Familial hyperhomocysteinemia, age and peripheral vascular diseases - an Italian study

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Abstract

Hyperhomocysteinemia is a widely recognized, although not yet entirely understood, risk factor for cardiovascular disease. Particularly, the complex relationships between age, hyperhomocysteinemia, predisposing genetic factors and peripheral vascular diseases have not been fully evaluated. Our contribution to this issue is a retrospective analysis of a large series of patients with peripheral arterial, venous and lymphatic disease, and of their blood relatives, with special reference to homocysteine plasma levels, age and methylenetetrahydrofolate reductase (MTHFR) polymorphisms. Serum homocysteine was measured in 477 patients (286 males, 191 females, age range 19-78 years) with various vascular clinical conditions: post-phlebitic syndrome (46) recurrent venous ulcers (78), arterial diseases (101) primary lymphoedema (87), secondary lymphoedema (161) and outlet thoracic syndrome (4), and in 50 normal controls. A MTHFR study for polymorphisms was carried on in the subjects with homocysteine values exceeding 15 mol/L. Serum homocysteine determination and MTHFR polymorphism studies were performed also in 1430 healthy blood related relatives (mainly siblings, descendents and sibling descendents) of the subjects with hyperhomocysteinemia and MTHFR polymorphisms. We found MTHFR polymorphisms in 20% of controls and in 69.3%, 69.5% and 53.8% of hyperhomocysteinemic subjects with arterial diseases, postphlebitic syndrome and venous ulcers, respectively. As expected, the percentage of hyperhomocysteinemia in patients with secondary lymphoedema and with thoracic outlet syndrome did not show significant differences compared to the control group. A MTHFR polymorphism was found in 116 out of the 214 hyperhomocysteinemic patients, *i.e.*, in the 54% of the overall patient population with hyperhomocysteinemia (214 patients). Interestingly 750 (52%) out of the 1430 blood relatives of the 116 patients with hyperhomocysteinemia and MTHFR polymorphisms showed at least one polymorphism in MTHFR gene. In this latter group of 750 healthy blood-related relatives bearing a MTHFR polymorphism the finding of hyperhomocysteinemia increased according to the age class from 1.6% in the age range <40 years up to 54.9% in the age range >60 years. The present study demonstrate that patients with peripheral arterial disease, post-phlebitic syndrome, venous ulcers and primary lymphoedema show a significantly higher incidence of hyperhomocysteinemia compared to controls, and adds further evidence to the causative role of hyperhomocysteinemia in the development of both arterial and venous disease. Moreover our data indicate a possible causative role of hyperhomocysteinemia in primary lymphoedema. In more than 50% of our hyperhomocysteinemic patients a polymorphism of MTHFR (C677T and/or A1298C) was detected. In subjects with these polymorphisms the frequency of hyperhomocysteinemia increases with age. We observed a quite similar frequency of the two polymorphisms in the studied population and therefore claim for the need to study both C677T and A1298C mutations in hyperhomocysteinemic patients.

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Introduction

The possible role of homocysteine high levels as a risk factor for vascular disease was described for the first time more than forty years ago,[1,2] and in the following decades a large amount of evidence has demonstrated that even mild increases in homocysteinemia are associated with an increased risk of cardiovascular diseases[3,4] including venous thromboembolic disease.[5] Hyperhomocysteinemia is frequently associated with MTHFR polymorphisms C677T and A1298C. MTHFR catalyzes the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, a co-substrate for homocysteine remethylation to methionine. Figure 1 summarizes homocysteine metabolism.[6-13] Figure 2 shows the pathophysiologic mechanism(s) of hyperhomocysteinemia-induced vascular damage.[14-18]

In the last few years, however, the causative role of hyperhomocysteinemia in cardiovascular disease has been questioned mainly based on the reports that failed to demonstrate a clinical benefit after lowering homocysteinemia levels with vitamins B6, B12 and folate.[19-21]

Particularly, it has been pointed out that treatment with homocysteine lowering agents, *i.e.* folic acid and B6 and B12 vitamins, over prolonged times, does not reduce the incidence of cardiovascular adverse effects,[19] although it has been reported that folate, vitamin B12 intake is associated with a reduction of the risk of ischemic stroke.[22,23] Therefore further studies have been advocated to address this issue.[24]

In order to understand the relationships between homocysteinemia and vascular disease we made a retrospective analysis of the patients who were referred to vascular disease rehabilitation program of our institute for peripheral arterial, venous and lymphatic disorders, paying special attention to homocysteine blood levels, MTHFR polymorphisms and age. The influence of MTHFR polymorphisms on homocysteine blood levels was also studied in the available healthy blood related relatives of hyperhomocysteinemic subjects with MTHFR polymorphisms.

Materials and Methods

A general description of the study is reported in Figure 3. Briefly, serum concentration of homocysteine was measured in 477 consecutive patients (286 males, 191 females, mean age 56.5 years, age range 19-78 years) with various vascular clinical conditions: post-phlebitic syndrome (46) recurrent venous ulcers (78), peripheral arterial diseases (101) primary lymphoedema (87), secondary lymphoedema (161) outlet thoracic syndrome (4) admitted to the rehabilitation program of our Hospital. Serum homocysteine determination was also performed in 50 normal control subjects (29 males and 21 females, mean age 55 years).

The quantitative determination of the

homocysteine serum levels was performed with an automated latex enhanced immunoassay (HemosIL, Homocysteine - 0020007800, Instrumental Laboratory SpA, Milano, Italy).

When the plasma homocysteine values exceeded 15 mol/L patients underwent MTHFR study for C677T and/or A1298C polymorphisms. MTHFR polymorphism analysis was performed after genetic amplification on venous blood EDTA treated samples by molecular genetic real time techniques in an associated laboratory (BIOS, Rome, Italy).

Homocysteine plasma determination and MTHFR polymorphism studies were performed also on 1430 healthy blood related relatives (mainly, siblings, descendents and sibling descendents) of the subjects with hyperhomocysteinemia and MTHFR polymorphisms.

Results

Patients

Hyperhomocysteinemia was found in 20% of controls and in 69.3%, 69.5% and 53.8 % of subjects with arterial diseases, postphlebitic syndrome and venous ulcers, respectively (Table 1). As expected, the percentage of hyperhomocysteinemia in patients with secondary lymphoedema and with thoracic outlet syndrome did not show significant differences compared to the control group (Table 1). At least one MTHFR polymorphism was found in 116 out of the 214 hyperhomocysteinemic patients *i.e.* in the 54% of the overall patient population with hyperhomocysteinemia (Table 2).

Blood related relatives of hyperhomocysteinemic patients bearing a methylenetetrahydrofolate reductase polymorphism

In 750 (52%) out of the 1430 blood relatives of the 116 patients with hyperhomocysteinemia and MTHFR polymorphisms at least one polymorphism in MTHFR gene was found. In this group of 750 asymptomatic subjects bearing a MTHFR polymorphism the frequency of the finding of hyperhomocysteinemia increased according to the age class from 1.6% in the age range <40 years up to 54.9% in the age range >60 years (Table 3). C677T polymorphism-associated hyperhomocysteinemia did not significantly differ from A1298C-induced hyperhomocysteinemia either in frequency or in hyperhomocys-

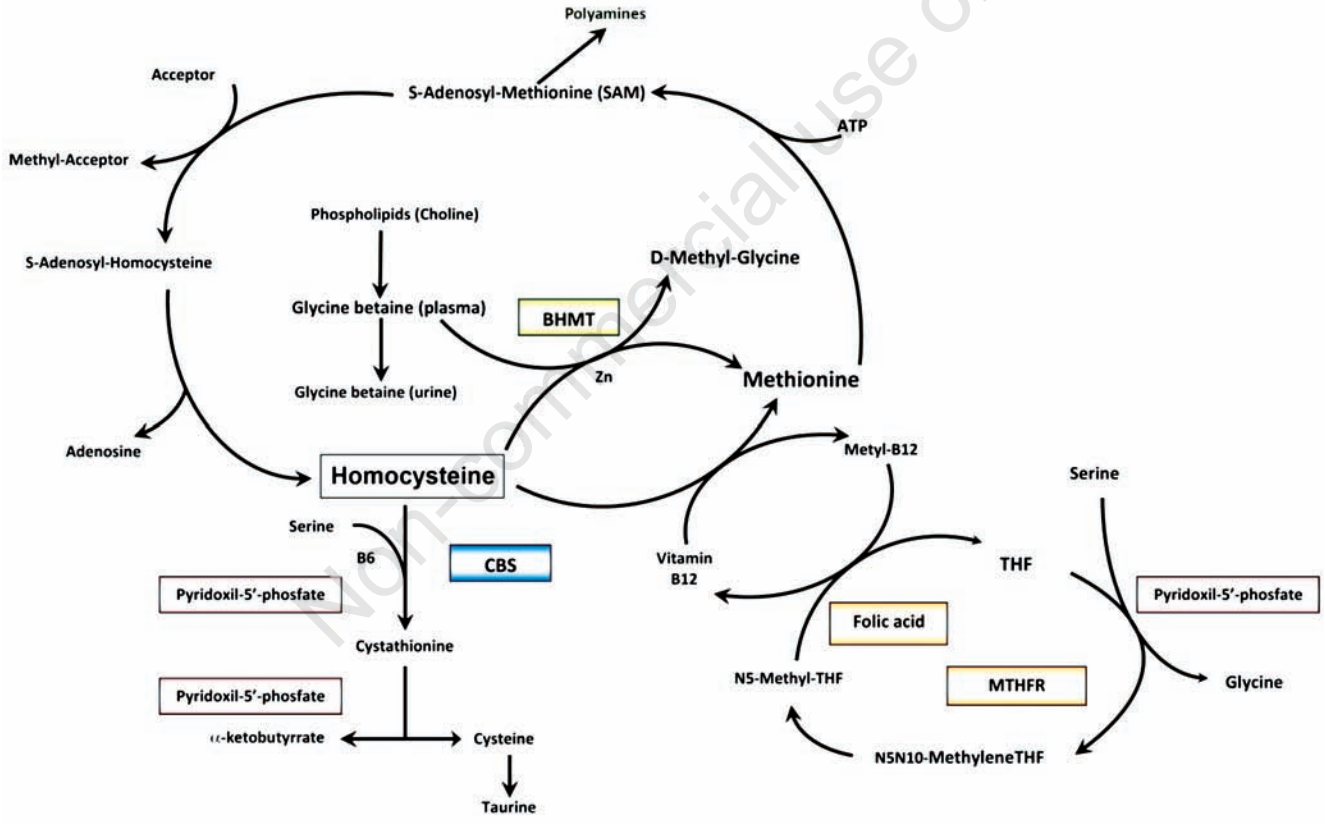

Figure 1. Homocysteine metabolism. Homocysteine is a sulphydryl amino acid derived from the intracellular demethylation of methionine. Homocysteine, when activated, yields a methyl group to different receivers (including creatine, steroid hormones, purine bases of DNA and RNA) and then it can be converted into homocysteine. Homocysteine may be, in turn, transformed irreversibly into cystathionine and then cysteine, or, in the absence of dietary methionine, remethylated to methionine. A series of enzymes and cofactors regulate these pathways. Homocysteine is produced through two possible pathways: remethylation or trans-sulphuration. The remethylation process converts back homocysteine to methionine (utilizing folate, vitamin B12 or trimethylglycine). The trans-sulphuration process utilizes vitamin B6, pyridoxal-5-phosphate, and catabolizes the homocysteine excess into metabolites that can be excreted from the organism. A mildly failure of the remethylation pathway (often due to reduced levels of folate, vitamin B12 or genetic defects) can increase significantly the homocysteine plasma levels. A mild failure in the trans-sulphuration pathway (caused by genetic defect or indadeguate levels of vitamin B6) can only increase slightly the homocysteine plasma concentration. There are several causes of hyperhomocysteinemia, some create a deficiency of the enzyme co-factors, and others reduce the activity of enzymes, involved in its metabolism. The deficiencies of vitamin B12, B6 or folic acid may be due to an inadequate diet, intake of drugs like methotrexate, nitroxide and levodopa or conditions involving hormonal changes like pregnancy and hypothyroidism.

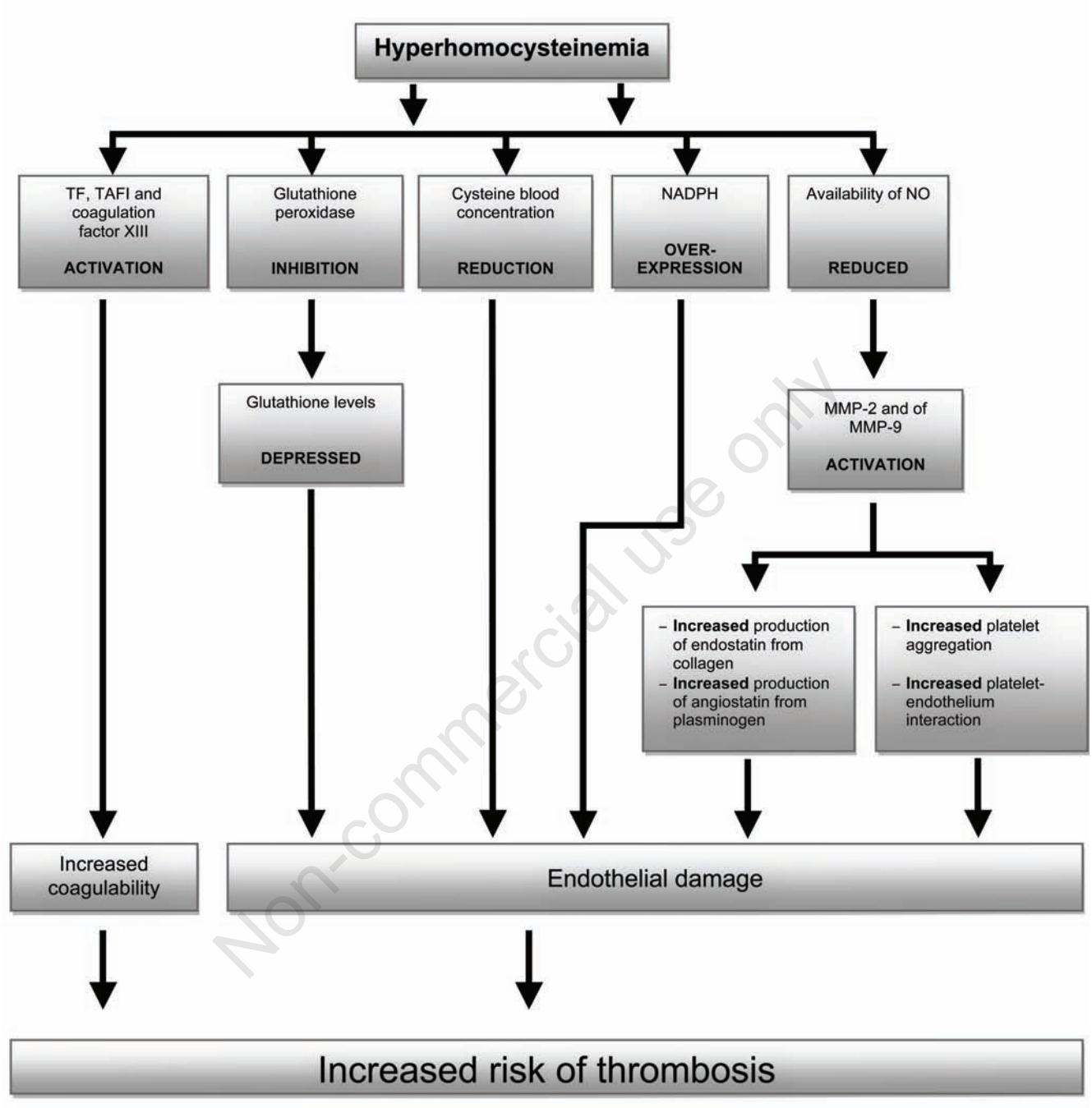

Figure 2. Hyperhomocysteinemia mechanisms of endothelial damage and thrombosis. Hyperhomocysteinemia induces an oxidative stress through both direct and indirect effects. Particularly important are the inhibition of glutathione peroxidase, the reduction of cysteine blood concentration, which results in depressed glutathione levels, the overexpression of NADPH and the reduced availability of nitric oxide. In its turn the depression of nitric oxide activity activates MMP-2 and MMP-9 with consequent increased aggregation of platelets and increased interaction between platelets and endothelium. Moreover MMP activation increases the production of endostatin from collagen and of angiostatin from plasminogen with consequent vascular damage. Thrombophilia is also sustained by the hyperhomocysteinemia-enhaced activation of TF, of TAFI and of factor XIII. All these data explain why abnormally elevated homocysteine blood levels result in endothelial damage and in a consequent increase of the risk of both arterial and venous thrombosis. TF, tissue factor; TAFI, thrombin activable fibrinolysis inhibitor; MMP-2, matrix metalloproteinase 2; MMP-9, matrix metalloproteinase 9.

teine induced levels (Table 4). Moreover, the association of the two polymorphisms in the same subject did not result either in an earlier appearance of hyperhomocysteinemia or in higher homocysteinemia levels. From a clinical point of view our findings indicate a quite similar frequency of the two polymorphisms in the studied population and therefore the need to study both C677T and A1298C mutations in hyperhomocysteinemic patients (Table 4).

Discussion and Conclusions

Despite some uncertainity due to the limited number of control subjects the present study shows a far higher incidence of hyperhomocysteinemia in patients with peripheral artery disease, post-phlebitic syndrome, venous ulcers and primary lymphoedema compared to controls, therefore adding further evidence to the causative role of hyperhomocysteinemia in the development of both peripheral thrombotic arterial and venous disease and of primary lymphoedema. The patients with primary lymphoedema recruited in the present study had no evidence of other vascular disease.

In more than 50% of our hyperhomocysteinemic patients a polymorphism of MTHFR (C677T and/or A1298C) was detected. It has been also observed that in subjects with MTHFR C677T and/or A1298C polymorphism the frequency of hyperhomocysteinemia increases with age. This latter finding is especially unexpected taking into account that genetic disorders usually, although not always, produce clinically evident disorders in the first decades of life. This could possibly be explained by an age related reduction of the defences against oxidative stress.

Another interesting observation is that C677T polymorphism-associated hyperhomocysteinemia did not significantly differ from A1298C-induced hyperhomocysteinemia either in frequency or in severity. Moreover, surprisingly, the association of the two polymorphisms in the same subject is not associated either with an earlier appearance of hyperhomocysteinemia or with higher homocysteinemia levels. The very similar frequency of the C677T and A1298C polymorphisms in the patient population implies the need to study both C677T and A1298C mutations in hyperhomocysteinemic patients. Particularly, it is known that in southern Europe, and especially in Italy, the prevalence of C677T homozygous polymorphism is quite higher compared with northern and central Europe.[25]

Finally, at authors' knowledge this is the first report indicating a possible causative role of hyperhomocysteinemia in primary lymphoedema.

Table 1. Homocysteine plasmatic values according to the underlying disease (data on 477 patients) and in the control group (50 subjects).

Test results	Groups	Arterial diseases 101	Post-phlebitic syndrome 46	Venous leg ulcers 78	Primary lymphoedema 87	Secondary lymphoedema 161	Thoracic outlet syndrome 4	Control subjects 50
5 to 15 μmol/L	Normal levels	31	14	36	61	117	4	40
15 to 30 μmol/L	Moderate hyperhomocysteinemia	44	17	30	19	28	0	9
30 to 100 μmol/L	Intermediate hyperhomocysteinemia	17	10	9	7	15	0	1
>100 μmol/L	Severe hyperhomocysteinemia	9	5	3	0	1	0	0
Hyperhomocysteinemic subjects (%)	-	69.3	69.5	53.8	29.9	27.3	0	20

Hyperhomocysteinemic subjects were significantly (P<0.001 at Chi-square test) more frequent in the groups of subjects with arterial diseases, postphlebitic syndrome, venous ulcers and primary lymphoedema compared to controls.

Table 2. Patients bearing A1298C and/or C677T polymorphisms (n=116), methylenetetrahydrofolate reductase polymorphism, homocysteinemia level and associated clinical disorders.

Mutation	Moderate hyperhomocysteinemia	Intermediate hyperhomocysteinemia	Severe hyperhomocysteinemia	Arteriopathy	Thrombo-phlebitis	Venous leg ulcers
C677T eterozygous 18 subjects	15	3	1	11	3	4
C677T homozygous 16 subjects	2	6	4	8	2	6
A1298C homozygous 28 subjects	17	10	1	19	5	4
A1298C homozygous 16 subjects	6	11	2	7	3	6
C677T eterozygous A1298C eterozygous 5 subjects	9	6	4	8	6	5
C677T eterozygous+A1298C homozygous 19 subjects	6	3	3	5	5	2
C677T homozygous+A1298C eterozygous 12 subjects	0	0	2	1	1	0
C677T homozygous+A1298C homozygous 2 subjects	2	2	1	1	3	1

The first three columns refer to the degree of hyperhomocysteinemia. The last three columns illustrate the associated vascular disease.

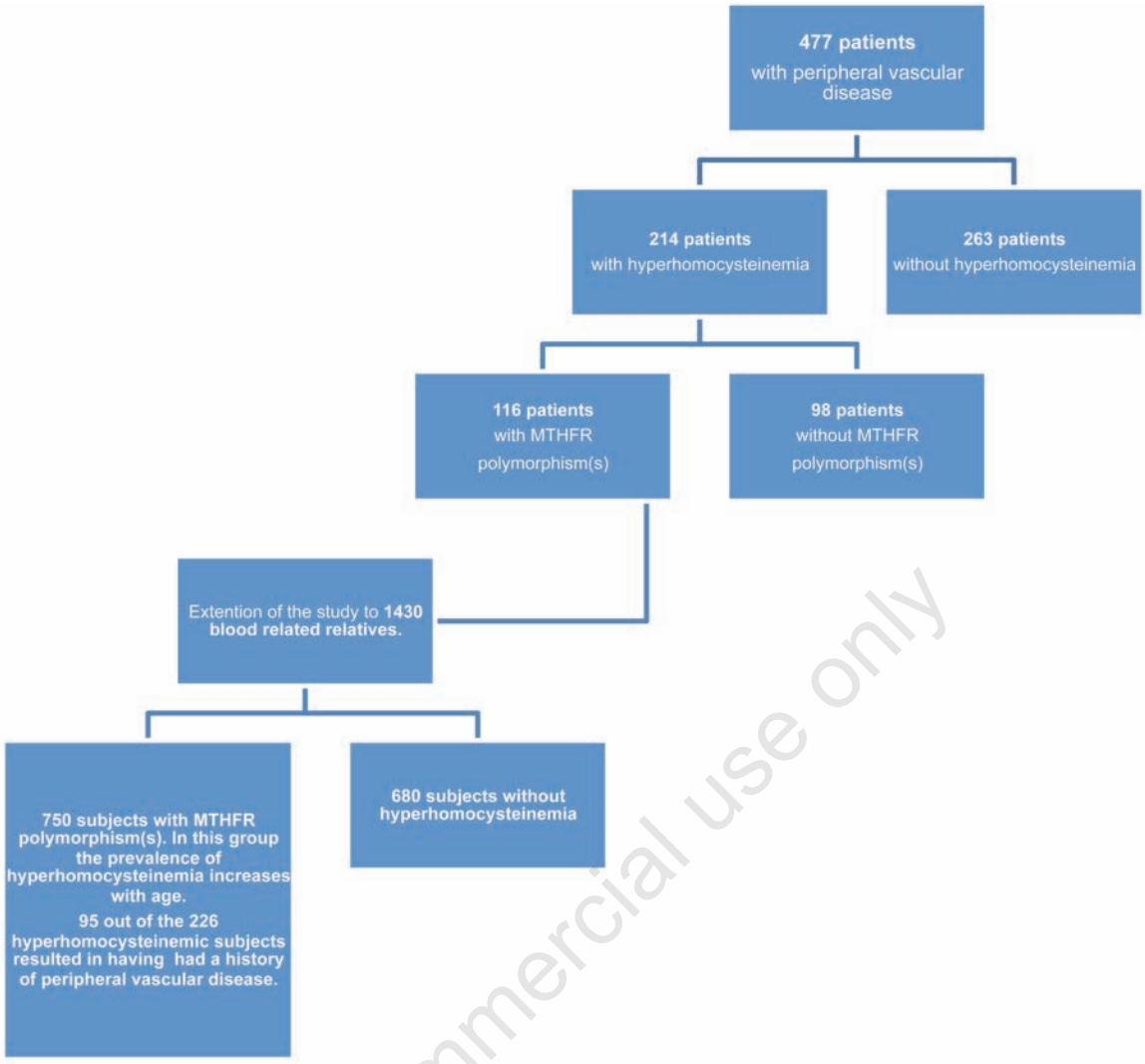

Figure 3. Study design, patients and subjects. MTHFR, methylenetetrahydrofolate reductase.

Table 3. Age related finding of hyperhomocysteinemia in asymptomatic methylenetetrahydrofolate reductase polymorphism bearers.

Age (years)	No. subjects	C677T eterozygotic mutation	C677T homozygotic mutation	A1298C eterozygotic mutation	A1298C homozygotic mutation	No. subjects with hyperhomo-cysteinemia	% subjects with hyperhomo-cysteinemia
≤39	83	24	16	28	19	2	1.66
40-49	254	66	48	89	55	34	13.4
50-59	211	56	45	61	56	79	37.4
≥60	202	53	42	63	55	111	54.9

The frequency of hyperhomocysteinemic subjects among age groups resulted always highly significant (P<0.001 at Chi-square test). Notice that in 38 cases a double polymorphism was found.

Table 4. Incidence of methylenetetrahydrofolate reductase (MTHFR) polymorphisms in the 750 blood relatives of the patients with MTHFR mutation and relative incidence and level of related hyperhomocysteinemia.

Mutation	Moderate hyperhomocysteinemia	Intermediate hyperhomocysteinemia	Severe hyperhomocysteinemia
C677T eterozygotic 199 subjects	20	23	8
C677T homozygotic 151 subjects	22	27	9
A1298C eterozygotic 241 subjects	23	25	9
A1298C homozygotic 185 subjects	28	26	6

Notice that 95 out of 226 (42%) bearers of a MTHFR polymorphism presented, at a carefully made clinical interview, a history for peripheral vascular disease.

References

1. Gibson JB, Carson NAJ, Neill DW. Pathological findings in homocystinuria. J Clin Path 1964;17:427-37.
2. McCully KS. Vascular pathology of homocysteinemia: implications for the pathogenesis of arteriosclerosis. Am J Pathol 1969;56:111-28.
3. Graham IM, O'Callaghan P. Vitamins, homocysteine and cardiovascular risk. Cardiovasc Drugs Ther 2002;16:383-9.
4. De Ruijter W, Westendorp RG, Assendelft WJ, et al. Use of Framingham risk score and new biomarkers to predict cardiovascular mortality in older people: population based observational cohort study. Br Med J 2009;338:a3083.
5. Ray JG. Meta-analysis of hyperhomocysteinemia as a risk factor for venous thromboembolic disease. Arch Int Med 1998;158: 2101-6.
6. Swift ME, Schultz DT. Relationship of vitamins B6 and B12 to homocysteine levels: risk for coronary heart disease. Nutr Rep Int 1986;34:1-14.
7. Selhub J, Miller JW. The pathogenesis of homocyst(e)inemia: interruption of the coordinate regulation by S-adenosylmethionine of remethylation and transsulfuration of homocysteine. Am J Clin Nutr 1991;55:131-8.
8. Miller JW, Ribaya-Mercado JD, Russell RM, et al. Total homocysteine in fasting plasma is not a good indicator of B6 deficiency, Am J Clin Nutr 1992;55:1154-560.
9. Miller JW, Nadeau MR, Smith D, Selhub J. Vitamin B6 deficiency vs folate deficiency: comparison of responses to methionine loading in rats. Am J Clin Nutr 1994;59: 1033-9.
10. Haagsma CJ, Blom HJ, van Riel PL, et al. Influence of sulphasalazine, methotrexate, and the combination of both on plasma homocysteine concentrations in patients with reumathoid arthritis, Ann Rheum Dis 1999;58:79-84.
11. Müller T, Werne B, Fowler B, Kuhn W. Nigral endothelial dysfunction, homocysteine, and Parkinson's disease. Lancet 1999;354:126.
12. Sorensen TK, Malinow MR, Williams MA, et al. Elevated second-trimester serum homocyst(e)ine levels and subsequent risk of preeclampsia. Gynecol Obstet Invest 1999;48:98-103.
13. Catargi B, Parrot-Rouland F, Cochet C, et al. Homocysteine, hypothyroidism, and the effect of thyroid hormone replacement. Thyroid 1999;9:1163-6.
14. Handy DE, Zhang Y, Loscalzo J. Homocysteine down-regulates cellular glutathione peroxidise (GPx1) by decreasing translation, J Biol Chem 2005;280:15518-25.
15. Ungvari Z, Csiszar A, Edwards JG, et al. Increased superoxide production in coronary arteries in hyperhomocysteinemia: role of Tumor Necrosis Factor- , NAD(P)H oxidase and inducible nitric oxide synthase. Arterioscler Thromb Vasc Biol 2003; 23:418-24.
16. Topal G, Brunet A, Millanvoye E, et al., Homocysteine induces oxidative stress by uncoupling of NO synthase activity through reduction of tetrahydrobiopterin. Free Radic Biol Med 2004;36:1532-41.
17. Fernandez-Patron C, Martinez-Cuesta MA, Salas E, et al. Differential regulation of platelet aggregation by matrix metalloproteinases-9 and -2. Thromb Haemost 1999; 82:1730-5.
18. Loscalzo J. Homocysteine-mediated thrombosis and angiostasis in vascular pathobiology. J Clin Invest 2009;119:3203-5.
19. Abraham JM, Cho L. The homocysteine hypothesis: still relevant to the prevention and treatment of cardiovascular disease? Cleveland Clinic J Med 2010;77:911-8.
20. Marcus J, Sarnak MJ, Menon V. Homocysteine lowering and cardiovascular risk: lost in translation. Can J Cardiol 2007;23:707-10.
21. Ray JG, Kearon C, Yi Q, et al. Homocysteine lowering therapy and risk for venous thromboembolism: a randomized trial. Ann intern Med 2007;146:761-7.
22. He K, Merchant A, Rimm EB, et al. Folate, vitamin B6 and B12 intakes in relation to risk of stroke among men. Stroke 2004;35: 169-74.
23. Saposnik G, Ray JG, Sheridan P, et al. Homocysteine-lowering therapy and stroke risk severity, and disability: additional findings from the HOPE 2 trial. Stroke 2009;40: 1365-72.
24. Smulders YM, Blom HJ. The homocysteine controversy. J Inherit Metab Dis 2011;34: 93-9.
25. Wilcken B, Bamforth F, Li Z, et al. Geographical and ethnic variation of the 677C>T allele of 5,10 methylenetetrahydrofolate reductase (MTHFR): findings from over 7000 newborns from 16 areas worldwide. J Med Genet 2003;40:619-25.