New cardiovascular risk factors: homocysteine and vitamins involved in homocysteine metabolism

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Among various cardiovascular risk factors, hyperhomocysteinemia has recently emerged as an important one. While there are currently no doubts on the relationship between severe hyperhomocysteinemia and vascular disease, some uncertainty still persists on the relationship between mild hyperhomocysteinemia and vascular disease. Several group B vitamins, namely vitamin B6, vitamin B12, and folate, influence homocysteine metabolism, being cofactors of the main metabolic pathways which allow the disposal of this amino acid. There are also, however, suggestions from the literature that group B vitamins, and in particular vitamin B6 (pyridoxine/pyridoxal-phosphate), are modulators of cardiovascular risk independent of homocysteine. The results of a recent study of ours, with a long follow-up, indeed suggest that homocysteine and vitamin B6 are independent and additive cardiovascular risk factors.

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Homocysteine as a cardiovascular risk factor - historical notes

Among risk factors for vascular diseases, hyperhomocysteinemia has gained increased importance in recent years. The first description of a relationship between hyperhomocysteinemia and atherosclerosis dates back to 1964, when a rare recessive autosomal syndrome, due to a deficiency of the enzyme cystathionine beta-synthase, was described. The disease was characterized by high urinary and plasma levels of homocysteine, and by a constellation of signs and symptoms including atherosclerosis, skeletal anomalies, as well as abnormalities of the central nervous system and the eye[1]. The relationship between severe hyperhomocysteinemia and atherosclerosis was first hypothesized, in a case-control study, by McCully[2] in 1969. This relationship was recently confirmed by prospective and retrospective case-control studies, showing the existence of a graded relationship between plasma levels of homocysteine and the risk for cardiovascular disease. It has been estimated that the incidence of coronary artery disease increases by a factor of 1.6 to 1.8 for every 5 μmol/l increase in fasting plasma homocysteine[3].

Slight-to-moderate hyperhomocysteinemia as a risk factor

On the basis of fasting homocysteine plasma concentrations, it is possible to distinguish four main conditions:
- normal values: 5-15 μmol/l;
- mild hyperhomocysteinemia: 16-30 μmol/l;
- moderate hyperhomocysteinemia: 31-100 μmol/l;
- severe hyperhomocysteinemia: > 100 μmol/l[4].

In the last few years, severe hyperhomocysteinemia has been conclusively accepted as an independent risk factor for coronary artery, cerebrovascular and peripheral vascular diseases[5]. For the much more frequent conditions of mild and moderate hyperhomocysteinemia, however, the relationship with cardiovascular diseases has yet to be conclusively proven. In a study of 482 dyslipidemic patients, Glueck et al.[6] showed an increase in the incidence of vascular disease in subjects with high levels of homocysteine, defined as > 16.2 μmol/l, with a relative risk of 2.8 in the subjects in the highest quintile of the distribution of homocysteine levels. In the Physicians' Health Study, the analysis of 271 men over a period of 5 years showed that homocysteine levels were significantly higher in subjects who later developed a

myocardial infarction, and that the relative risk for cardiovascular diseases was 3.4 times higher in subjects in the highest quintile[7].

Besides the above-mentioned studies, other prospective studies, such as the British United Provident Association (BUPA) study[8], the British Regional Heart Study[9], the Trømso study[10], as well as Nygard's study[11], have all shown the occurrence of a positive association between mild-to-moderate hyperhomocysteinemia and cardiovascular diseases. There are also, however, contrasting data from some prospective studies, such as the Multiple Risk Factor Intervention Trial (MRFIT)[12], the North Karelia Project[13] and the Atherosclerosis Risk in Communities (ARIC) study[14], which deny the existence of any association between moderate levels of hyperhomocysteinemia and vascular diseases in general and coronary artery disease in particular.

Homocysteine metabolism and gene variants of enzymes involved in its metabolism

Homocysteine is a sulfurated amino acid, which is produced as an intermediate in the metabolism of methionine. Homocysteine is structurally similar to cysteine: like cysteine, it has a sulfhydryl group, but the aliphatic chain is longer, containing one more carbon atom. Chemical characteristics are also similar, in that both amino acids may form disulfide bridges with other molecules possessing a sulfhydryl terminal group, for example another homocysteine, cysteine, or diamino acids, such as cysteinyl-glycine. When a disulfide bridge forms with another homocysteine molecule, the resulting molecule is termed homocystine. In the other cases, the resulting molecules are generically referred to as "mixed disulfides". Unlike cysteine, homocysteine is not incorporated in proteins and, as such, has no structural functions. It does, however, have a role in cellular metabolism in two important processes: the "activated methyl group cycle" (also called "remethylation pathway") and in the biosynthesis of cysteine, known also as the "trans-sulfuration pathway" (Fig. 1). Knowledge of the metabolic pathways involving homocysteine is of fundamental importance for an understanding of the etiology of the various forms of hyperhomocysteinemia. Homocysteine represents the connecting point of the two above-mentioned metabolic pathways. Homocysteine is formed as an intermediate in the cycle of the activated methyl group, or "remethylation cycle", which takes place in the cytoplasm and leads to the metabolization of methionine. Homocysteine itself is in part retransformed into methionine in this cycle, or is, in part, deviated toward the synthesis of cysteine (Fig. 1). The best and longest known enzymatic defect involving homocysteine metabolism is the lack of cystathionine beta-synthase, observable in 1 out of 333 500 live

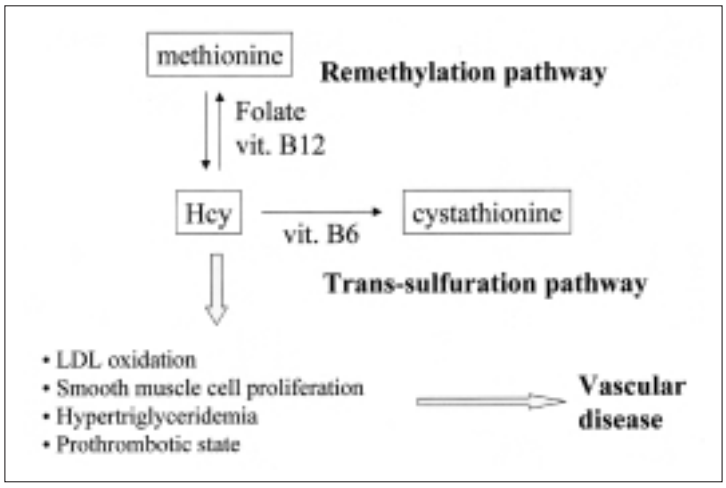

Figure 1. *The central role of homocysteine (Hcy) in the remethylation cycle and in the synthesis of cysteine, in an extremely simplified scheme. For normal biosynthetic reactions, the methyl groups need a greater activation energy than other single carbon units, for which the simple binding to tetrahydrofolate is sufficient for their activation. The remethylation cycle serves to synthesize a donor of methyl groups, the compound S-adenosylmethionine. The methyl group is associated with this molecule with very-high energy bonds, therefore making this molecule an ideal donor for the various acceptor substrates. The synthesis of the S-adenosylmethionine is catalyzed by the enzyme methionine-adenosyltransferase, with the hydrolysis of three phosphodiester bonds, which allows the formation of a high-energy bond in which the sulphur atom carries a positive charge, thus activating the methyl group. When this is released through the action of methyltransferases specific for various substrates, S-adenosylmethionine is generated. This, in turn, is reconverted to Hcy by hydrolysis. The cycle is closed by the intervention of either betaine (the product of oxidization of choline) or tetrahydrofolate, which transfers a methyl group to the sulfhydryl terminal group, regenerating methionine. These reactions are mediated by betaine-Hcy methyltransferase and 5-methyltetrahydrofolate-Hcy methyltransferase (also called methionine synthase, a vitamin B12-dependent enzyme), respectively. In turn, 5-methyltetrahydrofolate is generated by reduction from 5,10-methylenetetrahydrofolate through the enzyme 5,10-methyl/methylenetetrahydrofolate reductase, one of enzymes the genetic lack of which may determine hyperhomocysteinemia. About half of the Hcy produced through the remethylation cycle is redirected toward the synthesis of cysteine. This pathway is also called "trans-sulfuration pathway" since the sulfhydryl group of methionine is transferred to the backbone of the amino acid serine, forming a cysteine molecule via an intermediate represented by cystathionine. To catalyze the transformation of Hcy into cystathionine here intervenes the enzyme cystathionine beta-synthase, the genetic lack of which is the best known genetic defect leading to hyperhomocysteinemia.*

births[15], and transmitted as a recessive autosomal character. Cystathionine beta-synthase is the enzyme catalyzing the condensation of homocysteine and serine, and is responsible for the catabolism of around half of the homocysteine produced in the remethylation cycle[15]. It is therefore understandable how an enzymatic defect at this level may cause a marked increase in homocysteine in the cytoplasm, which then passes into the plasma. Also heterozygosity, estimated at between 0.3 and 1% in the general population[15,16], and causing a state of moderate homocysteinemia also associated with lesser degrees of vascular disease, is just as important as a medical problem. A second type of enzymatic defect, probably more frequent, but not yet quantitatively defined, consists in qualitative or quantitative alterations of the enzyme methylenetetrahydrofolate reductase (MTHFR)[17]. MTHFR catalyzes a rate-limiting reaction in the transformation of methionine into homocysteine and back through the transfer of the methyl group to and from methyltetrahydrofolate, respectively[18]. The total, genetically determined, absence of the enzyme represents one of the causes of severe hyperhomocysteinemia, with accelerated atherosclerosis, in young subjects. In 1988, Kang et al.[19] described a thermolabile variant of MTHFR, present in 5% of the population, associated with a reduction in enzyme activity and mild or moderate hyperhomocysteinemia. In 1995, Frosst et al.[20] identified the mutation responsible for this phenotypic variant in the substitution of cytosine with thymine at position 677. Studies aimed at verifying the association between moderate hyperhomocysteinemia due to the thermolabile MTHFR variant and vascular diseases have provided inconsistent results, despite the fact that the variant is constantly accompanied by an increase in homocysteine levels. The uncertainties in assessing the association of this genotype with cardiovascular risk also derive from the observation that homocysteine levels apparently increase after an ischemic event, suggesting that moderate hyperhomocysteinemia can be an epiphenomenon rather than a cause of the vascular event itself[21,22].

Mechanisms of vascular damage caused by homocysteine

The putative mechanisms through which homocysteine increases the risk for cardiovascular diseases are many. Several *in vitro* and *in vivo* studies have shown that homocysteine levels > 100 μmol/l, and therefore characteristic of homocystinuria, determine dysfunctions and damage to the endothelium[23,24], proliferation of vascular smooth muscle cells[25], platelet aggregation[26], and increased binding of lipoprotein(a) with fibrin[27]. Moreover, it has been demonstrated that concentrations of homocysteine between 10 and 50 μmol/l characteristic of moderate hyperhomocysteinemia, determine a reduced regeneration of endothelial cells, thus hampering the repair mechanisms of the damaged endothelium[28].

Vitamins, homocysteine metabolism, and vascular disease

Plasma levels of homocysteine are known to be related to vitamin status[29]. However, the relationship between the vitamin status (vitamin B6 [also called pyridoxine and, in its active form, pyridoxal-phosphate], vitamin B12 and folate) and cardiovascular disease has so far been highly equivocal[14,30-33].

A partial exception to this is vitamin B6. In two case-control studies, the vitamin B6 status has been associated with vascular disease (coronary artery disease, cerebrovascular stroke and peripheral arteriopathy), independent of homocysteine levels[34,35]. This was not the case, however, in two other studies[32,33]. In the Physicians' Health Study cohort, in which a relationship between the baseline levels of homocysteine and the risk for myocardial infarction and cardiac death at 5 years was found[7], a further analysis of the risk at 7.5 years related to the baseline levels of folate and pyridoxal-phosphate showed a trend toward the existence of associations, which did not however reach statistical significance[36]. In the ARIC study, Folsom et al.[14] carried out a prospective random-cohort analysis to evaluate the role of fasting homocysteine and group B vitamins on the incidence of ischemic heart disease at 3.3 years. At multivariate analysis, only vitamin B6 was significantly associated with the later occurrence of ischemic heart disease, with a relative risk for the uppermost vs the lowermost quintile of 0.28 (95% confidence interval 0.1-0.7). In the first randomized drug intervention study so far reported, the administration of vitamin B6 and folate reduced the levels of fasting and post-methionine load homocysteine and the number of positive ECG stress tests[37]. However this study did not clarify the possible contribution of such vitamins independent of the reduction of homocysteine. Indeed vitamin B6, without determining any significant changes in homocysteine plasma levels, significantly improved flow-mediated vasodilation in the brachial artery in a small, prospective, randomized, placebo-controlled study[38].

Taking into account existing data and hypotheses from the literature, we have re-examined the role of homocysteine and other cofactors involved in its metabolism, including vitamin B6, in a prospective, nested case-control study with a long follow-up. Since homocysteine can be influenced by smoking[39,40], hypertension[40-42], dyslipidemia[40,43,44], hyperglycemia[45], and high levels of C-reactive protein[46], in this study we matched each "case" of incident vascular (coronary or cerebrovascular) disease with controls, remained free of disease, also for these variables, besides age and sex normally considered.

Out of 1051 subjects (509 men and 542 women) recruited in 1987 into a study of a population in Friuli, North-Eastern Italy (the Martignacco study[47]), there were 66 first-ever coronary events and 43 first-ever cerebrovascular events (number of cases = 109). These were then matched, in a 1:1 ratio, with 109 control subjects (subjects without any ensuing events) with similar age, sex, smoking habit, history of hypertension, presence of dyslipidemia, and excess of weight. Serum samples obtained in 1987 and stored frozen at -80°C at the beginning of the study were used to measure concentrations of total homocysteine, folate, vitamin B6, and C-reactive protein. In synthesis, we found a concentration-dependent association between total homocysteine and the risk for events (odds ratio for the uppermost quartile vs the lowermost quartile 1.32, 95% confidence interval 1.01-1.73). Levels of folate and vitamin B6 did not significantly differ between cases and controls, but were negatively correlated ($p < 0.01$) with total homocysteine levels. Vitamin B6 did not show any correlations with baseline levels of total homocysteine, but was significantly different between cases and controls: for subjects in the uppermost quartile vs those in the lowermost quartile there was an odds ratio of 0.68 (95% confidence interval 0.49-0.95). The effects of high levels of homocysteine and low levels of vitamin B6 were at least additive, with an odds ratio for subjects in the lowermost quartile of vitamin B6 and the uppermost quartile of homocysteine of 2.79 (95% confidence interval 2.06-3.77). Cases and controls in this population did not differ with regard to baseline levels of C-reactive protein[48].

The results of our study therefore confirm a role of fasting homocysteine in the prediction of vascular events in the long term, but also show the adjunctive importance of the levels of vitamin B6, which therefore qualify as a new risk factor, independent of homocysteine and of C-reactive protein.

Riassunto

Tra i fattori di rischio cardiovascolare, l'iperomocisteinemia ha assunto negli ultimi anni un'importanza crescente. È stato infatti valutato che per ogni incremento di 5 μmol/l dei livelli plasmatici a digiuno di omocisteina, si realizza un aumento dell'incidenza di malattia coronarica da 1.6 a 1.8 volte.

Negli ultimi anni l'iperomocisteinemia grave è stata definitivamente accettata come fattore di rischio indipendente per malattia vascolare coronarica, malattie cerebrovascolari e malattie vascolari periferiche. Diverso è il caso dell'iperomocisteinemia lieve-moderata, assai più frequente, per la quale l'associazione con le malattie cardiovascolari non è stata tuttora definitivamente provata.

L'omocisteina ha un ruolo nel metabolismo cellulare in due importanti processi: il "ciclo del metile attivato" (via della rimetilazione) e nella via di biosintesi della cisteina, nota anche come "via di trans-sulfurazione", rappresentando il punto di collegamento delle due vie. Il difetto enzimatico più noto e caratterizzato da più lungo tempo è la carenza dell'enzima cistationina beta-sintetasi. Questo deficit enzimatico causa un notevole innalzamento dell'omocisteina citoplasmatica, che può passare nel plasma.

L'omocisteina aumenterebbe il rischio di malattia cardiovascolare determinando disfunzione e danno dell'endotelio, proliferazione delle fibrocellule muscolari lisce, aggregazione piastrinica, aumento del legame della lipoproteina(a) con la fibrina. Inoltre l'iperomocisteinemia moderata determinerebbe una ridotta rigenerazione di cellule endoteliali, ostacolando in tal modo i meccanismi di riparazione del danno endoteliale.

Diverse vitamine del gruppo B, principalmente B6, B12 e folato, influenzano il metabolismo dell'omocisteina, essendo cofattori delle principali vie metaboliche che permettono l'allontanamento dell'aminoacido.

Esistono tuttavia anche suggerimenti dalla letteratura che vitamine del gruppo B, e in particolare la vitamina B6 (piridossina, piridossal-fosfato), possano essere dei modulatori del rischio cardiovascolare (malattia coronarica, ictus cerebrovascolare e arteriopatia periferica) indipendenti dall'omocisteina.

Diversi studi non hanno però chiarito il possibile contributo di tali vitamine indipendentemente dall'effetto di riduzione dell'omocisteina. In verità la piridossina (vitamina B6), senza determinare alcuna variazione significativa delle concentrazioni plasmatiche di omocisteina, ha significativamente migliorato la vasodilatazione da flusso nell'arteria brachiale in un piccolo studio randomizzato, prospettico, controllato con placebo.

Noi abbiamo riesaminato il ruolo dell'omocisteina e di altre determinanti del suo metabolismo, compresa la vitamina B6, in uno studio prospettico con disegno "nested" caso-controllo, con un lungo follow-up. In 1051 soggetti reclutati nel 1987 in uno studio di popolazione in Friuli (lo studio di Martignacco), si sono verificati 66 primi eventi coronarici e 43 primi eventi cerebrovascolari (casi, n = 109); 109 soggetti di controllo (soggetti rimasti privi di eventi) sono stati dunque appaiati 1:1 ai casi, per età, sesso, abitudine al fumo, storia di ipertensione, presenza di dislipidemia e di eccesso di peso. Campioni di siero ottenuti nel 1987, all'inizio dello studio, sono stati usati per misurare i livelli di omocisteina totale, folato, vitamina B12 e vitamina B6, come pure le concentrazioni di proteina C reattiva. I risultati in sintesi hanno mostrato un'associazione concentrazione-dipendente tra omocisteina totale e rischio di eventi. La vitamina B6 non ha mostrato alcuna correlazione con i livelli basali di omocisteina totale, ma è risultata significativamente diversa tra casi e controlli. L'effetto di alti livelli di omocisteina e di bassi livelli di vitamina B6 era almeno additivo.

I risultati di questo studio confermano dunque un ruolo dell'omocisteina a digiuno nel predire eventi vascolari a lungo termine, ma mostrano anche l'importanza additiva dei livelli di vitamina B6, che si qualifica pertanto come un nuovo fattore di rischio, indipendente dall'omocisteina e dai valori di proteina C reattiva.

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