Familial hyperhomocysteinaemia and endothelium-dependent vasodilatation and arterial distensibility of large arteries

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Received 23 June 1998; accepted 2 November 1998

Abstract

Objectives: Mild hyperhomocysteinaemia, fasting as well as after a methionine load, occurs in families and is associated with premature atherosclerosis. We hypothesised that endothelial dysfunction plays a role in the relation between hyperhomocysteinaemia and clinical vascular disease. Methods: In this study flow-mediated, endothelium-dependent vasodilatation of the brachial artery and, as a marker of biophysical changes of the vessel wall such as increased smooth muscle cell tone or collagen formation, arterial distensibility of the common carotid artery were investigated in 123 healthy first-degree relatives of patients with mild hyperhomocysteinaemia and coronary, cerebral or peripheral artery disease. Results: In multiple linear regression analyses, the increase in the homocysteine concentration after a standard methionine load was a significant determinant of an impaired flow-mediated vasodilatation of the brachial artery (measured on a separate day). The only other predictors were the baseline vessel diameter and age. Fasting homocysteine level was not associated with flow-mediated vasodilatation in the brachial artery. There was no relationship between homocysteine levels and nitroglycerine-induced, endothelium-independent vasodilatation of the brachial artery. Arterial distensibility of the carotid artery was also not related to homocysteine levels. Conclusions: In healthy first-degree relatives of patients with mild hyperhomocysteinaemia, the increase in homocysteine level after a methionine load is an independent predictor of endothelial dysfunction. The results also suggest that fasting and post-methionine homocysteine levels may reflect distinct disturbances in methionine metabolism, which may be linked to vascular dysfunction through distinct mechanisms.

Keywords: Elasticity; Endothelial function; Nitric oxide; Homocysteine; Vasodilation

See Editorial of this article by Doshi et al. (pages 578–582) in this issue.

1. Introduction

Hyperhomocysteinaemia is an independent risk factor for premature atherosclerotic disease. This was first noted in children with homocystinuria who have homozygous cystathionine \( \beta \)-synthase deficiency, the enzyme required for the conversion of homocysteine to cystathionine [1]. Subsequently, it became clear that a moderate increase in homocysteine concentrations, such as might arise from heterozygosity for cystathionine \( \beta \)-synthase or from a thermolabile variant of methylenetetrahydrofolate reductase, is also associated with an increased risk of premature atherosclerosis [2].

In vitro studies suggest that the adverse vascular effects of hyperhomocysteinaemia are mediated, at least in part, by endothelial dysfunction [3,4], and specifically by the inability of the endothelium to produce adequate amounts of the endothelium-derived relaxing factor, nitric oxide...
(NO) [5]. Small in vivo studies in patients with premature peripheral arterial occlusive disease and hyperhomocysteinaemia [6], and in children with severe hyperhomocysteinaemia [7] have provided some evidence of endothelial dysfunction in subjects with hyperhomocysteinaemia. Recently, a disturbed endothelial-dependent vasodilatation has been observed in elderly subjects with hyperhomocysteinaemia, 40% of whom used antihypertensive medication [8]. Apart from the effects on endothelial function it also has been suggested that hyperhomocysteinaemia contributes to medial fibrosis with an increased formation of collagen and frayed elastic fibres [9], and promotes vascular smooth muscle cell proliferation [10].

It is not known, however, whether familial hyperhomocysteinaemia is associated with vascular dysfunction in the absence of clinical atherosclerotic disease. We therefore studied this issue in a large group of apparently healthy first-degree relatives of patients with mild hyperhomocysteinaemia and premature atherosclerotic disease. As estimates of vascular function we investigated the flow-mediated vasodilatation in the brachial artery (a measure of endothelium-dependent vasodilatation) and the distensibility of the common carotid artery (a measure of the intrinsic elasticity of the vascular wall).

2. Methods

2.1. Subjects

We recruited 139 healthy first-degree relatives (siblings; >90% of those eligible according to the inclusion criteria) of 60 patients with clinical coronary, peripheral or cerebral artery disease before the age of 55 years and hyperhomocysteinaemia (as defined by elevated homocysteine levels after methionine loading [11,12]). All fulfilled the following criteria: between 18 and 65 years of age, no pregnancy, serum creatinine <150 µmol/l, normal transaminase levels, and no history of venous thrombosis, myocardial infarction, stroke, or peripheral vascular disease. Sixteen subjects were subsequently excluded because they used medication with a known haemodynamic action (antihypertensive medication in twelve, cholesterol-lowering medication in two, insulin in one and ergotamine in one). None of the other subjects took any medication known to affect haemodynamic function or homocysteine levels. All subjects gave informed consent and the protocol was approved by the local ethics committee. The investigation conformed with the principles outlined in the Declaration of Helsinki [Cardiovase Res 1997;35:2–3].

Sixteen women were classified as post-menopausal (absence of menstrual bleeds for more than 1 year). Subjects were classified as smokers in case of current cigarette use (47 of the 123 participants). All subjects came fasting and refrained from smoking from 24.00 h the day before the laboratory investigations. Blood samples were taken for measurement of glucose, lipids, homocysteine, folate, pyridoxal-5-phosphate and vitamin B<sub>12</sub>. Six hours after an oral methionine load (0.1 g methionine per kg body weight), blood sampling for measurement of homocysteine levels was repeated.

2.2. Haemodynamic measurements

Haemodynamic studies were not performed on the same day as the blood sampling, so an acute increase in homocysteine concentration after methionine loading could not have influenced the vascular responses [13]. The arterial distensibility of the common carotid artery and the endothelium-dependent and -independent vasodilatation of the brachial artery were determined by a non-invasive echo-doppler method. All subjects had fasted and had refrained from smoking for more than 4 h. All measurements were performed by one observer, in a temperature-controlled room, with a vessel wall movement detector system (Wall Track System, Neurodata, Bilthoven, Netherlands) as described earlier [14]. This system consists of an ultrasound imager (Ultramark IV, ATL, Bothell, USA) connected to a data acquisition and processing unit.

Endothelium-dependent and -independent vasodilatation in the brachial artery of the right arm was determined as described in detail elsewhere [15]. In short, end diastolic diameter (D) measurements as described below for the common carotid artery were performed in the brachial artery and combined with determination of the peak systolic velocity (PSV). PSV was measured with doppler in the centre of the brachial artery at a 60° angle to the vessel. We used an acute increase in blood flow, which results in an increase in shear stress, as the stimulus for endothelium-dependent vasodilatation, and nitroglycerin (NTG) sublingually to obtain endothelium-independent vasodilatation, according to a recently described procedure [7,16]. After 15 min of rest in the supine position, baseline diameter and PSV were determined. This was followed by inflation of a blood pressure tourniquet around the forearm to 100 mmHg above the systolic blood pressure. After 4 min the cuff was released and the maximum PSV in the first 10 s was measured, followed by diameter recordings after 45–60 s. After another 15 min rest to allow the brachial artery diameter to return to baseline, PSV and diameter were determined before and 5 min after the administration of 400 µg NTG sublingually. The time points of measurement of the arterial diameter during both procedures have been validated and the coefficients of variation of baseline D, FMD and of the NTG-induced vasodilatation were 4.6, 5.5 and 7.7%, respectively [15]. Flow-mediated (endothelium-dependent) vasodilatation and NTG-induced vasodilatation were expressed as a percentage change in diameter relative to the baseline diameter. The change in PSV was expressed as a percentage of the baseline PSV. As the stimulus for the change
in diameter during reactive hyperaemia, changes in PSV are reported instead of changes in blood flow (which could have been calculated from \(D\) and PSV), because PSV is not independent of \(D\) [17] and because calculating flow from \(D\) and PSV (which is recorded in the centre of the vessel) overestimates the true flow [15,16]. We used the cutoff points proposed by the European Concerted Action Project (i.e. the upper quintile in the 800 control subjects in that study) to categorise the subjects as having normal or elevated fasting, post-methionine and \(\delta\) homocysteine levels [18]. To relate the flow-mediated dilatation (FMD) obtained in the present study to results in healthy persons without a family history of hyperhomocysteinaemia, we compared FMD in the 18 subjects in this study who had a normal increase in homocysteine level after methionine loading (<27 \(\mu\)mol/l, see above) with the FMD in a group of 18 healthy volunteers with similar age and sex distribution and without a family history of premature atherosclerotic disease or hyperhomocysteinaemia.

From M-mode, baseline vessel diameter can be determined with an accuracy of 0.1–0.2 mm and vessel wall displacement with a precision of 10 \(\mu\)m [19]. Vascular distension was measured 10 mm proximal of the right carotid artery bifurcation during a period of 5 s. Blood pressure, pulse pressure and heart rate were recorded on the left arm with an automatic device (BP-8800, Colin, Hayashi Komaki City, Japan). From end-diastolic diameter \((D)\), vascular distension during the heart cycle \((\delta D)\) and pulse pressure \((\delta P)\), distensibility \((DC)\) and compliance coefficients \((CC)\) were calculated as follows: \(DC = 2 \cdot \delta D/ D \cdot \delta P\), \(CC = \pi \cdot \delta D \cdot D^2/2 \cdot \delta P\).

Multiple definitions of arterial biophysical properties have been proposed [20]. The DC and the CC as defined above are equivalent to fractional change and change of cross-sectional area, respectively. DC is the inverse of Peterson’s elastic modulus, which is suitable for arterial stiffness measurements in vivo [21]. We found earlier that the within subjects coefficients of variation for \(D\), DC and CC were 3.1, 7.7 and 8.3\%, respectively [22], which is in accordance with the results of others [23].

We have earlier reported data on subclinical peripheral, carotid and coronary artery disease in the study subjects. An ankle-brachial pressure index (ABPI) according to a standard procedure was categorised as abnormal if <0.9 in one or both legs at rest. A treadmill exercise test was categorised as abnormal if the pressure dropped by 30 mmHg or more [12]. Persons with abnormal coronary or carotid artery disease were excluded because of the low number of cases [12].

### 2.3. Laboratory procedures

Plasma homocysteine levels were measured as total homocysteine (free plus protein bound) by using HPLC with fluorescence detection [24]. The increase in homocysteine level after methionine loading (delta \([\delta]\) homo-

cysteine) was defined as the post-load minus the fasting homocysteine level. Serum pyridoxal-5-phosphate was determined by HPLC with fluorescence detection. Serum vitamin B\(_12\) and folate were determined by radioassay (Becton Dickinson, France), serum glucose by a glucose oxidase method. Serum cholesterol and triglycerides concentrations were measured enzymatically (CHOD-PAP and CPO-PAP methods, Boehringer Mannheim, Germany). HDL-cholesterol levels were determined after precipitation of the VLDL and LDL with sodium phosphotungstate/magnesium. LDL-cholesterol levels were calculated using the Friedewald formula.

### 2.4. Statistics

Results are given as mean (median in case of a skewed distribution)±S.D. and range as indicated. Univariate and multivariate (forward and backward) stepwise regression analyses were used to determine predictors of flow-mediated and NTG-induced vasodilatation, and of the distensibility parameters. In these regression analyses, fasting, post-methionine and \(\delta\) homocysteine levels were entered in separate models both as continuous variables and as dichotomous variables according to the European Concerted Action Project cutoff values [18]. The presence or absence of an abnormal (one or more) peripheral artery test [12] was also entered as an independent variable in the models. The residuals were checked for a normal distribution and constancy of variance. Because baseline brachial artery diameter is an important determinant of the vascular responses [15,17], we adjusted for baseline \(D\) by taking the diameters after NTG and flow-mediated vasodilatation as the dependent variables, and the baseline diameter as one of the independent variables. We chose this procedure because relating the percentage vasodilatation to the baseline diameter predictably yields a negative relationship [25]. For the DC and CC, we adjusted for the mean arterial pressure (MAP) by taking \(\delta D/D\) and \(\delta D\cdot D\), respectively, as the dependent variable, and \(\delta P\) and MAP (together and separately) as independent variables. This was done because MAP is generally inversely related to DC and CC, but is not independent of the distending pressure \((\delta P)\), which is part of the formula of DC and CC. We used \(t\)-tests and Mann–Whitney tests, as appropriate, to compare the 18 subjects with a normal increase in homocysteine levels after methionine loading with control subjects without a family history of premature vascular disease or hyperhomocysteinaemia, and to compare subjects with normal to those with high fasting, post-methionine and \(\delta\) homocysteine levels according to the European Concerted Action Project cutoff points. All testing was two-sided with statistical significance accepted at \(P<0.05\). The statistical tests were performed with the spss, version 7.0 for Windows, statistical software package (SPSS, Chicago, IL, USA).
3. Results

Clinical and laboratory data of the study population are shown in Table 1. Thirty-four, two and one subjects, respectively, had pyridoxal-5-phosphate, vitamin B and folic acid levels below the lower limit of our reference ranges (<17 nmol/l, <120 pmol/l and <3.4 nmol/l, respectively). Correlations between homocysteine levels and demographic and laboratory data are shown in Table 2.

3.1. Endothelium-dependent and -independent vasodilatation

Mean FMD was 8.3 (74.4)% and mean NTG-induced dilation 14.4 (9.3)%. In one subject FMD was not obtained because of technical difficulties.

The upper quintiles of the fasting, post-methionine and δ homocysteine levels in the control subjects of the European Concerted Action Project were 12, 38 and 27 μmol/l, respectively [18]. When we used these cutoff values FMD was lower in subjects with elevated post-methionine (P<0.001) and δ homocysteine (P=0.097), but not in those with elevated fasting homocysteine (Fig. 1). NTG-induced vasodilatation did not differ between these groups (fasting homocysteine ≥12 μmol/l: 14.5 (9.2)% versus <12 μmol/l: 14.2 (9.5)%, P=0.84; δ homocysteine ≥27 μmol/l: 14.8 (9.5)% versus <27 μmol/l: 12.1 (8.0)%, P=0.26 and post-methionine homocysteine ≥38 μmol/l: 14.6 (9.5)% versus <38 μmol/l: 13.2 (8.3)%, P=0.54).

The baseline diameter, which was not different before the FMD and the NTG-induced vasodilatation [3.24 (0.61) and 3.24 (0.63) mm, respectively, P=0.99], was the most important predictor of the vascular responses in the brachial artery. Multiple linear regression analyses with the diameter after reactive hyperaemia as the dependent variable showed, however, that age and the increase in homocysteine level after methionine loading (δ homocysteine) were also independently related to the flow-

Table 1
Demographic and laboratory data of the study population

<table>
<thead>
<tr>
<th>Parameter</th>
<th>n/ Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Men/women (n)</td>
<td>68/55</td>
</tr>
<tr>
<td>Age (years)</td>
<td>45.0 (7.6; 24–62)</td>
</tr>
<tr>
<td>Body mass-index (kg/m²)</td>
<td>25.4 (3.7; 18.6–42.5)</td>
</tr>
<tr>
<td>Smokers yes/no (n)</td>
<td>47/76</td>
</tr>
<tr>
<td>Packyears (n²)</td>
<td>10.0 (13.3; 0–70)</td>
</tr>
<tr>
<td>Mean arterial pressure (mmHg)</td>
<td>92.6 (11.3; 70–139)</td>
</tr>
<tr>
<td>Abnormal peripheral vascular test yes/no (n)</td>
<td>42/81</td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>5.9 (1.2; 3.1–9.2)</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/l)</td>
<td>1.2 (0.4; 0.6–2.4)</td>
</tr>
<tr>
<td>LDL cholesterol (mmol/l)</td>
<td>4.1 (1.1; 1.5–7.0)</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>1.3 (0.9; 0.4–4.5)</td>
</tr>
<tr>
<td>Pyridoxal-5-phosphate (nmol/l)</td>
<td>22.5 (27.5; 4–191)</td>
</tr>
<tr>
<td>Folate (nmol/l)</td>
<td>272 (105; 102–626)</td>
</tr>
<tr>
<td>Vitamin B₁₂ (pmol/l)</td>
<td>11.7 (4.7; 3.1–28.7)</td>
</tr>
<tr>
<td>Homocysteine (μmol/l)</td>
<td>12.7 (9.3; 4.9–64.5)</td>
</tr>
<tr>
<td>Increase after methionine (δ)</td>
<td>44.5 (20.5; 4.0–116.6)</td>
</tr>
</tbody>
</table>

Values are mean (S.D.; range).

Table 2
Correlations of homocysteine levels and demographic and laboratory data

<table>
<thead>
<tr>
<th>Parameter</th>
<th>r</th>
<th>P</th>
<th>r</th>
<th>P</th>
<th>r</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male gender</td>
<td>+0.36</td>
<td>&lt;0.001</td>
<td>-0.06</td>
<td>0.49</td>
<td>-0.20</td>
<td>0.03</td>
</tr>
<tr>
<td>Age (years)</td>
<td>-0.25</td>
<td>0.006</td>
<td>-0.07</td>
<td>0.43</td>
<td>+0.04</td>
<td>0.70</td>
</tr>
<tr>
<td>Packyears (n)</td>
<td>+0.10</td>
<td>0.28</td>
<td>+0.09</td>
<td>0.92</td>
<td>-0.08</td>
<td>0.42</td>
</tr>
<tr>
<td>Cholesterol (mmol/l)</td>
<td>-0.26</td>
<td>0.007</td>
<td>+0.03</td>
<td>0.78</td>
<td>+0.12</td>
<td>0.22</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>-0.01</td>
<td>0.88</td>
<td>+0.06</td>
<td>0.50</td>
<td>+0.08</td>
<td>0.37</td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td>+0.001</td>
<td>0.99</td>
<td>-0.15</td>
<td>0.09</td>
<td>-0.13</td>
<td>0.17</td>
</tr>
<tr>
<td>Folate (nmol/l)</td>
<td>-0.36</td>
<td>&lt;0.001</td>
<td>-0.28</td>
<td>0.008</td>
<td>-0.13</td>
<td>0.18</td>
</tr>
<tr>
<td>P5-P (pmol/l)</td>
<td>-0.06</td>
<td>0.53</td>
<td>-0.21</td>
<td>0.03</td>
<td>-0.17</td>
<td>0.07</td>
</tr>
<tr>
<td>Vitamin B₁₂ (pmol/l)</td>
<td>-0.38</td>
<td>&lt;0.001</td>
<td>-0.10</td>
<td>0.28</td>
<td>+0.02</td>
<td>0.83</td>
</tr>
</tbody>
</table>

r, Spearman’s correlation coefficient. Hcy, homocysteine, δ, post-methionine minus fasting, BMI: body mass-index, MAP: mean arterial pressure, P5-P: pyridoxal 5-phosphate.
mediated, endothelium-dependent vasodilatation (standardised regression coefficients (stand r) for baseline diameter, δ homocysteine level and age, 0.94 (P<0.0001), −0.09 (P=0.08) and −0.07 (P=0.047), respectively). δ Homocysteine was not related to the NTG-induced vasodilatation. The determinants of the diameter after NTG were baseline diameter (stand r: 0.91, P<0.0001), MAP (stand r: 0.10, P=0.008) and age (stand r: −0.09, P=0.02). MAP was not related to FMD. Total cholesterol level (or levels of HDL-cholesterol, LDL-cholesterol and triglycerides), gender, current smoking (or pack year), body mass-index (BMI) and the presence or absence of one or more abnormal peripheral artery tests were not related to FMD or to NTG-induced vasodilatation. No interactions between variables included in the model were observed. The results for FMD are summarized in Table 3. Use of the European Concerted Action Project cutoff value for δ homocysteine in the regression models did not change the results (data not shown).

When post-methionine homocysteine levels were substituted for δ homocysteine levels, there was a trend for the former to similarly and inversely predict FMD (post-load homocysteine level, stand r: −0.06, P=0.07, baseline diameter, stand r: 0.94, P<0.0001, age, stand r: −0.07, P=0.06 in the same model as described above). Use of the European Concerted Action Project cutoff value for post-load homocysteine showed that increased post-load homocysteine was an independent predictor of FMD apart from baseline diameter (baseline diameter stand r: 0.95, P<0.001, increased post-load homocysteine stand r: −0.11, P=0.001) Fasting homocysteine levels (as continuous or dichotomous variable) were not related to FMD. There was no relation between the baseline PSV (or the change in PSV) and the homocysteine levels, whether fasting or after methionine loading.

When the subjects were divided according to the presence or absence of one or more abnormal peripheral artery tests there was no difference between the two groups for FMD or NTG-induced vasodilatation (FMD: abnormal test

7.4 (6.9%) versus normal test 9.3 (7.4%), P=0.19; NTG-induced vasodilatation 13.2 (7.4%) and 15.2 (10.2%), respectively, P=0.25).

When the group of women was analysed separately, postmenopausal status, in addition to the baseline diameter and the δ homocysteine, was a determinant of the FMD (stand r: −0.19, P=0.02; stand r: 0.79, P<0.0001 and stand r: −0.19, P=0.01, respectively), but not of the NTG-induced vasodilatation. No interaction between postmenopausal status and δ homocysteine was observed. Mean δ homocysteine in the post-menopausal women was 57.3 (16.6) versus 45.4 (20.7) μmol/l in the premenopausal women (P=0.03).

Fig. 2 shows the univariate relation between fasting homocysteine levels and FMD. Figs. 3 and 4 show the univariate relation between the δ homocysteine and FMD. Determinants of the brachial artery diameter after flow-mediated vasodilatation in multiple stepwise regression analysis were performed with δD/D as dependent variable and δD; MAP, age, gender, δ homocysteine (or fasting, or post-methionine homocysteine levels as continuous as well

3.2. Arterial distensibility

D, DC and CC at the carotid artery were 6.47 (0.70) mm, 18.2 (5.1)·10⁻³/kPa and 0.60 (0.18) mm²/kPa, respectively. In two subjects arterial distensibility measurements were not available because of technical difficulties. To investigate determinants of DC, multiple regression analyses were performed with δD/D as dependent variable and δD; MAP, age, gender, δ homocysteine (or fasting, or post-methionine homocysteine levels as continuous as well

![Fig. 2. Relationship between fasting plasma homocysteine concentrations and flow-mediated, endothelium-dependent vasodilatation in the brachial artery. (r: +0.016, P-value of the univariate regression coefficient: 0.83).](https://academic.oup.com/cardiovascres/article-abstract/42/3/743/283462/551x293)
as dichotomous variables), smoking habits, BMI and levels of cholesterol (or levels of HDL-cholesterol, LDL-cholesterol and triglycerides) as independent variables. Pulse pressure and age emerged as determinants of $\delta D/D$ (stand $r$: 0.25, $P=0.002$ and stand $r$: $-0.59$, $P<0.0001$). The other variables, including the $\delta$, the fasting and the post-methionine homocysteine levels, were not related to $\delta D/D$, although for MAP there was a trend towards an inverse relation (stand $r$: $-0.21$, $P=0.052$). To estimate determinants of the CC, $\delta D/D$ was taken as dependent variable, and otherwise the model remained unchanged. $\delta P$, age and male gender were determinants of $\delta D/D$ (stand $r$: 0.33, $P=0.0003$; stand $r$: $-0.31$, $P=0.0006$ and stand $r$: 0.39, $P<0.0001$; respectively); the other variables were not. Again a trend for an inverse relation between $\delta D/D$ and MAP was observed (stand $r$: $-0.18$, $P=0.08$).

4. Discussion

This study is the first to demonstrate that, in apparently healthy first-degree relatives of patients with mild hyperhomocysteinaemia and premature atherosclerotic disease, the increase in plasma homocysteine level after methionine loading is inversely associated with endothelial function as measured by flow-mediated, endothelium-dependent vasodilatation in the brachial artery. These results provide evidence for the role of the endothelium in the development of atherosclerotic disease in persons with familial mild hyperhomocysteinaemia.

It is now well established that mild hyperhomocysteinaemia is associated with an increased risk of premature cerebral artery disease [2,26], peripheral artery disease [2,26], coronary artery disease [2,27] and deep venous thrombosis [28]. On the basis of studies in vitro [3–5] and in animals [29], endothelial dysfunction has been proposed as an important mechanism linking hyperhomocysteinaemia to atherosclerosis and thrombosis. Clinical data to support this hypothesis have been limited however [6–8,30]; in particular, it had not been clarified if endothelial dysfunction was already present in familial hyperhomocysteinaemia in the absence of clinical vascular disease.

The results of the present study suggest that, in apparently healthy siblings of patients with premature atherosclerotic disease and hyperhomocysteinaemia, a large increase in plasma levels of homocysteine after methionine loading is associated with impaired endothelium-dependent vasodilatation. A flow-mediated increase in shear stress on the endothelium stimulates vasodilatation in conduit and resistance vessels [17], probably to maintain shear stress within a constant physiological range [31]. Although the endothelium produces many vasodilatory substances which could contribute to shear stress-induced vasodilatation [32], it has now been convincingly demonstrated that hyperaemia-induced, flow-mediated vasodilatation in the forearm is largely NO-mediated [33,34]. The validity of the method used is further supported by the demonstration of disturbances in endothelium-dependent vasodilatation, before clinically manifest disease occurs, in persons with risk factors for atherosclerosis such as hypercholesterolaemia and smoking [16]. Moreover, it has been shown that disturbances of the endothelium-dependent vasodilatation of the brachial artery parallel endothelial dysfunction of the coronary arteries [35].
Thus, our results suggest that in apparently healthy first-degree relatives of patients with hyperhomocysteinaemia and premature atherosclerotic disease the increase in homocysteine concentration after a standard methionine load is a significant, although relatively weak determinant of an impaired NO production and/or action. In the presence of a normal NTG-induced vasodilatation, these results point to a specific role for dysfunction of the endothelium.

4.1. Fasting and post-methionine homocysteine levels

We found that the increase in homocysteine level in response to a methionine load (δ homocysteine) was associated with endothelial dysfunction, as measured by flow-mediated vasodilatation, and that there was a trend (in the same direction) for the post-load homocysteine levels. No relation with FMD over the entire range of fasting homocysteine levels was established (Fig. 2). It might be argued that this was caused by selection of the study population, because the subjects studied were all siblings of patients diagnosed as hyperhomocysteinaemic on the basis of post-methionine homocysteine levels. This might obscure a relation between fasting homocysteine concentrations and FMD if fasting homocysteine levels were low on average and/or if lower fasting homocysteine levels were associated with larger increases in homocysteine after methionine loading. Neither was the case, however. We found no relation with FMD over the entire range of fasting homocysteine levels (Fig. 2), a result that did not change after exclusion of persons with elevated post-methionine homocysteine levels. A final possibility is that all subjects had a low FMD, thereby obscuring a relation with fasting homocysteine levels. We effectively excluded this by comparing the subjects with a relatively low (i.e., normal according to the cutoff values of the European Concerted Action Project [18]) δ homocysteine with subjects without a family history of hyperhomocysteinaemia, and found that FMD was similar in these groups. In view of these considerations, it is unlikely that a true inverse relation between FMD and fasting homocysteine levels was obscured in our study.

Alternatively, the post-methionine homocysteine level may be more strongly related to cardiovascular disease than the fasting level [36], although this has recently been questioned [18]. Nevertheless, fasting and post-methionine (or δ) homocysteine levels are likely to be determined by different pathways in the metabolism of methionine. An impaired ability to remethylate homocysteine to methionine has been shown to result in fasting hyperhomocysteinaemia, but a normal increase in response to methionine loading [37]. Conversely, a defect in trans-sulphuration is thought to result in an abnormally high increase of homocysteine levels after a methionine load, in the presence of normal fasting homocysteine levels [38]. This is in agreement with observations that hyperhomocysteinaemia post-methionine loading without elevated fasting homocysteine levels accounts for 35–50% of all cases of hyperhomocysteinaemia [11,18,39] Using the cutoff values of the large multicentre European Concerted Action Project study [18], we found that FMD in subjects with elevated post-methionine homocysteine levels was lower than that in those with normal levels, and that a similar trend was observed for the δ homocysteine but not the fasting homocysteine levels, also pointing to a difference between fasting and post-methionine (or δ) homocysteine levels. The post-load increase in homocysteine level may be a better reflection of methionine metabolism than the absolute post-load level, because the increase in homocysteine level is influenced less by differences in fasting homocysteine level than is the absolute level [40]. Thus, it is possible that, in the population we studied, a defect of homocysteine transsulfuration plays a major role, mainly resulting in elevated δ homocysteine (Fig. 1), and that this defect is related to an impairment of endothelial function. In a study by Tawakol et al., endothelial dysfunction was related to fasting homocysteine levels [8], suggesting distinct mechanisms of vascular impairment in relation to distinct disturbances in methionine metabolism in different study groups. This is important for the interpretation of the results of studies showing that post-methionine homocysteine levels are independently associated with atherosclerotic disease [18].

There is evidence that homocysteine can adversely
earlier there was no relation between homocysteine levels and the vascular responses. As reported, exercise did not influence the relation between homocysteine and NO, resulting in an increased oxidation of homocysteine and eventually in a decreased production of NO and an impaired vasodilatation [3,5].

It is important to note that the model of hyperhomocysteinaemia-induced oxidative stress as the prime cause of impaired NO synthesis and/or action cannot easily account for the inverse relation between FMD and fasting homocysteine levels that we observed, because average plasma levels during the day are probably much closer to fasting levels than to post-methionine levels. Therefore, alternative mechanisms linking post-methionine homocysteine levels to impaired endothelial function may have to be considered, such as an impairment of protein repair [42]. It is not known, however, whether such alternative mechanisms of homocysteine-associated endothelial injury are more closely related to fasting homocysteine levels than to fasting homocysteine levels, and this issue requires further study. Finally, subclinical atherosclerosis as measured by ankle-brachial pressure index or ankle pressure drop after exercise did not influence the relation between homocysteine levels and the vascular responses. As reported earlier there was no relation between homocysteine levels and the presence or absence of an abnormal peripheral artery test [12], suggesting that homocysteine is not an important factor in asymptomatic peripheral disease in siblings of young patients with vascular disease.

4.2. Arterial distensibility

Our finding that estimates of arterial distensibility were not disturbed may indicate that the predominant early effect of hyperhomocysteinemia is not on structural vascular properties, as would be expected if increased media fibrosis with smooth muscle cell proliferation and fragmented internal elastic membranes play a major role [9,43], or that any such effect is small compared to the observed influence of age and of mean arterial pressure. However, results in hyperhomocysteinemic minipigs with histologically verified aorta and carotid artery damage compatible with previously described findings in humans [9] showed that aortic stiffness was decreased as compared to control animals. In contrast, an increased smooth muscle tension was observed [44]. It has been suggested that localized elastic fibre splitting in the presence of smooth muscle cell hyperplasia accounts for these hemodynamic results. In humans NO inhibition in the radial artery by L-NMMA was associated with a paradoxical isometric smooth muscle relaxation, perhaps pointing to a compensatory vasodilatation [45]. Therefore, it is possible that such opposite effects of homocysteine on the various determinants of the vascular wall stiffness are responsible for the normal distensibility of the carotid artery observed in our study. In patients with end stage renal disease hyperhomocysteinaemia affected the arterial distensibility (measured as the pulse wave velocity) only in the lower lim and not in the upper limb, suggesting a preferential effect of homocysteine on the lower limb vessels [46]. Therefore, the conclusion that hyperhomocysteinaemia does not affect arterial distensibility must be viewed with caution.

In conclusion, in healthy first-degree relatives of patients with hyperhomocysteinaemia and premature atherosclerotic disease, the increase in homocysteine concentration after methionine loading is inversely predictive of flow-mediated, endothelium-dependent vasodilatation, but not of endothelium-independent vasodilatation. There was no correlation between fasting homocysteine levels and flow-mediated vasodilatation or arterial distensibility. This may explain, at least in part, why post-methionine hyperhomocysteinaemia is a risk factor for atherosclerotic and thrombotic disease. In addition, these findings suggest that fasting and post-methionine homocysteine levels reflect different metabolic pathways of methionine, which might be linked to vascular dysfunction through distinct mechanisms.

Acknowledgements

This study was supported by a grant from the Nederlandse Hartstichting (Dutch Heart Foundation). Dr. Stehouwer is a recipient of a Clinical Research Fellowship from the Netherlands Organisation for Scientific Research (NWO) and the Diabetes Fonds Nederland (Dutch Diabetes Foundation). We thank Servier Netherlands for financial support of the Wall Track System, and Mrs. E. Bulterijs for assistance with managing the study.

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