Cystatin C levels in plasma and peripheral blood mononuclear cells among hyperhomocysteinaemic subjects: effect of treatment with B-vitamins

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Homocysteine has been related to increased risk of CVD. Matrix degradation and inflammation may be involved in this link between hyperhomocysteinaemia and CVD. Recent studies suggest that cystatin C can modulate matrix degradation and inflammation. The present study measured cystatin C at protein (plasma) and mRNA levels (peripheral blood mononuclear cells (PBMC)) in hyperhomocysteinaemic individuals (n=37, female seven and male thirty, aged 20–70 years) before and after B-vitamin supplementation for 3 months in a randomised, placebo-controlled double-blind trial. In a cross-sectional study, seventeen of the hyperhomocysteinaemic subjects were age- and sex-matched to healthy controls (n=17). Our main findings were: (i) as compared with controls, hyperhomocysteinaemic subjects tended to have higher plasma concentrations of cystatin C and lower mRNA levels of cystatin C in PBMC; (ii) compared with placebo, treatment of hyperhomocysteinaemic individuals with B-vitamins significantly increased plasma levels of cystatin C and mRNA levels of cystatin C in PBMC; (iii) while plasma levels of cystatin C were positively correlated with plasma levels of TNF receptor-1, mRNA levels of cystatin C in PBMC were inversely correlated with this TNF parameter. Taken together, our findings suggest that disturbed cystatin C levels may be a characteristic of hyperhomocysteinaemic individuals, potentially related to low-grade systemic inflammation in hyperhomocysteinaemic subjects, and that B-vitamins may modulate cystatin C levels in these individuals.

Homocysteine: B-vitamins: Cystatin C: Inflammation: Atherosclerosis

Epidemiological studies have established that elevated plasma levels of homocysteine are associated with an increased risk of ischaemic stroke, myocardial infarction and venous thromboembolism(1–3). In addition, animal models of hyperhomocysteinaemia have shown abnormalities of vascular structure and function(4). Paradoxically, however, clinically controlled trials failed to show that lowering homocysteine with vitamin B therapy as secondary prevention reduced risk of CVD or mortality(5–7). In contrast, the recently reported improvement in stroke mortality observed after folic acid fortification in the United States and Canada, but not in England and Wales (where fortification is not mandatory), is consistent with the hypothesis that folic acid fortification helps to reduce deaths from stroke(8). These findings are supported by a recent meta-analysis, showing that folic acid supplementation can effectively reduce the risk of stroke in primary prevention(9). Thus, the homocysteine hypothesis in CVD is not dead(10), and the precise mechanism by which hyperhomocysteinaemia is related to atherogenesis needs to be further elucidated.

Inflammation and matrix degradation play important roles in the pathogenesis of atherosclerosis and plaque destabilisation. Previously, we have shown that hyperhomocysteinaemic subjects are characterised by raised serum levels of inflammatory cytokines and matrix metalloproteinases, potentially reflecting a pathogenic loop between inflammation and matrix degradation in the development of hyperhomocysteinaemia-related CVD(11–15).

The main determinants of elevated plasma concentration of homocysteine are deficiency of vitamins B12, B6 and folate, polymorphism in the methyl tetrohydrofolate reductase gene and impaired renal function. There is an inverse relationship between homocysteine and glomerular filtration rate throughout the whole range of renal function(14,15). Plasma concentration of cystatin C, a low molecular weight protein produced by all nuclear cells, has been considered to be a better marker of glomerular filtration rate than plasma creatinine(14,15). Interestingly, recent reports suggest a pivotal role for cystatin C in plaque stability potentially involving interaction with matrix degradation(16–18). Cystatin C is the most...
abundant endogenous inhibitor of cysteine proteases, in particular of cathepsins S and K. Local deficiency of cystatin C in human atherosclerotic and aneurysmal aortic lesions has been reported, suggesting an imbalance between cystatin C and the cathepsins that would favour matrix degradation\(^{(17–19)}\). Moreover, data from knock-out mice models of cystatin C point to a role of cystatin C as an anti-atherogenic protein, protecting against enhanced elastin degradation\(^{(18)}\).

In contrast to reports on low levels of cystatin C at the cellular levels within the atherosclerotic lesion, conflicting data are reported regarding the association of plasma levels of cystatin C and risk of CVD\(^{(19–26)}\). However, few studies, if any, have compared plasma levels of cystatin C with its accompanying intracellular expression within the same individuals. We hypothesise a role for cystatin C in the pathogenesis of hyperhomocysteinaemia-related CVD, and in the present study we examined cystatin C levels in plasma and in peripheral blood mononuclear cells (PBMC) from the same hyperhomocysteinaemic individuals as well as in normohomocysteinaemic control subjects. We also examined the ability of B-vitamins to modulate these parameters in hyperhomocysteinaemia.

**Subjects and methods**

**Subjects**

Thirty-seven adults of 20–70 years of age with hyperhomocysteinaemia (fasting plasma total homocysteine concentration >15 µmol/l at screening) were recruited at the Lipid Clinic and the Department of Medical Biochemistry, Oslo University Hospital, Rikshospitalet and at Department of Clinical Chemistry, Oslo University Hospital, Ullevål, Oslo, Norway. In the cross-sectional study, seventeen of the hyperhomocysteinaemic subjects were sex and age matched to healthy control subjects (n 17), who were health care workers with no history of hypertension, diabetes, CVD or other acute or chronic illness, consecutively recruited in the same period and from the same area of Norway (eastern part). The study was conducted according to the Declaration of Helsinki, and all procedures involving the subjects were approved by the Regional Committee of Medical Ethics and by the Norwegian Medicines Control Authorities. Written informed consent was obtained from all subjects.

**Vitamins B\(_12\), B\(_6\) and folic acid (TrioBe\(^{®}\)) therapy in hyperhomocysteinaemic subjects**

Study design and inclusion/exclusion criteria have been published previously\(^{(27)}\). Thirty-eight hyperhomocysteinaemic subjects completed the study\(^{(27)}\). In the present study, serum and plasma samples were available from all but one participant in the TrioBe\(^{®}\) group (n 37; n 18 and n 19 in the placebo and B-vitamin groups, respectively), and PBMC were available from twenty-nine participants (fourteen in the placebo group and fifteen in B-vitamin group). The hyperhomocysteinaemic subjects were randomised to receive either TrioBe\(^{®}\) (cyanocobalamin 0·5 mg, pyridoxine hydrochloride 3·0 mg and folic acid 0·8 mg; 1 tablet/d; Recip AB, Arsta, Sweden) or an identical-appearing placebo tablet (1 tablet/d; Recip AB) for 3 months in a double-blind fashion. Reduced renal function according to plasma creatinine concentration was an exclusion criterion. Compliance as judged by pill count of returned, unused pills was 91 (sd 7) % and 87 (sd 6) % (P=0·11) in the TrioBe\(^{®}\) and placebo groups, respectively.

**Blood sampling protocol**

Venous blood samples were collected after an overnight fast and without medication ingestion in the morning of sampling. Plasma and serum were processed and stored at −80°C. All analyses, except the routine laboratory assays, were performed after the last patients had completed the treatment period. To avoid run-to-run variability, serial samples from a given subject were analysed at the same time.

**Cell isolation**

PBMC were obtained from heparinised blood by gradient centrifugation in Isopaque–Ficoll (Lymphoprep, Nycomed, Oslo, Norway). PBMC pellets for RNA analysis were immediately frozen and stored at −80°C.

**Quantitative real-time RT-PCR**

Total RNA was isolated from PBMC pellets as described previously\(^{(29)}\). To detect gene expression of cystatin C, 0·1 µg total RNA from each sample was reverse transcribed by TaqMan high-capacity reverse transcription reagent kit (Applied Biosystems, Foster City, CA, USA). For quantitative real-time RT-PCR amplification, sequence specific PCR primers for cystatin C were designed using the Primer Express software version 1·5 (Applied Biosystems): forward primer: 5’-AGACCCAGCCCA-ACTTGGA-3’, reverse primer: 5’-AGCAGAATGCTTTCC-TTTTCAGA-3’. The β-actin was used as a housekeeping gene for normalisation (Applied Biosystems).

**Enzyme immunoassays**

Plasma concentrations of cystatin C and TNF receptor (TNFR)-1 were quantified by enzyme immunoassays from R&D Systems (Minneapolis, MN, USA). According to the manufacturer, cystatin C concentration in EDTA plasma from thirty-six individuals ranged from 560 to 1173 ng/ml, mean 774 (sd 155) ng/ml.

**Routine laboratory assays**

Concentrations of homocysteine were measured on the Abbott IMx analyzer (Abbott Laboratories, Abbott Park, IL, USA); serum folate and serum vitamin B\(_12\) on the Wallac AutoDelfia analyzer (Wallac Oy, Turku, Finland); total cholesterol, LDL cholesterol, HDL cholesterol, TAG, creatinine and C-reactive protein were measured using the Modular-P platform (Roche Diagnostics, F. Hoffmann-La Roche Ltd, Basel, Switzerland); and fibrinogen by STA-R Evolution (Diagnostica Stago, Asnieres, France).

**Statistical analysis**

Data are given as means and standard deviations or median (minimum–maximum) if not otherwise stated. Data from patients and controls and differences in changes between
Cystatin C in hyperhomocysteinaemia

Results

Cystatin C in hyperhomocysteinaemic subjects and age- and sex-matched healthy control subjects – cross-sectional testing

Characteristics of the participants in the cross-sectional study are given in Table 1. The expected differences in homocysteine and folate levels were observed. Although controls showed significantly higher creatinine levels compared with homocysteine subjects, all creatinine values were within the normal range (Table 1). While there was a tendency towards higher plasma concentration of cystatin C in hyperhomocysteinaemic patients as compared with controls (Table 1; Fig. 1(a); P<0·05), an opposite pattern was seen in PBMC with a tendency towards lower mRNA levels of cystatin C in cells from those with hyperhomocysteinaemia (Table 1; Fig. 1(b); P=0·060).

Effect of B-vitamin therapy on cystatin C levels in hyperhomocysteinaemia

Next, we conducted a randomised, placebo-controlled double-blind trial with B-vitamin therapy for 3 months in the hyperhomocysteinaemic subjects (n 37). There were no significant differences between the TrioBe® treatment group (n 19) and the placebo group (n 18) at baseline (Table 2). Whereas 3 months of TrioBe® treatment increased serum levels of folate from 7·5 (SD 2·9) nmol/l to 37·8 (SD 14·9) nmol/l, and of vitamin B12 from 227 (SD 81) pmol/l to 425 (SD 177) pmol/l, P<0·001, the plasma concentration of homocysteine was reduced from 19 (13–65) μmol/l to 9 (6–20) μmol/l, n 19; P<0·001. In contrast, no significant differences in these parameters occurred within the placebo group (data not shown).

Table 1

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Hyperhomocysteinaemic subjects (n 17)</th>
<th>Control subjects (n 17)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>Mean 45·2 Median 12·7 sd 1·2 Min–max 16–69</td>
<td>Mean 43·8 Median 10·0 sd 1·0 Min–max 7–14</td>
</tr>
<tr>
<td>Male (n)</td>
<td>13</td>
<td>13</td>
</tr>
<tr>
<td>CVD (n)</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Statin treatment (n)</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>Current smokers (n)</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
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<td>23·9</td>
</tr>
<tr>
<td>Homocysteine (μmol/l)</td>
<td>8·7*</td>
<td>13·2</td>
</tr>
<tr>
<td>Folate (nmol/l)</td>
<td>250</td>
<td>276</td>
</tr>
<tr>
<td>Vitamin B12 (pmol/l)</td>
<td>4·9</td>
<td>5·3</td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>3·1</td>
<td>3·6</td>
</tr>
<tr>
<td>LDL cholesterol (mmol/l)</td>
<td>1·4</td>
<td>1·5</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/l)</td>
<td>1·0</td>
<td>0·4</td>
</tr>
<tr>
<td>TAG (mmol/l)</td>
<td>77†</td>
<td>88</td>
</tr>
<tr>
<td>Creatinine (μmol/l)</td>
<td>1·0</td>
<td>1·0</td>
</tr>
<tr>
<td>C-reactive protein (mg/l)</td>
<td>0·4–2·7</td>
<td>0·2–4·0</td>
</tr>
<tr>
<td>Cystatin C (ng/ml)</td>
<td>1054†</td>
<td>893</td>
</tr>
<tr>
<td>mRNA cystatin C:β-actin</td>
<td>0·77§</td>
<td>0·89</td>
</tr>
</tbody>
</table>

min, Minimum; max, maximum.

For mRNA data, n 14 in both groups.

* P=0·05 v. control subjects.
† P=0·01 v. control subjects.
‡ P=0·05 v. control subjects.
§ P=0·06 v. control subjects.

There were no significant differences in plasma concentrations of cystatin C between the two treatment groups at baseline (P=0·331; Table 2). While no significant changes in cystatin C were observed within the placebo group (P=0·528), TrioBe® treatment for 3 months significantly increased plasma concentration of cystatin C (840 (SD 253) v. 969 (SD 300) ng/ml, P=0·010) without any changes in creatinine levels (data not shown), resulting in a significant difference in changes between the two treatment groups (P=0·013; Fig. 2(a)).

At baseline, there were no significant differences in mRNA levels of cystatin C in PBMC between the two treatment groups (P=0·275; Table 2; n 14 and n 15 in the placebo and TrioBe® groups, respectively). While no significant changes occurred in the placebo group (P=0·433), TrioBe® treatment was accompanied by a significant increase in mRNA levels of cystatin C in PBMC (0·77 (SD 0·19) v. 0·84 (SD 0·20), P=0·041), resulting in a significant difference in changes between the two treatment groups (P=0·029; Fig. 2(b)).

Correlations between cystatin C and clinical and inflammatory parameters in hyperhomocysteinaemic subjects in the TrioBe® study – baseline data

Plasma concentrations of cystatin C were significantly correlated to creatinine levels (n 37, r 0·729; P<0·001) and BMI
Dysregulated cystatin C metabolism in hyperhomocysteinaemia, characterised by elevated plasma levels of cystatin C and low levels of cystatin C mRNA in PBMC. In both compartments, cystatin C levels seem to be related to increased activity in the TNF system with a positive correlation to plasma and a negative correlation to intracellular cystatin C levels. Moreover, our findings also suggest a role for B vitamins in the modulation of cystatin C levels in hyperhomocysteinaemic individuals.

Whereas normal arteries express abundant cystatin C, human atherosclerotic lesions have relatively low levels of cystatin C (19). Thus, data from knock-out mice models of cystatin C point to a role of cystatin C as an anti-atherogenic protein, protecting against enhanced elastin degradation (18).

In contrast, most epidemiological studies support a relationship between high circulating levels of cystatin C and CVD (21–26). In the present study, we found that the hyperhomocysteinaemic patients tended to have lower mRNA levels of cystatin C in PBMC and higher plasma levels of cystatin C as compared with matched healthy control subjects. This pattern may suggest different origin of cystatin C levels in plasma and in mononuclear leukocytes. Since cystatin C is synthesised by all nucleated cells, plasma levels of cystatin C were positively correlated with plasma levels of TNFR-1, mRNA levels of cystatin C in PMBC were inversely correlated with this marker of activity in the TNF system. Taken together, these findings may suggest a dysregulated cystatin C metabolism in hyperhomocysteinaemia, characterised by elevated plasma levels of cystatin C and low levels of cystatin C mRNA in PBMC. In both compartments, cystatin C levels seem to be related to increased activity in the TNF system with a positive correlation to plasma and a negative correlation to intracellular cystatin C levels. Moreover, our findings also suggest a role for B vitamins in the modulation of cystatin C levels in hyperhomocysteinaemic individuals.

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atherosclerosis through impaired inhibition of matrix degradation. Our findings that plasma levels but not cellular mRNA levels are positively correlated to creatinine may be in line with this notion. Furthermore, consistent with this hypothesis, leukocyte-specific expression of cystatin C in apoE-knock-out mice was actively involved in matrix remodelling associated with plaque regression (29). Thus, although further studies are needed, it is not inconceivable that the decreased expression of cystatin C in PBMC from hyperhomocysteinaemic subjects, at least partly, could contribute to the increased risk of CVD in these individuals. Moreover, the ability of TrioBe® to increase cystatin C mRNA levels in PBMC may suggest a beneficial effect of B-vitamins in hyperhomocysteinaemia. It is well documented that B-vitamin therapy to lower plasma homocysteine significantly reduces cardiovascular risk in patients with homocystinuria(30).

Inflammation is suggested to be a pathophysiological link between cystatin C and CVD(26). Thus, inflammatory cytokines such as TNFα have been found to reduce cystatin C expression in vascular endothelial cells(16). Furthermore, associations between plasma markers of inflammation and plasma levels of cystatin C were observed among subjects with and without coronary artery disease(31–33). In fact, large epidemiological studies have documented a significant association between plasma cystatin C and mildly increased C-reactive protein levels, the hallmark of the chronic inflammatory state associated with atherosclerosis(26), and a similar pattern was also seen in the present study. Moreover, while we found that plasma levels of TNFRI were positively correlated with circulating cystatin C levels, this reliable marker of TNF activity was inversely correlated with cystatin C mRNA levels in PBMC. Previously, we have shown that hyperhomocysteinaemic subjects are characterised by enhanced inflammatory response(11–13). Our findings in the present study may suggest that disturbed cystatin C levels in these individuals, at least in part, may reflect a response to systemic and local inflammation with different effect on extracellular v. intracellular cystatin C level.

In the Vitamins to Prevent Stroke Study, B-vitamins had no significant effect on serum cystatin C levels among stroke patients with homocysteine levels mostly within the normal range(34). In our patients with elevated plasma homocysteine levels, the lowering of homocysteine levels during B-vitamin therapy was accompanied by enhanced plasma levels of cystatin C. The reason for this apparently conflicting data is not clear, but as cystatin C is produced by all nucleated cells(19,26), it is possible that B-vitamin supplementation in hyperhomocysteinaemic individuals could induce a more global increase in cystatin C levels, which also influences plasma cystatin C concentrations. In fact, it is tempting to speculate that while an increase in cystatin C levels secondary to impaired renal function is maladaptive, an increase in plasma levels of cystatin C during therapy, which is not related to impairment of renal function as in the present study, may be beneficial, reflecting an increase in the anti-protease capacity.

The present study has the limitation that relatively few patients were included, and the role of cystatin C in the hyperhomocysteinaemia-related CVD should be further investigated in larger study populations. Such studies should also try to
relate cystatin C levels to clinical manifestations of CVD in these individuals. Strengths of the present study, however, were that plasma levels and mRNA levels of cystatin C in PBMC were measured in the same individuals, the effect of homocysteine-lowering therapy was evaluated in subjects with hyperhomocysteinemia, and that impaired renal function as judged by creatinine was an exclusion criterion.

Our findings suggest that disturbed cystatin C levels may be a characteristic of hyperhomocysteinemic individuals, potentially related to low-grade systemic inflammation in these individuals. Further studies are needed to examine whether cystatin C could contribute to the increased risk of CVD that are observed in hyperhomocysteinemia. Our findings also suggest a role for B-vitamins in the modulation of cystatin C levels in hyperhomocysteinemic subjects.

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