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Plasma and aqueous humour levels of homocysteine in exfoliation syndrome

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Abstract *Background:* Recent studies have suggested that the relationship between elevated plasma homocysteine (Hcy) and increased risk of vascular disease holds also for certain diseases of the eye with vascular aetiology. Elevated plasma Hcy levels have been noted among patients with exfoliation syndrome (XFS). The purpose of this study was to establish whether subjects with XFS have higher plasma and aqueous humour Hcy levels values than non-XFS subjects, particularly in relation to vitamin B status. *Methods:* Using a cross-sectional study design, 36 subjects with XFS and 36 non-XFS subjects with intraocular pressure (IOP) lower than 23 mmHg, matched by age and gender, were first selected. The participant exclusion criteria included parameters known to alter Hcy metabolism. In the XFS group, 11 subjects had a concurrent diagnosis of exfoliative glaucoma (XFG). Fasting plasma and aqueous humour Hcy samples were collected, along with erythrocyte folate (E-Fol) and serum vitamin B6 and B12 samples. The Hcy samples were analysed using a fluorescence polarization immunoassay method. *Results:* Plasma Hcy level was significantly higher (*P*=0.020, after Bonferroni correction for multiple testing) in the XFS group than in the controls. The Hcy concentrations in the aqueous humour did not differ statistically between the two groups. Plasma and aqueous humour Hcy concentrations were not statistically significantly correlated within the groups of exfoliation-positive and -negative subjects. E-Fol, and serum vitamin B6 and B12 levels did not differ statistically between the XFS group and the control group. *Conclusions:* The finding that subjects with XFS are more prone to elevated plasma Hcy emphasizes exfoliation as a clinical sign and a marker of thromboembolic vasculopathies induced by hyperhomocysteinaemia.

Introduction

Raised plasma level of homocysteine (Hcy), an intermediary sulfur-containing amino acid formed during the conversion of the amino acid methionine to cysteine, is reportedly a risk factor for retinal arterial and venous occlusive disorders [1, 6, 7, 47], although some studies do not support this for the aetiology of central retinal vein occlusion (CRVO) [5, 20, 29]. In addition, positive correlation has been noted between plasma Hcy levels and non-arterial ischaemic optic neuropathy [17, 29]. Three recent studies found a correlation between open-angle glaucoma and Hcy, one in both chronic primary open-angle glaucoma (POAG) and exfoliation glaucoma (XFG) [4], the other two in XFG alone [21, 46]. Vessani and co-workers also noted that plasma Hcy levels were significantly higher in non-glaucomatous patients with XFS [46].

Hyperhomocysteinaemia can be genetic in origin or mediated by nutritional factors. Because the metabolism

of methionine via Hcy to cysteine is a complex pathway involving enzymes dependent on cobalamin (vitamin B12), pyridoxal phosphate (derived from vitamin B6) and folic acid, among other co-factors, genetically inherited defects or deficiencies in these rate-limiting enzymes are probably the most important determinants of elevated levels of Hcy.

Hcy appears to have a prominent role in vascular pathogenesis. It promotes endothelial injury, myointimal hyperplasia, hypertrophy, deposition of sulfated glycos-aminoglycans, collagen synthesis, and fibrosis and calci-fication of atherosclerotic plaques [24]. Within the vessel wall Hcy is bound to extracellular matrix (ECM) com-ponents [42, 45], and in hyperhomocysteinaemia Hcy is thought to induce adverse changes in turnover and remodelling of ECM [10, 43, 44]. On the other hand, the vascular damage reported in serious hyperhomocys-teinaemia might result from the oxidative metabolism of Hcy to Hcy thiolactone [15, 24], or to other sulfated compounds such as Hcy sulfinic acid and homocysteic acid [28]. It has been suggested that Hcy thiolactone, by increasing free radical oxidants, induces endothelial cell apoptosis in a concentration-dependent manner [25] and makes alterations to mitochondrial function resulting in hyperplasia and fibrosis of smooth muscle cells [24].

Exfoliation syndrome (XFS) is known to be the most common identifiable cause of open-angle glaucoma worldwide [33]. Although XFS manifests clinically as small whitish deposits of fibrillar–granular flecks in the anterior segment of the eye, this ECM material may also be found in extraocular tissues, including skin and the connective tissue portions of various visceral organs, e.g. heart, lung, liver, kidney, gall bladder, and cerebral meninges [34, 40]. Nevertheless, despite its wide preva-lence in the older population [11, 41], the pathogenic mechanism of XFS and its clinical importance remain poorly understood.

XFS and hyperhomocysteinaemia-related vascular dis-eases have some characteristics in common. Exfoliation is significantly associated with a history of coronary disease or arterial hypertension, or a combined history of angina and acute myocardial infarction or stroke [26]. Schu-macher and co-workers observed an association between aneurysms of the abdominal aorta and ocular exfoliation [36]. On the other hand, later, a high incidence of elevated Hcy levels was found in patients who suffered an ab-dominal aortic aneurysm [38, 50].

Regarding the role of vascular pathology in distur-bances of ocular haemodynamics, some studies have indicated lowered orbital blood flow in patients with XFS [31, 52], suggesting probable vascular or flow ab-normalities in the development of exfoliation. Repo et al. described generalized peripheral iris transluminance in XFS, suggesting a possible role of iris hypoxia in the development of XFS [30]. Later, vasculopathy of iris blood vessels leading to obliteration of the vascular lumen

with concurrent iris hypoperfusion was observed in XFS eyes [13]. In a further study, Repo et al. observed in iris specimens of XFS eyes strongly fibrotized muscle tissue devoid of normal morphology with fibrillar material around the blood vessels, as well as vacuolization and significantly decreased amounts of mitochondria [32]. In contrast, a histopathological study on eyes with simulta-neous anterior segment XFS and ischaemic CRVO found no morphologically evident XFS vasculopathy in the central retinal vessels within or immediately behind the lamina cribrosa [8].

The literature contains no clear documentation on the amino acid content of the human aqueous humour. The blood–aqueous barrier (BAB) restricts protein access from the circulation into the aqueous humour, but in ex-perimentally induced anterior segment ischaemia, dis-ruption of BAB could be detected with laser flare pho-tometry [14]. There are divergent opinions on the likeli-hood that XFS enhances the aqueous protein concentra-tion. Some studies have shown that impairment of BAB with increased aqueous protein concentration is a feature of XFS [18, 49], but one report failed to support this [3].

Assuming that Hcy metabolism influences the intra-cellular synthesis of exfoliation material and its transition into ECM, we set out to measure and compare the plasma and aqueous humour concentrations of Hcy in subjects with exfoliation in the anterior segment of the eye. Sub-jects with no sign of XFS served as controls. In addition, erythrocyte folate (E-Fol) and serum vitamins B6 and B12 were determined in both groups.

Materials and methods

Subjects and controls

The study participants were patients attending the University Hospital, Kuopio, and the North Karelia Central Hospital, Joensuu, both in eastern Finland, for routine cataract surgery.

The use of these volunteers followed the tenets of the declara-tion of Helsinki and received approval from the local research ethics committees. All the enrolled subjects signed written consent forms and were told in detail about the purpose of the investigation and the method used to obtain aqueous humour for research pur-poses. The diagnosis of XFS was confirmed in 36 patients prior to the cataract operation, using biomicroscopic examination in my-driasis; these subjects formed the study group. The same number of patients with no sign of exfoliation material in the anterior segment of the eye, and matched by age and gender, were chosen as con-trols. In the XFS group, 11 patients had a diagnosis of XFG. The controls had an IOP below 23 mmHg, and they had no medical history or clinical sign of any other eye disease than cataract. All the participants had the anterior segment of the eye examined by biomicroscope in mydriasis, and the posterior segment with Volk lenses of 60 and 90 dioptres. Otherwise, the exclusion criteria for parameters known to interfere with Hcy metabolism were: medical history of major systemic illness (diabetes, vasculitis, renal and hepatic disease), thromboembolic diseases (including stroke, myo-cardial infarction and peripheral arterial or venous occlusions) during the past half year, evidence of chronic alcohol abuse, anti-convulsant and immunosuppressive therapy, hormone substitution,

cholesterol-lowering agents (statin drugs), antidepressants, recent antimicrobial therapy and intake of vitamin supplements, natural products or fish oil.

In the study population, both the XFS/XFG group and the control group comprised 28 female and 8 male patients. The mean age of the XFS/XFG group was 77.4 years (SD 6.0) and of the controls 77.2 years (SD 5.4).

Variables examined

Blood samples to test for plasma Hcy and E-Fol and serum vitamins B6 and B12 were collected in the fasting state just before the patients were prepared for cataract surgery. The samples were taken to the laboratory immediately, centrifuged within 1 h and stored at −70°C. The aqueous humour samples were taken at the onset of the surgical procedure via corneal paracentesis, and an amount of 100–200 μl (depending on the structural volume) was aspirated from the anterior chamber and injected into a specimen container (Microtainer, UCSD Healthcare, CA, USA). These tubes were immediately frozen and stored at −70°C. Serum vitamin B6 was analysed using a HPLC system and a Chromsystems Vitamin B6 assay (Munich, Germany) (reference range 15–73 nmol/l). The levels of plasma and aqueous Hcy were measured using a fluorescence polarization immunoassay (Abbott IMx Homocysteine Assay, Abbott Laboratories, USA). For this assay the normal values of the plasma Hcy are 5–15 μmol/l according to the manufacturer. In contrast, the literature gives no information on Hcy contents of the aqueous humour for reference value. E-Fol and serum vitamin B12 concentrations were measured using an AutoDelfia Folate Assay and AutoDelfia B12 Vitamin Assay, based on a time-resolved fluoroimmunoassay method (Wallac, Perkin Elmer, Turku, Finland).

Statistics

A matched-pairs study design with cases and controls of the same gender and age was applied. Because the variables were not normally distributed in either of the main groups (tested by Kolmogorov–Smirnov and Shapiro–Wilk methods), the Wilcoxon matched-pairs signed-ranks test was used to perform the analysis in comparisons between the groups with regard to the above-mentioned variables. For the subgroups, the Mann–Whitney U test for continuous variables with a skewed distribution was applied. Associations between parameter concentrations in various environments were calculated with Spearman's rank-order correlation. As testing a lot of variables interferes with some probable correlations among them, we considered a simple Bonferroni correction of the P values. The significance level for comparisons between parameters was set at α=0.05.

Results

Homocysteine

As shown in Table 1, significantly higher plasma Hcy levels were found in patients with XFS/XFG (17.8± 6.7 μmol/l, range 9.6–38.2 μmol/l, median 16.8 μmol/l) compared with the control group (15.8±6.5 μmol/l, range 8.3–35.7 μmol/l, median 13.9 μmol/l) (P=0.020, Wilcoxon matched-pairs signed ranks test, after making a Bonferroni-type correction). In contrast, the Hcy concentrations in the aqueous humour were almost identical, with no statistically significant difference between the two groups (P=0.995, Wilcoxon matched-pairs signed-ranks test). Nor did the plasma Hcy levels show any correlation with the aqueous Hcy concentrations in the groups of exfoliation-positive or -negative subjects (P=0.976 and P=0.731, respectively, Spearman's rank-order correlation). Regarding the XFS and XFG subgroups, the plasma Hcy levels were found to be higher in XFG than non-glaucomatous XFS patients (20.3±8.0 μmol/l, range 12.4–38.2 μmol/l, median 17.7 μmol/l vs 16.7±5.9 μmol/l, range 9.6 – 37.7 μmol/l, median 14.8 μmol/l), though the difference was not statistically significant, at least partly due to the small number of patients (n=11 and n=25, respectively; P=0.112, Mann–Whitney U test).

Erythrocyte folate and serum vitamins B6 and B12

Table 1 shows the measured levels for E-Fol and serum vitamins B6 and B12 in the XFS/XFG and control groups. There were no statistical differences in these three parameters between the two main study groups (respective P values: 0.441, 0.600 and 0.911, Wilcoxon matched-pairs signed-ranks test).

When the XFS and XFG subgroups were compared, the mean serum vitamin B12 concentration was lower in the subjects with XFG (303±158 pmol/l, range 166–747 pmol/l, median 255 pmol/l vs 318±77 pmol/l, range 121–560 pmol/l, median 325 pmol/l). The difference remained slightly below the level of significance (P=0.069, Mann–Whitney U test), again at least partly due to the small number of individuals in this subgroup. Neither were there any statistical differences in E-Fol and serum vitamin B6 between the subgroups.

Table 1 Plasma (*P-Hcy*) and aqueous (*A-Hcy*) homocysteine, erythrocyte folate (*E-Fol*), and serum B6 (*S-B6*) and B12 (*S-B12*) vitamin levels in patients with exfoliation syndrome (including exfoliative glaucomas; *XFS/XFG*) compared to controls (data expressed as mean ± SD)

Patients	Number	P-Hcy (μmol/l)	A-Hcy (μmol/l)	E-Fol (nmol/l)	S-B6 (nmol/l)	S-B12 (pmol/l)
XFS/XFG	36	17.8±6.7*	0.87±0.86[ns]	466±227[ns]	33.3±20.1[ns]	313±106[ns]
Controls	36	15.8±6.5	0.8±0.54	531±318	37.9±28.2	308±95

*P=0.020 significantly different from the controls (Wilcoxon matched-pairs signed-ranks test) adjusted with Bonferroni correction
[ns] Not significantly different from the control group (Wilcoxon matched-pairs signed-ranks test)

Table 2 Plasma (*P-Hcy*) and aqueous (*A-Hcy*) homocysteine levels in male and female patients determined separately for exfoliation-positive and exfoliation-negative subgroups (data expressed as mean ± SD; *ns* not significantly different from the control group, Mann–Whitney *U* test) *XFS/XFG* Patients with exfoliation syndrome and/or exfoliative glaucoma

Patients	Number	P-Hcy (μmol/l)	A-Hcy (μmol/l)
XFS/XFG-positive			
Males	8	19.9±5.8[ns]	0.55±0.77[ns]
Females	28	17.2±6.9	0.97±0.87
XFS/XFG-negative			
Males	8	16.8±8.1[ns]	1.05±0.82[ns]
Females	28	15.6±6.1	0.73±0.43

[ns] Not significantly different from the control group (Wilcoxon matched-pairs signed-ranks test)

The influence of gender on homocysteine level

The influence of gender on plasma and aqueous Hcy was examined within the subgroups of XFS-positive and XFS-negative subjects (Table 2). In neither case was the difference statistically significant, though the mean plasma Hcy levels were slightly higher in males than females. In the XFS- positive group, the mean plasma Hcy concentration was 2.7 μmol/l higher in males than females (median 18.9 μmol/l vs 15.6 μmol/l), but, due at least partly to the small number of individuals in this subgroup, the difference did not reach statistical significance ($P=0.069$, Mann–Whitney *U* test). Nor was there any difference in the aqueous Hcy levels between males and females in the XFS-positive and XFS-negative subgroups ($P=0.094$ and $P=0.987$, respectively, Mann–Whitney *U* test).

Discussion

The results of the present study demonstrate a statistically significant association between the presence of raised plasma Hcy and XFS. However, in terms of aqueous humour levels of Hcy, and measurements of E-Fol and serum vitamins B6 and B12, no statistically significant differences could be found between the index and control patients. The mean aqueous concentrations of Hcy were about 1/20 those in plasma (Table 1), which implies an active functioning of the BAB and a balancing role regarding amino acid access into the intraocular space. According to our results, there was no correlation between the plasma and aqueous concentrations of Hcy, which again suggests an active BAB function.

In vascular pathogenesis, endothelial dysfunction in conduit and resistance vessels may underlie the associations between Hcy and atherosclerosis, and increased oxidative stress contributes a pathophysiological role to the deleterious endothelial effects of Hcy [16, 51]. In addition, Hcy has several harmful effects on haemody-

namics. It enhances thromboxane A2 formation and platelet aggregation [9], impairs the ability of endothelial cells to inhibit platelet aggregation and, by forming S-nitrosothiols, reduces nitric oxide (NO) bioavailability [39] and has procoagulant effects [22]. These facts could help explain the increased risk of ocular thromboembolism, but also suggest how the eye becomes predisposed to slowly developing ischaemic alterations.

In iris vasculopathy among patients with XFS, there is a gradual and persistent degeneration of the cells in the affected vessel walls associated with the production of excess ECM, including exfoliative fibres, and these alterations apparently develop under hypoxic conditions [13, 31]. This hypothesis is supported by an observation that oxygen partial pressure (pO_2) values measured in the anterior chamber in patients with XFS were lower than in those without XFS [13]. In the present study, however, the fact that Hcy was not elevated in the aqueous humour in XFS contradicts its direct relationship with the pathobiology of the abnormal ECM formation process, which involves all aqueous-bathed ocular structures, and implies a relationship only with XFS-associated systemic and ocular vasculopathy.

In the pathogenesis of exfoliation material, there may be an association between Hcy and matrix metalloproteinases (MMPs). Hcy decreases endothelial NO availability by generating nitrotyrosine [39]. In an experimental induction study, a progressive rise in nitrotyrosine levels was shown at 4 and 8 weeks after Hcy administration, and the consequent decrease in NO concentration resulted in increased MMP activity [37]. In another experimental study, a homocysteinic medium increased elastinolytic gelatinase A and B (MMP-2 and -9) in the vessel wall and, in addition, provided more marked labelling for caspase, a cytoplasmic protease involved in mediating some forms of apoptotic degradation [27]. In a recent report by Schlötzer-Schrehardt et al. [35], the aqueous humour of patients with XFS or XFG had significantly higher concentrations of MMP-2 and -3 than control eyes. Simultaneously, TIMP-1 and -2, as representatives of tissue inhibitors of MMPs, were also detected at higher level. In XFG patients, the ratio of MMP-2 to its principal inhibitor TIMP-2 was decreased, resulting in an excess of TIMP-2 over MMP-2. The authors suggest that complex changes in the local MMP–TIMP balance and reduced MMP activity in the aqueous humour may promote the abnormal matrix accumulation characteristic of XFS with evident implication in the pathogenesis of XFG. Both MMPs and TIMPs are widely expressed in the anterior uvea, and their differential localization in the ciliary body in particular suggests they may have a physiologic role in maintaining homeostasis in the uveal tract [19].

As it has been shown that Hcy-induced mitochondrial abnormalities follow the toxic exposure to hydrogen peroxide (H_2O_2) generated during oxidative reactions [2],

it is evident that Hcy and H_2O_2 act synergistically to promote mitochondrial damage. A novel observation is that H_2O_2 increases MMP-2 activity, too, leading to stimulation of Ca^{2+} ATPase during the oxidation process [23]. This finding is parallel with the observation that the aqueous humour of patients with XFS or XFG had significantly higher concentrations of MMP-2 than that in control eyes [35].

In the present study, plasma Hcy concentrations were found to be rather high in both cases and controls, i.e. 17.8 μmol/l (SD 6.7) in patients with XFS and 15.8 μmol/l (SD 6.5) in controls; this is somewhat above the upper reference limit of 15 μmol/l. In a study population of 326 eastern Finnish men aged 42–60 years, the mean plasma Hcy concentration was 11.2 μmol/l (SD 3.1) [48]. However, under normal physiologic conditions, Hcy blood concentrations increase gradually with age and, in addi-

tion, men have plasma Hcy levels more than 20% higher than women [12]. The fact that our study population comprised mainly elderly subjects (median age of cases 78.5 years and controls 78.0 years) would explain the high basic plasma Hcy levels. Furthermore, the study design was not modelled to use any cut-off values in evaluating hyperhomocysteinaemia.

Among previous studies there are a few reports of elevated plasma Hcy associated with XFS, implying that hyperhomocysteinaemia may have an important influence on the pathogenesis of glaucomatous optic neuropathy and may also partly explain the increased risk of vascular diseases among patients with XFS [4, 21, 46]. However, the basic mechanism by which these two clinical findings are interlinked remains unclear, and more detailed investigations are warranted to resolve the underlying associations.

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