Influence of methylenetetrahydrofolate reductase genotype, exercise and other risk factors on endothelial function in healthy individuals

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ABSTRACT
Cardiovascular disease has a multifactorial aetiology that is influenced by both genetic and environmental factors. Endothelial dysfunction is a key event in the pathogenesis of vascular disease that occurs before structural vascular changes or clinical symptoms are evident. Conventional risk factors, for example hypertension and diabetes mellitus, are associated with endothelial dysfunction, but the influence of other putative risk factors is not clear. The methylenetetrahydrofolate reductase (MTHFR) C677T genotype, a common polymorphism that induces hyperhomocysteinaemia, has been proposed as being a genetic risk factor for cardiovascular disease. A total of 126 healthy adults recruited by MTHFR C677T genotype (42 of each genotype, i.e. CC, CT and TT) underwent assessment of endothelial function. Brachial artery endothelium-dependent flow-mediated dilatation (FMD) was measured using high-resolution ultrasonic vessel ‘wall-tracking’. Using multiple regression analysis, MTHFR genotype and 21 other subject and subject-lifestyle variables were investigated as potential predictors of endothelial function. FMD was influenced positively by frequency of aerobic exercise and by hormone replacement therapy, and negatively by increases in systolic blood pressure. MTHFR C677T genotype and the associated variation in plasma homocysteine levels did not influence FMD. Additionally, other factors, including plasma cholesterol and self-supplementation with either antioxidant vitamins or cod liver oil, showed no significant relationship with FMD, although these findings are compromised by the narrow range studied for cholesterol and the small number of subjects taking supplements. These observations have implications for risk factor management in the primary prevention of cardiovascular disease in healthy individuals.

INTRODUCTION
Cardiovascular disease has a multifactorial aetiology that is influenced by a variety of genetic and environmental factors. Risk factors that cannot be changed include age, male gender and a genetic predisposition to premature cardiovascular disease. Conventional ‘modifiable’ risk factors include hypertension, diabetes mellitus, the post-menopausal state in women, cigarette smoking, dietary habit, obesity and physical inactivity. The fact that a significant proportion of cardiovascular disease is unexplained by conventional risk factors has encouraged investigation to identify other causal factors. It has been proposed that the amino acid homocysteine may have a

Key words: atherosclerosis, genetics, primary prevention, risk factors, vascular endothelium.
Abbreviations: FMD, flow-mediated dilatation; HRT, hormone replacement therapy; MTHFR, methylenetetrahydrofolate reductase; SBP, systolic blood pressure.
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role in the pathogenesis of cardiovascular disease. Elevated plasma homocysteine levels are associated with increased cardiovascular risk, although whether the relationship is causal remains unclear [1–4]. If homocysteine were causally related to cardiovascular disease, then the common C677T mutation of the enzyme methylenetetrahydrofolate reductase (MTHFR), which results in elevated plasma homocysteine levels, would be expected to be associated with increased risk of cardiovascular disease. Individuals homozygous for this mutation (those having the TT genotype) typically exhibit plasma homocysteine levels ~ 25% greater than those in individuals with a normal genotype (CC) or those heterozygous for the mutation (CT genotype) [5,6].

Endothelial dysfunction is an early feature of atherosclerotic vascular disease associated with established cardiovascular risk factors [7]. In the presence of functional endothelium, increased blood flow stimulates the endothelial production of nitric oxide (NO), which causes smooth muscle relaxation and vasodilatation, a phenomenon termed flow-mediated dilatation (FMD). This response is impaired when the endothelium is dysfunctional. Assessment of endothelium-derived NO-mediated dilatation is widely regarded as a surrogate marker of vascular health. Measurement of peripheral artery endothelial function has been shown to accurately reflect coronary endothelial function [8], and coronary endothelial dysfunction is a long-term predictor of the development of atherosclerosis and cardiac events [9,10].

Strategies to reduce cardiovascular risk require a multifactorial approach combining modification of lifestyle (e.g. regular exercise, cessation of cigarette smoking and changes in diet) with appropriate pharmacological interventions (e.g. administration of lipid-lowering and anti-hypertensive drugs). Intervention therapies known to reduce cardiovascular risk are typically accompanied by improved endothelial function in both coronary and peripheral vessels [11–14]. Here we report the extent of the influence of a variety of factors on brachial artery FMD in healthy adult volunteers. The study is derived from a subsidiary analysis of baseline data collected for an investigation specifically designed to examine the effects of increased folate intake, homocysteine and MTHFR C677T genotype on endothelial function. It aimed to clarify the role of the MTHFR C677T genotype and the resulting variation in plasma homocysteine levels on FMD in the absence of conventional vascular risk factors.

METHODS

Subject recruitment
Volunteers aged between 18 and 65 years were recruited from blood donor sessions and workplaces in South Wales. Exclusion criteria included hypertension (diastolic blood pressure > 100 mmHg), diabetes mellitus, smoking, pregnancy, active supplementation with B vitamins or folic acid, and previous treatment for cardiovascular disease. Potential recruits were informed of the study requirements before providing written consent. The study was approved by the Local Research Ethics Committee (Bro Taf Health Authority) and was carried out in accordance with the Declaration of Helsinki (1989) of the World Medical Association.

In order to recruit equal numbers of individuals with each MTHFR C677T genotype (CC, CT and TT), eligible volunteers (n = 634) were screened before entry. DNA was extracted from inner cheek buccal cells collected using a cytobrush, and genotype was determined by heteroduplex analysis [15]. A total of 126 subjects (42 with each MTHFR C677T genotype) were evaluated on a single occasion at which they completed a ‘lifestyle’ questionnaire, underwent assessment of endothelial function and provided a fasting venous blood sample. (These subjects subsequently took part in a folic acid intervention study reported elsewhere [16].)

Lifestyle questionnaire
The lifestyle questionnaire was designed to establish subject medication and nutritional supplement consumption, exposure to passive smoking, and exercise habits. Activities such as running, cycling, rowing, swimming and aerobics, but not walking, were categorized as aerobic exercise.

Assessment of endothelial function
FMD was assessed in the brachial artery using the method described previously by our research group [17] with the commercial Vadirec® “wall-tracking” system (Vadirec, Oosterbeck, The Netherlands). An ultrasound image of the brachial artery is obtained using a 7.5 MHz linear phased-array transducer. For each measurement, 10 s of digitized radio frequency signal is sampled at 1 kHz. The operator marks the positions of the anterior and posterior walls of the brachial artery on the waveform of the first radio frequency signal. The Vadirec system tracks vessel movement automatically over the 10 s of recording and calculates the mean end-diastolic diameter to a theoretical accuracy of ±3 μm. Repeat measurements in the same subject have a between-day coefficient of variation of 12.8%.

Blood flow was measured with an 8 MHz continuous-wave Doppler probe (SciMed Dopstation®; SciMed, Bristol, U.K.) mounted at 60° to the vessel. Blood flow was calculated as the product of the Doppler time velocity integral, heart rate and brachial artery diameter measured by wall tracking. Blood pressure was measured by photoplethysmography (Finapres®; Ohmeda, Louisville, CO, U.S.A.) via a cuff on the middle finger of the arm being studied.
Following baseline measurements, ischaemia was induced in the hand by inflation of a wrist cuff to suprasystolic blood pressure for 5 min. Cuff release stimulated reactive hyperaemia in the hand, resulting in a secondary increase in blood flow along the brachial artery. The stimulus for brachial artery dilation is increased shear stress exerted by this increased blood flow. End-diastolic diameter, blood flow and pressure were recorded at 40 s intervals for 240 s after cuff release, and at 6, 8 and 10 min (Figure 1). The maximum absolute change in end-diastolic diameter (µm) observed for each individual subject during the first 200 s after cuff release was taken as the measure of FMD. Values obtained following occlusion of the wrist are smaller than values reported after upper-arm occlusion, and have been shown to be mediated exclusively by NO [18].

### Blood analysis

Venous blood was centrifuged within 10 min of collection and plasma was stored at $-70 \, ^\circ C$ until analysis. Plasma lipids, glucose and creatinine were determined using standard laboratory procedures. Plasma homocysteine and plasma folate concentrations were measured using Abbott IMx methods.

### Statistics

A total of 23 subject variables were investigated (see Table 1). Each factor was initially investigated independently for possible differences between the three

### Table 1: Potential predictor variables of brachial artery FMD

<table>
<thead>
<tr>
<th>Parameter</th>
<th>CC</th>
<th>CT</th>
<th>TT</th>
<th>All subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Dependent variable</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FMD (µm)</td>
<td>94 (66)</td>
<td>93 (68)</td>
<td>106 (85)</td>
<td>98 (73)</td>
</tr>
<tr>
<td><strong>Predictor variables</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>42 (12)</td>
<td>37 (11)</td>
<td>40 (11)</td>
<td>39 (12)</td>
</tr>
<tr>
<td>Gender (male/female)</td>
<td>13/29</td>
<td>19/23</td>
<td>21/21</td>
<td>53/73</td>
</tr>
<tr>
<td>Genotype</td>
<td>42</td>
<td>42</td>
<td>42</td>
<td>126</td>
</tr>
<tr>
<td>Baseline end-diastolic diameter (mm)</td>
<td>3.56 (0.83)</td>
<td>3.61 (0.74)</td>
<td>3.62 (0.69)</td>
<td>3.40 (0.68)</td>
</tr>
<tr>
<td>Basal blood flow (ml/min)</td>
<td>30 (19)</td>
<td>35 (29)</td>
<td>32 (28)</td>
<td>32 (26)</td>
</tr>
<tr>
<td>Peak blood flow (ml/min)</td>
<td>121 (66)</td>
<td>125 (75)</td>
<td>119 (80)</td>
<td>122 (73)</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>119 (16)</td>
<td>115 (15)</td>
<td>121 (15)</td>
<td>118 (15)</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>68 (11)</td>
<td>70 (12)</td>
<td>71 (9)</td>
<td>70 (11)</td>
</tr>
<tr>
<td>Heart rate (beats/min)</td>
<td>65.0 (9.5)</td>
<td>62.7 (8.5)</td>
<td>62.4 (8.7)</td>
<td>63.4 (8.9)</td>
</tr>
<tr>
<td>Plasma total cholesterol (mmol/l)</td>
<td>4.82 (1.01)</td>
<td>5.00 (0.91)</td>
<td>4.89 (0.85)</td>
<td>4.91 (0.92)</td>
</tr>
<tr>
<td>Plasma triacylglycerol (mmol/l)</td>
<td>0.95 (0.49)</td>
<td>1.08 (0.46)</td>
<td>0.97 (0.35)</td>
<td>1.00 (0.44)</td>
</tr>
<tr>
<td>Plasma high-density lipoprotein (mmol/l)</td>
<td>1.27 (0.31)</td>
<td>1.26 (0.32)</td>
<td>1.27 (0.29)</td>
<td>1.26 (0.30)</td>
</tr>
<tr>
<td>Plasma creatinine (µmol/l)</td>
<td>78.4 (8.4)</td>
<td>78.8 (11.2)</td>
<td>81.4 (9.3)</td>
<td>79.5 (9.7)</td>
</tr>
<tr>
<td>Plasma glucose (mmol/l)</td>
<td>5.34 (0.45)</td>
<td>5.28 (0.45)</td>
<td>5.32 (0.40)</td>
<td>5.31 (0.43)</td>
</tr>
<tr>
<td>Plasma total homocysteine (µmol/l)</td>
<td>8.8 (2.5)</td>
<td>9.3 (2.5)</td>
<td>12.5 (5.7)</td>
<td>10.2 (4.2)</td>
</tr>
<tr>
<td>Plasma folate (µg/l)</td>
<td>9.1 (3.5)</td>
<td>7.6 (3.1)</td>
<td>6.7 (3.3)</td>
<td>7.8 (3.4)</td>
</tr>
<tr>
<td>Aerobic exercise (sessions/week)</td>
<td>1.0 (1.5)</td>
<td>1.4 (1.6)</td>
<td>1.8 (2.0)</td>
<td>1.4 (1.7)</td>
</tr>
<tr>
<td>Exposure to passive smoking (h/week)</td>
<td>5 (10)</td>
<td>9 (16)</td>
<td>10 (17)</td>
<td>8 (15)</td>
</tr>
<tr>
<td>Antioxidant vitamin supplementation</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>9</td>
</tr>
<tr>
<td>Cod liver oil supplementation</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>HRT</td>
<td>3</td>
<td>1</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>Contraceptive pill</td>
<td>2</td>
<td>5</td>
<td>4</td>
<td>11</td>
</tr>
</tbody>
</table>
MTHFR C677T genotype groups by one-way ANOVA followed by the Tukey test. Potential predictors of FMD were subsequently sought among all other variables using multiple regression analysis (SPSS Inc., Chicago, IL, U.S.A.). Administration of cod liver oil, antioxidant vitamins, hormone replacement therapy (HRT) and the oestrogen-containing contraceptive pill were noted as being either present or absent. The three MTHFR C677T genotypes were investigated both as a linear series and with CC and CT genotypes combined. A regression model was built by backwards elimination of variables to a limit of $P = 0.05$. Residuals from the resulting model were checked for normality, and the partial regression plots were checked for non-linearity among the independent variables.

RESULTS

Subject characteristics of the separate MTHFR C677T genotype groups are detailed in Table 1. Of the variables tabulated, significant differences between genotype groups were demonstrated only for plasma total homocysteine ($P < 0.001$) and folate ($P < 0.01$) levels. Differences were as expected for MTHFR mutations. The level of homocysteine in the homozygous TT group was greater than in the other two groups, and the plasma folate level in the TT genotype group was lower than in the CC genotype group, which had the highest value ($P < 0.05$; Tukey tests).

Multiple regression analysis identified significant relationships between FMD and three subject variables ($P < 0.001$). The calculated model incorporated aerobic exercise ($P = 0.001$), HRT ($P = 0.03$) and systolic blood pressure (SBP) ($P = 0.04$). The model was:

$$FMD \ (\mu m) = 187 + [12.3 \times \text{aerobic exercise (sessions/week)}] + (65.7 \times \text{HRT})$$

$$+ [-0.93 \times \text{SBP (mmHg)}]$$

in which HRT is given a value of 0 (if absent) or 1 (if present).

Residuals for the three factors appeared to be normally distributed and the relationships were linear. There was a weak correlation between HRT and SBP ($r = 0.3$, $P < 0.01$). Regression coefficients varied by $< 40\%$ when each was considered as sole independent variable. Thus, for every session of aerobic exercise per week, FMD increased by $12 \mu$m, while for every $10$ mmHg increase in SBP FMD decreased by $9 \mu$m. Women taking HRT increased their FMD by $66 \mu$m.

Of the variables not incorporated within the final model, MTHFR C677T genotype, homocysteine and cholesterol showed negligible relationships with FMD ($P = 0.94$, $P = 0.96$ and $P = 0.91$ respectively), whereas gender and age came closer to reaching significance ($P = 0.34$ and $P = 0.16$ respectively). The brachial artery parameters of basal diameter, peak flow and basal flow were also close to achieving significance ($P = 0.21$, $P = 0.18$ and $P = 0.07$ respectively).

DISCUSSION

In a group of healthy adults at no known increased risk of vascular disease and recruited specifically to include equal numbers of individuals with each MTHFR C677T genotype, we demonstrate that, of the variables investigated, those that appeared to significantly influence brachial artery FMD were aerobic exercise, SBP and HRT. There was no relationship between FMD and either MTHFR C677T genotype or plasma homocysteine concentration.

The beneficial effects of exercise in both the primary and secondary prevention of cardiovascular disease are well established [11]. Within the general population, levels of physical activity and incidence of cardiovascular disease are inversely related. Additionally, among patients with established cardiovascular disease, mortality is lower among those who undertake regular exercise. One mechanism by which exercise has been proposed to mediate its cardiovascular benefit is by enhancement of endothelial function [19]. Endothelium-dependent NO-mediated dilatation can be improved by exercise training in individuals both with and without vascular disease [20–22].

Hypertension is a major risk factor for vascular disease, with even moderate hypertension being associated with increased cardiovascular risk. Individuals with hypertension typically exhibit endothelial dysfunction. The aetiology is multifactorial with, among others, reduced synthesis and accelerated breakdown of NO and alterations in the architecture of resistance vessels being involved [23]. Treatment with anti-hypertensive drugs can correct the dysfunction, depending on the ability of the drugs to counteract the various mechanisms [14].

The cardiovascular benefits of oestrogens are widely documented, although whether HRT (usually a combination of oestrogens and progestogens) offers cardiovascular protection remains controversial [24]. However, pre-menopausal women typically have a lower incidence of atherosclerosis, and have been shown in some studies to have greater FMD responses, than do age-matched men, and oestrogen replacement therapy improves FMD in post-menopausal women [25]. Use of oral contraceptives by premenopausal women had no effect on FMD. The present study was not designed specifically to investigate the endothelial effects of HRT and, as a consequence, the number of subjects receiving such therapy was limited (seven out of 126). Thus our findings in relation to HRT must not be over-interpreted. In assessing the significance and scale of the effect in this small group, it should be noted that those taking HRT
were significantly older than other women in the study [mean (S.D.) values of 57 (4) and 37 (11) years respectively; \( P < 0.001 \) by \( t \)-test).

Our study was intended to examine the endothelial effects of MTHFR C677T genotype. Predetermination of genotype enabled equal numbers of individuals with each genotype to be studied, rather than the unbalanced ratios found within the general population, where approx. 12% of individuals are homozygous for the mutation (gene frequency \( \sim 0.32 \)) [15]. We can therefore report with confidence that, in healthy subjects, MTHFR C677T genotype and the associated variation in plasma homocysteine levels do not influence endothelial function significantly. The case for either factor having a causal role in the pathogenesis of vascular disease is therefore weakened. Indeed, a previous study has reported there to be no relationship between cardiovascular disease and MTHFR C677T genotype [26]. Mildly elevated plasma homocysteine levels may act synergistically with other risk factors but, in the absence of the latter, may cause little vascular damage [27,28]. Subjects for the present study were recruited specifically to be free of other known cardiovascular risk factors.

Cholesterol, within the narrow range studied, showed no relationship with vascular endothelial function. The apparent lack of effect of administration of antioxidant vitamins or cod liver oil also must not be over-interpreted, as the present study is limited by the relatively small numbers of individuals regularly taking these supplements (nine and six respectively). However, beneficial endothelial effects of either antioxidant vitamins or cod liver oil have only ever been reported previously in individuals with vascular disease, and not in healthy individuals [29–31].

The present study provides new information about the degree of influence that various factors have on endothelial function and, by inference, cardiovascular health in a healthy population. Regular aerobic exercise and the presence of a low blood pressure enhance endothelial function. Additionally, HRT may be advantageous to post-menopausal women. It is interesting to note that the three factors found here to significantly influence FMD have all been proposed to regulate NO production by directly affecting endothelial NO synthase. Exercise, which increases blood flow and hence shear stress exerted on endothelial cells [32,33], and oestrogens, which are a component of HRT, have both been reported to increase the expression of endothelial NO synthase [34]. In contrast, increased blood pressure down-regulates NO synthase activity [35]. Importantly, MTHFR C677T genotype and plasma homocysteine levels, within the range studied here, appear not to influence FMD in healthy individuals. These observations have implications for risk factor management in the primary prevention of cardiovascular disease. Effort in healthy individuals on normal diets should be directed towards modification of exercise patterns and blood pressure.

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REFERENCES


C. H. Pullin and others


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