be important for cell adhesion or cell migration into fibrin clots. After tissue injury, a blood clot formation serves the dual role of restoring vascular integrity and serving as a temporary scaffold for the wound healing process. Fibrin and plasma FN, the major protein components of blood clots, are essential to perform these functions. In the blood clotting process, after fibrin deposition, plasma FN-fibrin matrix is covalently crosslinked, and it then promotes fibroblast adhesion, spreading, and migration into the clot (Lee *et al.,* 2010).

Fibronectin has free cysteine residues and numerous disulphide bonds that could interact with homocysteine *via* oxidative and/or thiol/disulphide exchange reactions.

Majors *et al.* (2002) showed that homocysteine binds to several human plasma proteins, including fibronectin. The authors hypothesized that homocysteine binds to fibronectin *via* a disulphide linkage and that this binding results in a functional change, namely, the inhibition of fibrin binding by fibronectin. This inhibition may lead to a prolonged recovery from a thrombotic event and contribute to vascular occlusion.

## THE ROLE OF ANTIOXIDANTS IN HYPERHOMOCYSTEINEMIA

Plasma homocysteine level depends among other things on sex, age, smoking, function of the liver and kidneys, physical activity and diet. Proper diet, abstaining from tobacco smoking, and optimal physical activity may influence the level of this amino acid. There are also various compounds which lead to the reduction of Hcy levels. Folic acid, vitamins $B_6$ and $B_{12}$ should be mentioned, because some pathways of Hcy metabolism are correlated with the level of these vitamins. Results of Pietrzik and Bronstrup (1998) showed that daily supplementation with folic acid in the range of 0.5–5 mg, and with about 0.5 mg of vitamin $B_{12,}$ decreased Hcy concentration in blood. However, the results of Bogers *et al.* (2007) showed that increased fruit and vegetable consumption may be insufficient to change plasma Hcy concentration. Other experiments indicate that elevated homocysteine and folate deficiency are associated with oxidative stress. Oxidant injury has been suggested as a potential mechanism of atherogenesis in hyperhomocysteinemia (Sauls *et al.,* 2007). Results of Kolling *et al.* (2011) demonstrated that supplementation of folic acid can be used as an adjuvant therapy in cardiovascular alterations caused by Hcy. They observed that Hcy induced oxidative-nitrative stress in a rat heart while folic acid had protective properties.

Epidemiologic studies indicate that regular consumption of polyphenolic antioxidants, the secondary plant metabolites, is correlated with a decreased risk of cardiovascular diseases, diabetes, arthritis and cancer. Moreover, on the basis of various observations, it is proposed that Hcy and HTL may act as oxidants in the model system *in vitro* and *in vivo* (Carluccio *et al.,* 2007; Olas *et al.,* 2008), but some dietary polyphenolic antioxidants can attenuate the oxidative damage; induced by hyperhomocysteinemia (Carluccio *et al.,* 2007). A variety of well-known antioxidants, including polyphenolic antioxidants, have been shown to exert a protective action against Hcy toxicity (Schoecksnadel *et al.,* 2005; Carluccio *et al.,* 2007; Noll *et al.,* 2009a; 2009b). However, the mechanisms of protection provided by many exogenous compounds against Hcy action are still unknown. Red wine-derived polyphenolic compounds at low doses significantly reduced plasma Hcy levels and restored the hepatic and plasma paraoxonase-1 activity decreased in chronic hyperhomocysteinemia (Noll *et al.,* 2009a; 2009b). Moreover, Noll *et al.* (2009a) observed that the aortic expression of proinflammatory cytokines and adhesion molecules as well as the level of the soluble lectin-like oxidized low-density lipoprotein receptor-1 were reduced in hyperhomocysteinemic mice fed with the red wine polyphenolic extract. Fu *et al.* (2003) reported that red wine prevents homocysteine — induced endothelial dysfunction in porcine coronary arteries. Our results showed that the well-studied polyphenol — resveratrol (3,4',5-trihydroxystilben), which is an integral component of human diet found naturally in fruits, nuts, flowers, seeds, red wine and bark of different plants, strongly, but not completely, reduced platelet apoptosis induced by Hcy or HTL, suggesting that pathways other than reactive oxygen species generation were also involved (Olas *et al.,* 2010). Resveratrol exhibits a wide range of biological effects, including antiplatelet, anti-inflammatory, anticancer, antimutagenic and antifungal properties. It is also a potent antioxidant, reactive oxygen species scavenger and metal chelator. Resveratrol reduces lipid peroxidation; as well as oxidation and nitration of platelet and plasma proteins (Olas & Wachowicz, 2005). Resveratrol and other polyphenolic compounds may also increase nitric oxide bioavailability, thereby antagonizing the development of endothelial dysfunction, decrease blood viscosity, improve insulin sensitivity, counteract platelet hyperactivity, inhibit platelet adhesion to fibrinogen-coated surfaces, as well as decrease plasma levels of von Willebrand factor, fibrinogen, and coagulation factor VII (Lippi *et al.,* 2010). Furthermore, resveratrol may protect plasma proteins against modifications (measured by the level of thiol and ε-amino groups) caused by homocysteine or its thiolactone (Malinowska & Olas, 2010). Resveratrol is also able to reduce the toxic action of Hcy and HTL on the hemostatic properties of fibrinogen and human plasma (Malinowska & Olas, 2010). Since Hcy and its derivatives promote free radical production and oxidative damage to proteins (Sibrian-Vazques *et al.,* 2010), it has been suggested that resveratrol, which can change the level of reactive oxygen species or reactive nitrogen species (specially that of nitric oxide), may be responsible for the reduction of protein modifications induced by Hcys or HTL. Results obtained by Malinowska and Olas (2011) also suggest that, due to such properties, resveratrol, may be responsible for the inhibition of platelet aggregation (stimulated by thrombin) during hyperhomocysteinemia.

Grape seeds are one of the richest plant sources of phenolic substances, and Kolodziejczyk *et al.* (2011) observed that the grape seed extract reduced the toxic effect of Hcys and HTL on fibrinolysis. The grape seed extract (12.5–50 μg/ml) supported plasminogen to plasmin conversion inhibited by Hcys or HTL. For example, in the presence of grape seed extract (at the highest tested concentration — 50 μg/ml) the increase of about 78% (for human plasminogen-treated with Hcys) and 56% (for human plasma-treated with Hcys), was found. These studies also demonstrated in the *in vitro* model system, that the grape seed extract (12.5–50 μg/ml) diminished the reduction of thiol groups and of lysine ε-amino groups in plasma proteins (as determined *via* colorimetric methods) treated with Hcys (0.1 mM) or HTL (1 μM). In the presence of the grape seed extract at the concentration of 50 μg/ml, the level of reduction of thiol groups reached about 45% (for plasma treated with Hcys) and about 15% (for plasma treated with HTL).Very similar protective effects of the grape seed extract were observed in the measurements of lysine ε-amino groups in plasma proteins treated with Hcys or HTL (Kołodziejczyk *et al.,* 2011). Our preliminary results indicate that the extract from berries of *Aronia melanocarpa* (a rich source of phenolic substances) reduces the toxic effects of Hcy and HTL on the hemostatic properties of fibrinogen and plasma. These findings indicate a possible protective action of the *A. melanocarpa* extract in hyperhomocysteinemia-induced cardiovascular disorders. Moreover, our experiments showed that the extract from berries of *A. melanocarpa,* due to its antioxidant action, significantly attenuated

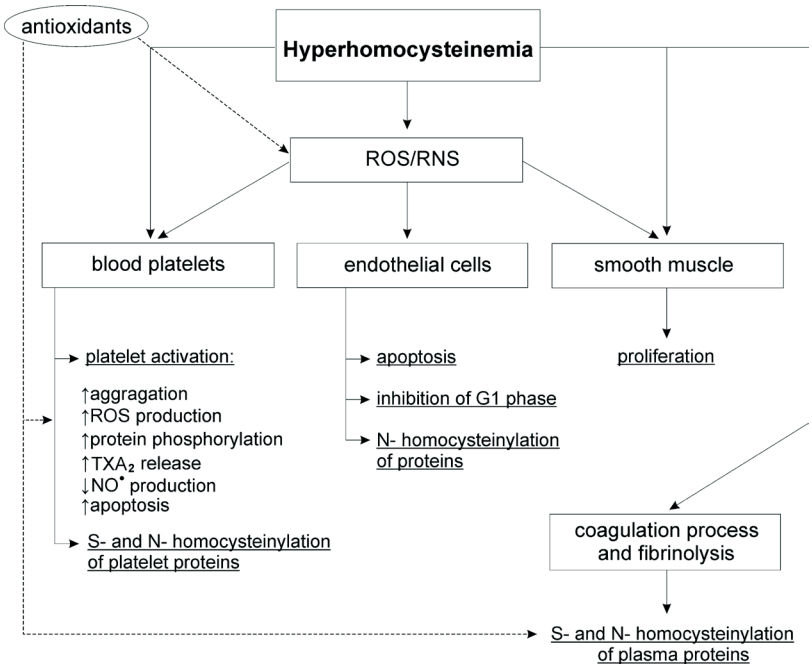


**Figure 5. Proposed model for the protective role of phenolic antioxidants on selected elements of hemostasis during hyperhomocysteinemia.**
Abbreviations: RNS, reactive nitric species; ROS, reactive oxygen species; ·NO, nitric oxide, $TXA_2$ – thromboxane $A_2$.

the oxidative stress (assessed by measuring of the total antioxidant status — TAS) in plasma in a model of hyperhomocysteinemia (Malinowska *et al.,* 2011). In comparative studies, the extract from berries of *A. melanocarpa* and resveratrol had similar protective properties (Malinowska *et al.,* 2011). Other available data suggest that high dietary intake of antioxidative vitamins (vitamin A, beta-carotene and vitamin C) could be a protective factor against atherosclerosis and the pro-oxidative effect of Hcy on low density lipoproteins (LDL) (Seo *et al.*, 2010). It has been established that Hcy alters glutamate uptake, $Na^+/K^+$-ATPase activity and the oxidative status in rat hippocampus, while vitamin C prevents these effects (Machado *et al.,* 2011). More information about the role of diet in prophylaxis and treatment of hyperhomocysteinemia has been provided by Malinowska *et al.* (2010) and Manolescu *et al.* (2010).

## CONCLUSIONS

The contribution of hyperhomocysteinemia to hemostatic abnormalities is complex and still unclear. Hcy or its derivatives, e.g. its thiolactone, may modulate the signal transduction in different types of blood cells and sometimes act in opposite ways. Homocysteine and HTL also cause changes in the level of the reactive oxygen and nitrogen species (particularly $^{\bullet}NO$) and may be responsible for the modification of hemostasis induced by these compounds. Moreover, the biological significance of hemostatic protein modifications (fibrinogen and other coagulation factors) induced by Hcy or HTL is only partly recognized. It has been established, however, that in particular, N-homocysteinylation induced by HTL may play an important role in different pathophysiological anomalies leading to cardiovascular diseases. This review article describes the current knowledge on the prevention of the negative consequences of hyperhomocysteinemia. It seems that various antioxidants (present in human diet), including phenolic compounds, may reduce the toxic effects of Hcy or its derivatives on hemostasis (Fig. 5). These findings give hope for the development of dietary supplements, which will be capable of preventing thrombosiss which occurs under pathological conditions, observed also in hyperhomocysteinemia, such as plasma procoagulant activity and oxidative stress.

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## Conflicts of interest disclosure

The Authors state that they had no interests which might be perceived as posing a conflict or bias.

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