

# **HOMOCYSTEINE AND ENDOTHELIAL FUNCTION**

by

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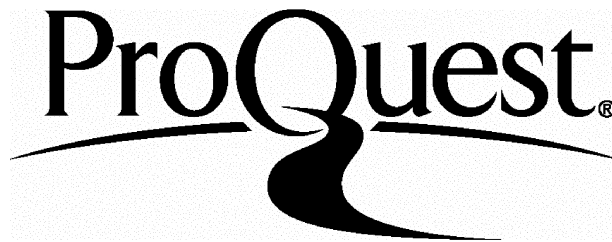
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## Abstract

Dysfunction of the endothelium is an early event in the pathophysiology of atherosclerosis. Elevated plasma homocysteine (Hcy) is a risk factor for vascular disease which may be mediated by endothelial injury. The thesis describes a series of clinical and laboratory experiments to investigate the relationship between Hcy and endothelial function.

Non-invasive measurement of endothelial dysfunction in humans was assessed in the forearm as an impaired increase in brachial artery diameter during reactive hyperaemia (shear stress-induced, nitric oxide-mediated vasodilatation). Responses were compared with endothelium-independent vasodilatation following sublingual glyceryl trinitrate. Flow-mediated brachial artery dilatation (FMD) using this method, was shown to be an endothelium-dependent mechanism and normal values for the population established with the addition of regression modelling to define an appropriate index to represent endothelium-dependent and -independent responses.

An association between hyperhomocysteinaemia and FMD was demonstrated in (i) homozygous homocystinuria, (ii) following an acute elevation of plasma Hcy after oral methionine loading (an effect that was partially reversed by the prior oral administration of vitamin C), and (iii) in subjects with low B<sub>12</sub>/folate status (where elevated Hcy resulted from impaired remethylation). However, in a cross-sectional population study across the range of Hcy concentrations no correlation between Hcy and FMD was observed. In healthy subjects with mild hyperhomocysteinaemia, oral folic acid supplementation (5mg daily for 6 weeks) enhanced FMD and lowered Hcy, providing preliminary evidence that folate may have beneficial cardiovascular effects in adults with elevated Hcy levels.

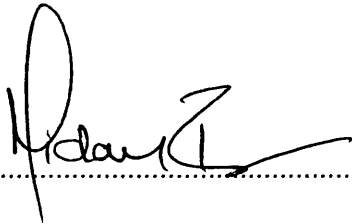
In vitro, endothelium-dependent relaxation in isolated rabbit aortic rings to both receptor-dependent and -independent agonists was impaired by Hcy. Prior incubation with antioxidants and the intracellular superoxide scavenger, Tiron, attenuated the inhibitory effect of Hcy supporting the hypothesis that Hcy-related endothelial injury may be mediated in part by oxidative stress and alterations in intracellular redox status.

## **Declaration**

The work described in this thesis, except where indicated otherwise, is my own and was carried out in the Departments of Cardiology and Pharmacology and Therapeutics, University of Wales College of Medicine (UWCM), Cardiff between May 1995 and July 1997.

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## LIST OF ABBREVIATIONS

Ach	- Acetylcholine
ACE	- Angiotensin converting enzyme
ADMA	- Asymmetric dimethyl arginine
BL	- Baseline
CBS	- Cystathionine $\beta$ -synthase
CAT	- Catalase
Chol	- Total serum cholesterol
Creat	- Creatinine
CV	- Coefficient of variation
DBP	- Diastolic blood pressure
+E	- Endothelium intact
-E	- Endothelium denuded
EDD	- End-diastolic diameter
$\Delta$ EDD	- Change in end-diastolic diameter
$\Delta$ EDDmax	- Maximum change in end-diastolic diameter
EDRF	- Endothelium derived relaxing factor
ELISA	- Enzyme-linked immunosorbent assay
$\Delta$ Flow	- Change in arterial blood flow
FMD	- Flow-mediated vasodilatation
Gluc	- Serum glucose
GMP	- Guanosine monophosphate
GTN	- Glyceryl trinitrate
Hb	- Haemoglobin
Hcy	- Plasma homocysteine

H <sub>2</sub> O <sub>2</sub>	- Hydrogen peroxide
HPLC	- High performance liquid chromatography
L-Arg	- L-arginine
LDL	- Low density lipoprotein
L-NMMA	- N <sup>G</sup> -monomethyl L-arginine
Lp(a)	- Lipoprotein (a)
MCV	- Macrocytic volume
MTHFR	- Methylenetetrahydrofolate reductase
NADH	- Nicotinamide adenine dinucleotide (reduced)
NADPH	- Nicotinamide adenine dinucleotide phosphate (reduced)
NO	- Nitric oxide
ecNOS	- Endothelial constitutive nitric oxide synthase
NS	- Non significant
OR	- Odds ratio
O <sub>2</sub> <sup>•-</sup>	- Superoxide anion
ONOO <sup>•</sup>	- Peroxynitrite radical
PCA	- Perchloric acid
PE	- Phenylephrine
QC	- Quality control
r	- Correlation coefficient
RF	- Radiofrequency (of ultrasound)
Rmax	- Maximum relaxation response
SAH	- S-adenosylhomocysteine
SAM	- S-adenosylmethionine
SBD-F	- Ammonium 7-fluoro-2-oxa-1,3-diazole-4-sulphonate
SBP	- Systolic blood pressure

SD	- Standard deviation
SDMA	- Symmetric dimethyl arginine
SEM	- Standard error of the mean
SNP	- Sodium nitroprusside
SOD	- Superoxide dismutase
SST	- Serum separation tube
THF	- Tetrahydrofolate
Vadirec	- Vascular distension recorder
VWF-Ag	- Von Willebrand factor antigen

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## **CHAPTER 1**

### **Introduction**

#### **1.1 Background**

The pattern of cardiovascular disease in the United Kingdom is only partly explained by the known major risk factors for atherosclerosis, which do not account for all vascular events. The search for 'novel' risk factors is important in order to identify individuals who may benefit from primary or secondary prevention strategies, and may also provide insights into the aetiology of vascular disease and possible pathophysiologic mechanisms. In recent years, homocysteine has become increasingly implicated in the development of vascular disease with plausible mechanisms proposed for its role in promoting atherosclerosis. A large number of epidemiological studies have now demonstrated it to be an independent and potentially reversible risk factor and clinical trials are underway to determine the effect of homocysteine lowering on cardiovascular endpoints.

Homocysteine is a sulphur containing amino acid derived from the metabolism of dietary methionine. It was originally discovered by du Vigneaud<sup>1</sup> in 1932 and recognised some 30 years later as a cause of an inborn error of metabolism. Carson and Neill<sup>2</sup> described children and infants with mental retardation who displayed high levels of homocysteine in the urine (homocystinuria) in association with other congenital abnormalities and precocious thromboembolism.

Vascular disease is a major pathological feature of homocystinuria<sup>3</sup>. A direct pathogenic role of homocysteine is implicated by the observation that similar histological appearances occur in several different inborn errors of homocysteine metabolism, in which intermediate metabolites occur in different patterns, all leading to severe hyperhomocysteinaemia<sup>4</sup>. In the commonest cause of homocystinuria, due to defective cystathionine  $\beta$ -synthase (1 in 200,000), thromboembolism is a common cause of death and the incidence of vascular complications is reduced when homocysteine is lowered by pyridoxine therapy, in those individuals with a pyridoxine sensitive enzyme defect<sup>5</sup>. The observation of vascular damage in homocystinuria in combination with some animal experiments led McCully to propose a new theory of atherosclerosis in which homocysteine was implicated<sup>6</sup>.



The concentration of plasma homocysteine in homocystinuria, is markedly elevated usually in excess of ten times that of normal individuals (normal ca. 10  $\mu\text{mol/L}$ ). In the general population homocystinuria is rare but mild hyperhomocysteinaemia (15 to 30  $\mu\text{mol/L}$ ) is common. Great interest lies in the proposal that mild elevations in plasma homocysteine may promote vascular disease and be potentially reversed by lowering plasma levels with vitamin supplements.

## 1.2 Metabolism of homocysteine and its regulation

### 1.2.1 Nomenclature (see Fig. 1.1)

*Homocysteine* (Hcy) is often used in contexts that encompass several (oxidised and reduced) species. Thus, *free homocysteine* in plasma is the acid-soluble, non-protein bound fraction. Only trace amounts ( $<0.3\mu\text{mol/l}$ ) of reduced Hcy can be detected in plasma, whereas most Hcy exists as various disulphide forms. About 70% is bound to albumin (protein-bound homocysteine or Hcy-albumin mixed disulphide), whereas the remaining 30% exists as mixed disulphides with other thiols and the cysteine-Hcy disulphide is the most abundant species.

*Total homocysteine* (tHcy) includes all (free and protein-bound) forms of Hcy in plasma.

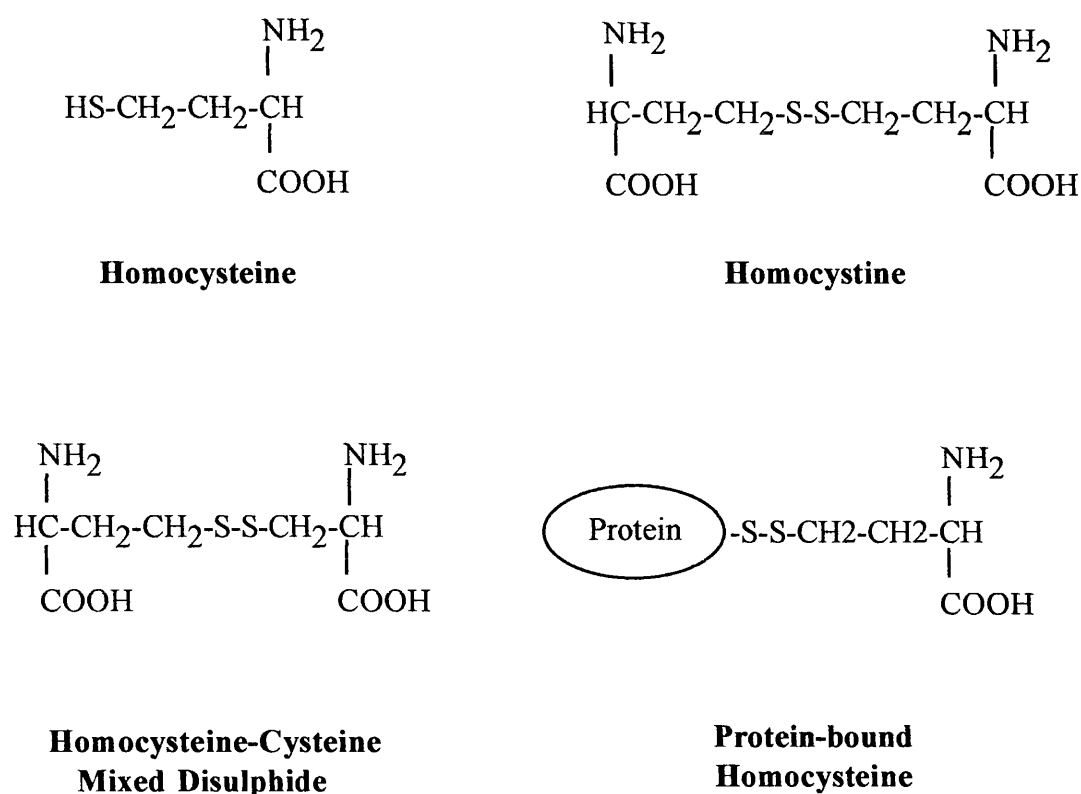
*Homocystine* is formed when two molecules of Hcy are linked by a disulphide bridge.

*Homocystinuria* refers to genetic diseases characterised by massive urinary excretion of Hcy and its derivatives. A *homocystinuric* is a patient with homocystinuria.

*Hyperhomocysteinaemia* refers to elevations of plasma total Hcy above normal values.

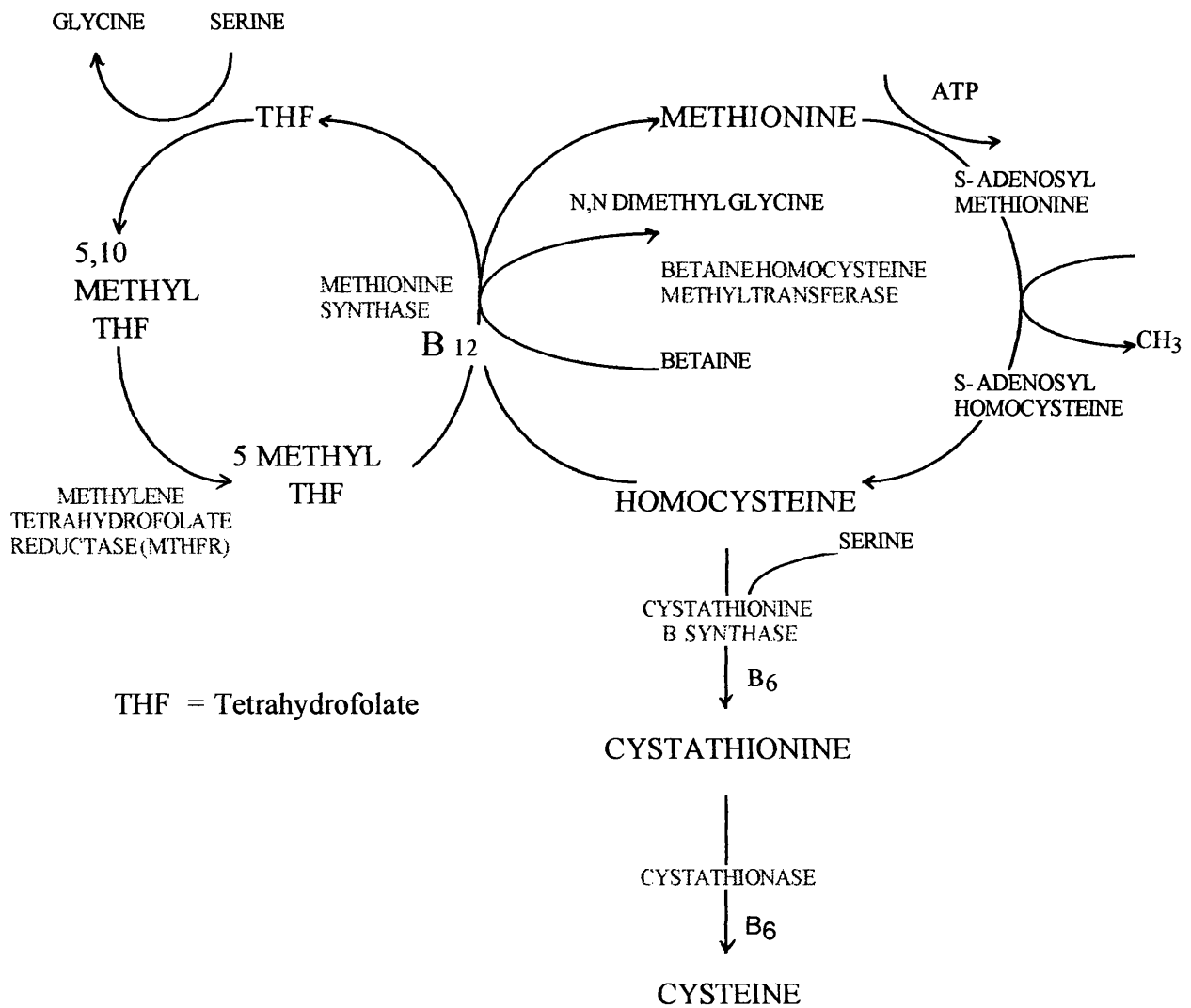
### 1.2.2 The methionine cycle and transsulphuration pathway

The metabolism of Hcy is shown in Fig. 1.2. Methionine is an essential sulphur-containing amino acid that is supplied through catabolism of dietary proteins. The average daily intake of the Western male is approx. 15-35 mg/kg<sup>7</sup>. Methionine that is not incorporated into proteins may be catabolised through transamination<sup>8</sup>, but most is converted to S-adenosylmethionine (SAM), catalysed by methionine adenosyltransferase. Only a small fraction of formed SAM is used



**Fig 1.1 Chemical structures of homocysteine and related compounds**

Homocysteine is a sulphur containing amino acid. It may be converted to homocystine or alternatively to homocysteine-cysteine mixed disulphide. Homocysteine also binds to the sulphydryl-moiety of plasma proteins via a disulphide bond and this forms the majority of circulating homocysteine found in plasma.



**Fig 1.2 The transsulphuration and remethylation pathways of homocysteine metabolism**

for the synthesis of polyamines and most functions as a methyl donor in various transmethylation reactions. S-adenosylhomocysteine (SAH), the demethylated product of SAM, is further hydrolysed to adenosine and Hcy, catalysed by the enzyme S-adenosylhomocysteine hydrolase. This reaction is the only known source of Hcy in vertebrates<sup>4</sup>.

Homocysteine is catabolised to cysteine through two vitamin B<sub>6</sub>-dependent reactions that complete the transsulphuration pathway. In the first of these reactions, homocysteine is condensed with serine to form cystathionine by the enzyme cystathionine  $\beta$ -synthase. The reaction is irreversible under physiological conditions, and at this point, Hcy is committed to the transsulphuration pathway. Cystathionine is further metabolised to cysteine, catalysed by the B<sub>6</sub>-dependent enzyme  $\gamma$ -cystathionase.<sup>9</sup>

Remethylation of Hcy to methionine is catalysed either by 5-methyltetrahydrofolate-homocysteine methyltransferase (methionine synthase) or betaine-homocysteine methyltransferase. The former enzyme, which is widely distributed, requires 5-methyltetrahydrofolate (the reduced circulating form of folate) as a methyl donor and vitamin B<sub>12</sub> as a cofactor. Betaine-homocysteine methyltransferase is confined to the liver and only minor activity has occasionally been found in kidney and adrenal glands<sup>9</sup>. Both these reactions conserve methionine.

### 1.2.3 Regulation of the cycle

Homocysteine is an important branch-point metabolite that connects the metabolism of diverse compounds like methionine, cysteine, cobalamin, reduced folates, and vitamin B<sub>6</sub>. This system represents an important regulatory locus<sup>9</sup>; Hcy may be directed into different anabolic or catabolic pathways.

During methionine excess, methionine is catabolised through the transmethylation-transsulphuration pathway to cysteine and finally sulphate. Excess methionine increases SAM and decreases 5-methyltetrahydrofolate in liver. Adaptation involves increased flux of Hcy into cystathionine synthesis and the rate of remethylation is low, the latter through inhibition of 5-methyltetrahydrofolate synthesis by SAM.

Metabolite levels and enzyme activities are changed in essentially opposite directions during methionine deficiency, and this regulatory response ensures efficient methionine conservation through enhanced Hcy remethylation<sup>10</sup>.

### **1.3 Assessment of homocysteine status**

In an adult population, the normal plasma total homocysteine (tHcy) as defined by 95% of healthy subjects is approximately 5-15  $\mu\text{mol/L}$ . Median concentrations are slightly higher in males than females (i.e. 8.5 $\mu\text{mol/L}$  and 7.2 $\mu\text{mol/L}$ , respectively)<sup>11</sup>. Hyperhomocysteinaemia is usually defined as a plasma tHcy >15 $\mu\text{mol/L}$ , and is defined here as mild (15-30 $\mu\text{mol/L}$ ), moderate (30-100 $\mu\text{mol/L}$ ) or severe (>100 $\mu\text{mol/L}$ ), although at present there is no consensus for defining these terms in absolute concentrations.

#### **1.3.1 Methionine loading**

The methionine loading test was originally introduced to detect heterozygosity for cystathionine  $\beta$ -synthase deficiency<sup>12,13</sup>. It is presently used to stress the Hcy metabolising pathways and is often included in clinical studies on vascular disease. The procedure involves oral intake of a standard dose of methionine (0.1g/kg) with tHcy measurement after a fixed interval of 4 or 6 hours post-load<sup>11</sup>.

The post-load tHcy concentration is probably more sensitive than the fasting concentration to disturbances in the transsulphuration pathway. Thus subjects with impaired ability to remethylate Hcy (cobalamin and folate deficiency) have hyperhomocysteinaemia during fasting, but may have a normal increase in tHcy after methionine loading. In contrast, subjects with a mild disturbance of the transsulphuration pathway often have a normal fasting tHcy but are methionine intolerant<sup>14</sup>. Fasting and post-load tHcy are significantly correlated: they discriminate between vascular patients and controls equally well, but the results do not completely overlap<sup>15</sup>. Fasting tHcy alone may fail to identify a proportion of all subjects with methionine intolerance<sup>16</sup>.

#### **1.3.2 Determinants of plasma homocysteine**

Women have lower tHcy concentrations than men and tHcy increases with age. This may be due to differences in vitamin status between the sexes and in different age groups<sup>17</sup> and to the higher muscle mass in men. Renal function has a central role in the elimination of Hcy from plasma<sup>18</sup>. Urinary excretion of Hcy,

however, is very low and extensive metabolism of Hcy probably takes place in the kidneys<sup>19</sup>. Diet and lifestyle influence tHcy concentration. Intake of vitamins B<sub>6</sub>, B<sub>12</sub> and folate in food or supplements is inversely correlated to plasma tHcy<sup>17</sup>. Smoking and coffee consumption cause a shift of the distribution towards higher tHcy values whereas physical activity is associated with lower levels<sup>20,21</sup>.

#### **1.4 Pathogenesis of hyperhomocysteinaemia (Table 1.1)**

##### **1.4.1 Inherited and congenital defects**

Homocystinuria encompasses a heterogeneous group of diseases caused by inherited defects of Hcy, cobalamin or folate metabolism. The most common form is caused by cystathionine  $\beta$ -synthase deficiency and is described below. Homocystinuria due to inherited defects of cobalamin transport and metabolism, although less frequent, may be the result of a wide variety of defects that involve cobalamin coenzyme synthesis, methionine synthase mutations and abnormalities of cobalamin transport<sup>22</sup>. All lead to similar clinical syndromes, regardless of the underlying mechanism.

In cystathionine  $\beta$ -synthase (CBS) deficiency, the enzyme defect responsible was identified by Mudd et al in 1994. The inactivation of CBS results in blockage of the transsulphuration pathway and intracellular accumulation of Hcy, which is then exported to the circulation (see reference<sup>4</sup> for comprehensive review). CBS deficiency is inherited as an autosomal recessive trait,<sup>23</sup> three classes of CBS mutants have been identified according to the level of enzyme activity: (i) no detectable enzyme activity; (ii) reduced activity and normal affinity for coenzyme; and (iii) reduced activity and reduced affinity.<sup>24</sup>

Clinical manifestations and complications are thought to be the direct consequence of hyperhomocysteinaemia, rather than to any other associated biochemical abnormalities that are known to occur as a result of the metabolic block in Hcy metabolism. The most compelling evidence that incriminates Hcy as the agent directly responsible for the vascular and other complications of hyperhomocysteinaemia comes from a comparison of the metabolic derangements seen in the various disorders leading to elevated Hcy levels. For example, methionine levels are diametrically different in individuals with defects of the transsulphuration

Table 1.1:

**Causes Of Hyperhomocysteinaemia**

**A: Inherited and Congenital Defects**

1. *Enzyme deficiencies*
  - a. Cystathionine  $\beta$  synthase
  - b. Methylenetetrahydrofolate reductase (MTHFR)
  - c. Methionine synthase
  - d. Cobalamin coenzyme synthesis
2. *Transport Defects*
  - a. Transcobalamin II deficiency
  - b. Cobalamin lysosomal transporter

**B. Acquired Disorders**

1. *Nutritional*
  - a. Folic acid deficiency
  - b. Cobalamin deficiency
  - c. Pyridoxine deficiency
2. *Metabolic*
  - a. Chronic renal disease
  - b. Hypothyroidism
  - c. Psoriasis
3. *Drug induced*
  - a. Methotrexate
  - b. Nitrous oxide
  - c. Azaribine
  - d. Oestrogen antagonists

pathway (CBS deficiency) and those of the remethylation pathway (methionine synthase or methylenetetrahydrofolate reductase). The clinical manifestations of homocystinuria include skeletal abnormalities, lens dislocation, mental retardation and occlusive vascular disease including thrombosis and precocious atherosclerosis<sup>2</sup>. The underlying mechanisms that give rise to these complications are not known. The vascular complications are a major cause of morbidity and mortality. Several mechanisms have been proposed to explain the vascular damage and predisposition to thrombosis and are outlined below (Section 1.7).

Methylenetetrahydrofolate reductase (MTHFR) is a key enzyme in the metabolism of Hcy (see Fig. 1.2), and catalyses the reduction of 5,10-methylene tetrahydrofolate to 5-methyl tetrahydrofolate which is the carbon donor for the remethylation of Hcy to methionine. Severe deficiency is rare, but partial MTHFR deficiency is much more common affecting approximately 5-15% of the general population<sup>25</sup>. This enzyme variant is characterised by its distinctive thermolability under specific conditions of heat inactivation<sup>26</sup>. The thermolabile MTHFR mutation is caused by a single base change in DNA (C to T substitution at nucleotide 677)<sup>27</sup> and can now be easily detected by readily available genotyping techniques<sup>28</sup>. Inheritance of the mutation leads to moderate elevation in plasma tHcy and there is some preliminary evidence that this thermolabile MTHFR mutation may be over-represented in patients with coronary artery disease but this remains controversial<sup>25,29</sup>.

## **1.4.2 Acquired disorders**

### **1.4.2.1 Nutrient deficiency**

Folate, cobalamin and pyridoxine are required as cofactors or substrates in the metabolism of Hcy. Deficiency of one (or more) of the three vitamins, arising either from nutritional deficiency, malabsorption, increased utilization or inactivation caused by various chemicals or drugs, interferes with Hcy metabolism causing an increase in the serum level of Hcy. Several studies have shown that plasma concentrations of folate, vitamin B<sub>12</sub> and pyridoxal 5'-phosphate are inversely associated with plasma tHcy concentrations<sup>17,30</sup>. The degree of rise in tHcy caused by these nutrient deficiencies is somewhat variable but is sometimes extreme and may attain the levels encountered in patients with homozygous CBS deficiency.



Individuals with suboptimal intake of folate and cobalamin leading to low-normal levels may also have elevated tHcy levels.

*(a) Folate deficiency*

Dietary folate deficiency accounts for a major proportion of all causes of folate deficiency, the prevalence of folate deficiency being highest among poor, malnourished populations and frequently affects the elderly and alcoholics. Folate, in the form of 5-methyltetrahydrofolate, is an obligatory co-substrate in the conversion of Hcy to methionine catalysed by methionine synthase. Consequently folate deficiency, whatever the cause, decreases Hcy conversion through this pathway increasing plasma Hcy levels in most cases. It has been estimated that, under normal circumstances, approximately half of all available Hcy is metabolised through remethylation<sup>31</sup>. Studies have reported elevated concentrations of tHcy in patients with folate deficiency<sup>32</sup>, however, not all patients with folate deficiency have elevated tHcy levels, which may in part be explained on the basis that the alternative pathway for transmethylation of Hcy through betaine-homocysteine methyltransferase is not folate dependent. Moreover, Hcy may be metabolised through the transsulphuration pathway, which is vitamin B<sub>6</sub> dependent.

Other causes of folate deficiency include a variety of diseases of the small intestine that result in malabsorption, for example tropical and non-tropical sprue as well as extensive inflammatory bowel disease<sup>33</sup>. Folate deficiency may also occur in conditions associated with increased rates of cellular proliferation as a result of greater utilisation of folate (e.g. chronic haemolysis, myeloproliferative disorders and psoriasis)<sup>34</sup>. Antifolate drugs result in a state of functional folate deficiency, the most widely used being methotrexate. Other drugs associated with folate deficiency include trimethoprim, phenytoin, phenothiazines and tricyclic antidepressants. Long-term use of oral contraceptives has also been reported to be associated with folate deficiency<sup>33</sup>; the mechanism may be complex and may possibly also act by an effect on pyridoxine metabolism<sup>35</sup>.

Vitamin supplementation may lower tHcy concentrations and folate is the most potent tHcy-lowering agent. Folate has been used in daily doses ranging from 0.65 to 10mg daily and it seems that in apparently healthy volunteers, a low daily dose of 0.65mg or less may be sufficient to maintain plasma tHcy concentrations within the normal reference range<sup>36</sup>. This low folate dose may, however, be

insufficient in various pathological conditions predisposing towards coronary heart disease, such as patients with chronic renal failure.

*(b) Cobalamin deficiency (vitamin B<sub>12</sub>)*

Cobalamin is also required for the major metabolic pathway of Hcy remethylation. However, folate is required as a co-substrate for the methionine synthase reaction, whereas cobalamin in the form of methylcobalamin, functions as an essential cofactor for methionine synthase (see Fig. 1.2). Cobalamin deficiency, whatever the cause, is associated with an increase in plasma tHcy concentration<sup>37</sup>. Serum cobalamin and folate levels show a statistically significant inverse correlation with tHcy in groups of normal volunteers<sup>38</sup>.

Although folate is the most potent Hcy-lowering agent, vitamin B<sub>12</sub> supplementation has a small but significant effect on circulating tHcy concentrations<sup>36</sup>. Moreover, it has been shown that folic acid supplementation is ineffective in reducing tHcy in patients with vitamin B<sub>12</sub> deficiency.

*(c) Pyridoxine deficiency (vitamin B<sub>6</sub>)*

Both enzymes involved in the two reactions of the transsulphuration pathway require vitamin B<sub>6</sub>. Nutritional deficiency is rare in humans but several studies have established a link between pyridoxine deficiency and atherosclerosis<sup>39,40</sup>. Drugs that may interfere with pyridoxine metabolism include isoniazid, hydralazine and penicillamine.

Owing to its important role in treating CBS deficiency, pyridoxine may be regarded as the obvious choice of treatment of hyperhomocysteinaemia. However, high-dose vitamin B<sub>6</sub> supplementation, without folate and vitamin B<sub>12</sub>, does not lower fasting homocysteine levels significantly in subjects with other causes of raised tHcy<sup>36</sup>. It has been postulated that low folate and/or vitamin B<sub>12</sub> status results in low S-adenosylmethionine concentrations. SAM is required to activate CBS and the supplementation of only vitamin B<sub>6</sub> does not appear to activate the transsulphuration pathway, as the essential activator SAM will remain low owing to the inadequate folate and vitamin B<sub>12</sub> status. Only when the latter two vitamins are in abundance will remethylation proceed unimpeded with a subsequent rise in SAM concentrations and activation of the transsulphuration pathway<sup>41</sup>. Vitamin B<sub>6</sub> will however lower the post-methionine load tHcy peak concentration.

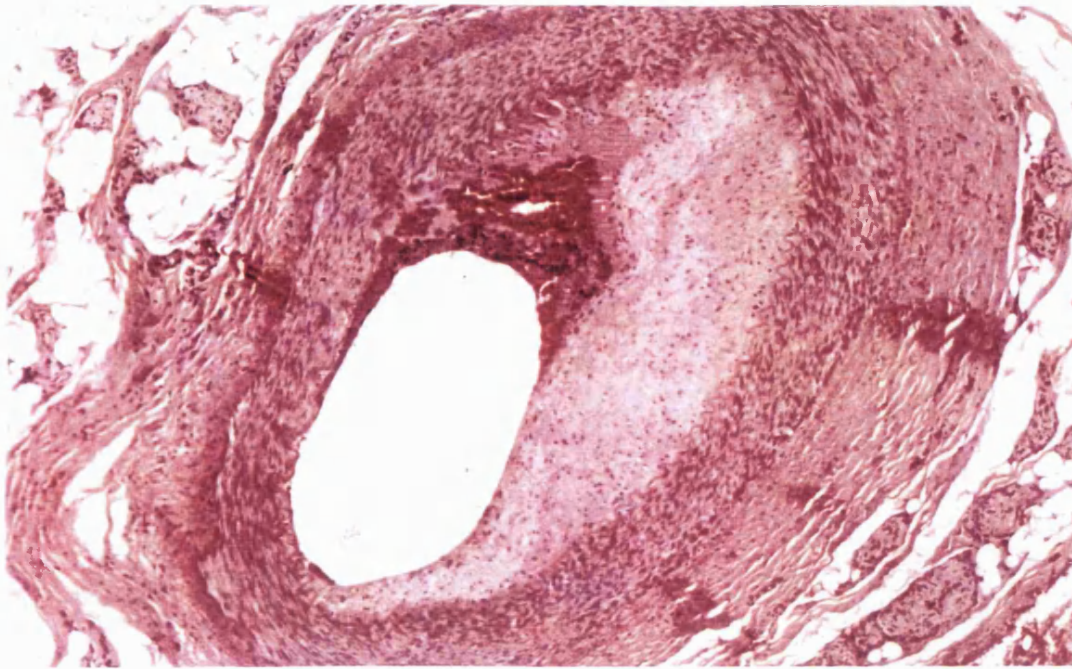
#### 1.4.2.2 Metabolic abnormalities

Moderate hyperhomocysteinaemia may also occur in chronic renal disease<sup>42</sup>. Since renal excretion of Hcy accounts for 1% of the total Hcy produced in the body<sup>31</sup>, it can be concluded that raised levels of tHcy in renal failure may not be attributed solely to reduced renal excretion. Metabolism of Hcy is increased in chronic renal failure through the transsulphuration pathway and it is likely that remethylation is impaired through lack of folate, in accordance with evidence that there may be an intracellular lack of folate, particularly in patients on dialysis<sup>43</sup>.

#### 1.5 Vascular pathology in homocystinuria (Fig 1.3)

Pathological features of vascular damage in homocystinuria affect both arterial and venous systems. Venous thrombosis is frequently identified as the cause of death although, histologically, the veins are generally normal except for the presence of thrombus<sup>3</sup>. In contrast, histological abnormalities of arteries are widespread affecting most arterial systems. The lesions are characterised by intimal and medial hyperplasia, with intimal fibrosis, disruption of the internal elastic lamina and thickening of the media being prominent features. There is a notable lack of lipid, in contrast to the classical appearances of atheromatous vascular disease, which is characterized by plaques containing cholesterol crystals and lipid deposits. This histological distinction indicates that homocystinuria is not simply causing accelerated atherosclerosis.

An international survey of 629 patients with CBS deficiency<sup>5</sup> was reported in 1985. Peripheral venous thrombosis with subsequent pulmonary embolism was relatively common accounting for 42 of 59 deaths; the next most frequent occurrence was cerebral vascular accidents (32%). Peripheral arterial thrombosis and myocardial infarction were reported in 4%.



**Fig 1.3: Histological findings in homocystinuria**

Typical appearances of a blood vessel at post mortem from a patient with homocystinuria. The major findings are:-

- (a) thrombus present in the lumen
- (b) intimal and medial hyperplasia
- (c) disruption of the internal elastic lamina

## **1.6 Cardiovascular disease and mild hyperhomocysteinaemia. Cause or indicator?**

The high incidence of vascular complications in severe hyperhomocysteinaemia in homozygotes for CBS deficiency has focused attention upon Hcy as an atherogenic and thrombophilic agent. For two decades since McCully and Wilson<sup>6</sup> proposed the “homocysteine theory of atherosclerosis” there has been accumulating evidence of mild hyperhomocysteinaemia as a risk factor for vascular disease although interest has waxed and waned.

Evidence that hyperhomocysteinaemia is an independent risk factor for vascular disease (coronary, cerebral and peripheral) has now come from a large number of published epidemiological studies as recently summarised in a meta-analysis<sup>44</sup> of 27 studies up to 1994. The summary odds ratio (OR) as an estimation of the relative risk in people with elevated tHcy levels was 1.7 (95% CI 1.5-1.9) for coronary artery disease; 2.5 (95% CI 2.0-3.0) for cerebrovascular disease; and 6.8 (95% CI 2.9-15.8) for peripheral vascular disease. These ORs were calculated from all included homocysteine determinations, i.e. fasting, basal or post-load, but did not substantially differ from the values found if the calculations were restricted to those studies that measured exclusively fasting levels. This indicates that elevated fasting tHcy levels constitute as strong an excess risk of cardiovascular disease as do the post-load determinations.

The majority of epidemiological evidence is derived from case-control studies but these do not distinguish between cause and effect, i.e. the presence of the disease may have produced, and not caused, the elevated tHcy. Prospective cohort studies, which in general provide the most rigorous evidence, have provided the strongest data to date showing a clear association between tHcy level and risk of ischaemic heart disease<sup>45,46</sup> and importantly, that the dose-response relationship is continuous<sup>47</sup>. However, a few prospective studies have not yielded a positive association<sup>48,49</sup>.

In order to address the need for a large case-control study with sufficient numbers of patients and controls included to establish the association between mild hyperhomocysteinaemia and arterial occlusive disease with more validity, nineteen centres from 11 European countries started their European Concerted Action Project supported by the European Community (Comac)<sup>50</sup>. Low cut-off levels for defining hyperhomocysteinaemia were used, either in the fasting state or after a methionine

load (> upper quintile [20%]), thereby having more relevance to those levels found in the general population. Despite this selection criteria, with elevated fasting and post-load levels defined to be abnormally high when above 12 and 38  $\mu\text{mol/L}$  respectively, the relative risk for all vascular disease (coronary, cerebral and peripheral) for mild fasting and post-load hyperhomocysteinaemia was 2.1 (95% CI 1.6-2.7). After adjustment for other risk factors no change in the OR was observed indicating homocysteine was an independent factor for all vascular disease, equivalent as a risk factor to hypercholesterolaemia and smoking; however, hypertension proved to be a greater risk. Significantly, the interaction between mild hyperhomocysteinaemia and smoking and hypertension indicated a strong synergistic effect of these factors if jointly present in an individual.

In summary, from numerous studies it is clear that mild hyperhomocysteinaemia is strongly and independently related to arterial vascular disease. This relationship has not, however, been conclusively shown to be one of cause and effect, although circumstantial evidence is persuasive. Hcy interacts with other conventional risk factors, notably with hypertension and smoking. Thus vascular patients with combined risk factors leading to synergism in their joint effects may benefit most from interventions aimed at lowering Hcy using inexpensive vitamin supplements, a policy that may have widespread public health implications.

### **1.7 Putative mechanisms for vascular injury**

The mechanism by which Hcy may contribute to vascular damage is an active field of investigation. Damage to endothelium appears to be a key early event in many vascular diseases<sup>51</sup> and may play a central role in the promotion of atherosclerosis by Hcy. The endothelium is a monolayer of cells sited at the interface between blood and vessel wall forming a continuous lining throughout the vascular tree. Endothelium-derived relaxing factor (EDRF), shown to be nitric oxide (NO), is synthesized from L-arginine within the endothelial cell by the constitutive enzyme nitric oxide synthase<sup>52</sup>. NO is released from the endothelium in response to various stimuli of which an important physiological stimulus is pulsatile flow. NO plays a vital role in controlling vascular reactivity, vascular remodelling and coagulation. Endothelial dysfunction, leading to reduced NO activity, may occur without histological evidence of endothelial damage or significant atheroma<sup>53</sup>.

The 'response to injury hypothesis' identifies endothelial injury as a critical initiating event in the development of atherosclerosis<sup>54</sup>. Following endothelial damage, proliferation of smooth muscle cells, increased synthesis of sulphated proteoglycosaminoglycans, fibrosis, calcification and deposition of lipoprotein are important processes in the development of atherosclerosis. Homocysteine could potentially be involved in several of these processes. Indeed, a variety of possible mechanisms for Hcy-mediated vascular injury have been proposed and are reviewed below.

#### 1.7.1 Cellular toxicity and endothelial injury

Homocysteine may exert an effect by direct toxicity to any cellular component of the arterial wall. The effect of Hcy on endothelial function has been studied in various ways. Studies using endothelial cells *in vitro* have shown evidence of cellular injury in the presence of Hcy. Thus Hcy can induce cellular damage as indicated by <sup>51</sup>Cr release from labelled human endothelial cell monolayers in a dose-dependent manner<sup>55</sup>. However, the significance of these data must be questioned in that the effect is not specific to Hcy as it has been shown by others that cysteine can also induce the detachment of endothelial cells at the concentrations used (ca. 5mM)<sup>56</sup>.

Several studies *in vivo* have demonstrated endothelial abnormalities following Hcy infusion. One such study<sup>57</sup>, demonstrated endothelial desquamation in baboons infused with Hcy thiolactone for 5 days. Sustained infusion (3 months) produced eccentric fibromuscular lesions containing intracellular lipids and foam cells, resembling early atherosclerotic lesions in humans.

Proliferation of vascular smooth muscle cells is a prominent feature of atherosclerosis<sup>58</sup>. This is another possible mechanism for Hcy-induced vascular injury. In support of this proposal Hcy has been shown to have a direct growth promoting effect on vascular smooth muscle cells in culture<sup>59</sup>.

The function of endothelium and vascular smooth muscle are intimately related in several respects including the control of vascular tone and blood flow. NO released by endothelium in response to pulsatile flow<sup>60</sup> acts locally to produce vascular smooth muscle cell relaxation via a cyclic GMP-dependent mechanism, modulating arterial distensibility. Alterations in the concentration of bioavailable NO

may therefore affect these functions. The bioavailability of nitric oxide may be decreased by reacting with reactive oxygen species such as superoxide, or its activity may be conserved in the presence of reduced thiols forming nitrosothiols. S-nitrosothiols have potent vasodilatory and anti-platelet effects<sup>61</sup>. In the presence of Hcy, nitric oxide forms S-nitrosohomocysteine.

Adverse effects of Hcy may occur when the endothelium is unable to support nitrosothiol formation due to endothelial injury. Under these conditions, Hcy may then generate H<sub>2</sub>O<sub>2</sub> and undergo conversion to Hcy-thiolactone, which may further contribute to endothelial dysfunction. Homocysteine-mediated endothelial cell toxicity has been shown to be dependent on the duration of exposure to Hcy<sup>62</sup>, brief (15 min) exposure of endothelial cells to Hcy resulted in the formation of S-nitrosohomocysteine whereas prolonged (>3 hrs) exposure resulted in impaired NO-mediated responses.

Results, however, from *in vitro* experiments need to be interpreted with caution. Most have used high concentrations of Hcy well above the range for mild hyperhomocysteinaemia and even homocystinuria. Lentz and co-workers have recently reported the effect of more relevant levels of Hcy on endothelial function<sup>63</sup>. This primate model demonstrated vascular dysfunction in monkeys with hyperhomocysteinaemia, achieving an increase in plasma tHcy from 4 to 10 µM by dietary modification (methionine feeding, folate deficient). *In vivo* changes in blood flow to the leg after infusion of endothelium-dependent and-independent agonists together with vasomotor responses measured *ex vivo* in carotid arteries were determined. Relaxation to endothelium-dependent vasodilators was impaired in the hyperhomocysteinaemic monkeys as was the endothelium-independent response to nitroprusside, although to a lesser extent. These data suggest that endothelial function and possibly also smooth muscle cell function may be impaired by mild hyperhomocysteinaemia.

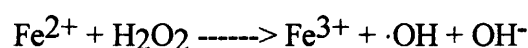
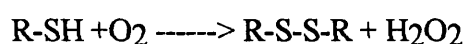
The most persuasive evidence is likely to come from studies in man. Confirmation that endothelial dysfunction occurs in homocystinuria comes from a clinical study by Celermajer and colleagues using non-invasive external vascular ultrasound<sup>64</sup>. Flow-mediated dilatation of the brachial artery is endothelium-dependent and is a measure of the flow-related NO response in that artery<sup>65</sup>. In this study, brachial or femoral artery responses to reactive hyperaemia (flow-mediated, endothelium-dependent) were measured in 9 children (4 to 17 yrs) with homozygous



homocystinuria. Although plasma homocysteine concentrations were not measured, flow-related endothelial function was markedly impaired in these children but not in their heterozygous parents or normal controls. These data, if confirmed with other studies, would strongly support the proposal that hyperhomocysteinaemia can cause endothelial damage in humans.

### 1.7.2 Free-radical mediated damage

It has been proposed by many authors that a molecular mechanism for Hcy-mediated damage may involve free radical production<sup>66</sup>. At the simplest level of redox reactions it should be noted that Hcy in plasma can exist in its reduced form but is usually rapidly auto-oxidized to homocystine, homocysteine-mixed disulphide and homocysteine-thiolactone. Olszewski and McCully have proposed chemical reactions whereby Hcy could lead to production of free radicals<sup>67</sup>: -



The sulphydryl group of Hcy could act catalytically with ferric or cupric ions in a mixed function oxidation system to generate hydrogen peroxide, hydroxyl radicals and homocysteinyl radicals. Free radicals produced by this or other reactions could cause pathological damage by several mechanisms including direct cellular toxicity, lipid peroxidation and reactions with nitric oxide.

Homocysteine is acutely cytotoxic to cultured endothelial cells, an effect that may be prevented by the addition of catalase, implicating hydrogen peroxide<sup>68</sup>. However, this cytotoxicity is dependent on the presence of cupric ions suggesting a direct role of additional oxidants. *In vitro* high levels of Hcy can generate superoxide anions ( $\text{O}_2\cdot^-$ ) and hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) via auto-oxidation. Attention has focused on these oxidative products in mediating the cytotoxic properties of Hcy. Radicals generated intracellularly may pose a greater threat to cellular function.  $\text{H}_2\text{O}_2$  freely crosses the cell membrane and is normally converted to  $\text{H}_2\text{O}$  by catalase or glutathione peroxidase<sup>69</sup>. Intracellular glutathione peroxidases are potent antioxidants and their depletion may decrease the cell's ability to combat oxidative stress. Homocysteine is unique in that it inhibits glutathione peroxidase

activity *in vitro* unlike other thiols, providing another possible explanation for endothelial cell toxicity<sup>70</sup>.

The hydroxyl radical is highly reactive and can initiate lipid peroxidation reactions. Hence, it has been proposed that this could cause oxidative modification of low-density lipoprotein and actively promote atherosclerosis. Although this is an attractive hypothesis, the experimental evidence is not supportive. No increase in lipid peroxides could be found in plasma from patients with severe homocystinuria when assayed by highly specific techniques nor indeed was there consumption of ubiquinol<sup>71</sup>. Furthermore, LDL isolated from patients with homocystinuria was not more susceptible to oxidation *in vitro*<sup>72</sup>.

Alternative free radical mechanisms (other than lipid peroxidation) could operate to increase NO catabolism, particularly the formation of the powerful oxidant peroxynitrite<sup>73</sup> (ONOO<sup>-</sup>), the adduct formed from the combination of superoxide anions and nitric oxide. This reaction is more than three times faster than catabolism of superoxide by superoxide dismutase<sup>74</sup>. Because peroxynitrite is formed optimally from equimolar concentrations of NO and superoxide<sup>75</sup>, it is unlikely that peroxynitrite would achieve high concentrations under normal conditions. In fact low concentrations of peroxynitrite have been shown to behave very similarly to NO and cause vasorelaxation<sup>76,77</sup>. In contrast, higher concentrations of peroxynitrite may be very toxic. It can form the cytotoxic peroxynitrous acid<sup>78</sup>, cause hydroxyl radical toxicity<sup>79</sup> and cause protein fragmentation by nitration of amino acids<sup>80</sup>. Hypercholesterolaemia, as a risk factor for atherosclerosis, has been shown to impair the glutathione detoxification mechanism against peroxynitrite<sup>81</sup> and this may also potentially occur in hyperhomocysteinaemia<sup>70</sup>. The addition of antioxidants may have a beneficial role in these circumstances reducing NO consumption and excessive peroxynitrite formation.

Thus the hypothesis that homocysteine may promote vascular damage by free radical mechanisms is plausible, in some respects, but as yet lacks convincing experimental backing.

### 1.7.3 Homocysteine and thrombosis

The endothelial surface plays a major role in thrombosis and fibrinolysis. This aspect in relation to Hcy has been reviewed in detail<sup>82</sup>. In summary,

hyperhomocysteinaemia appears to be able to promote thrombosis by increasing Factor V and Factor XII activity, decreasing protein C activation, inhibiting thrombomodulin expression and suppressing heparin sulphate expression. Thrombin formation is the final common pathway.

Most of these effects have been demonstrated by high concentrations of Hcy *in vitro* and their application *in vivo* remains unproven. Of particular recent interest is the demonstration that patients with concurrent homocystinuria and factor V Leiden have an increased risk of thrombosis<sup>83</sup>. Factor V Leiden produces an amino acid substitution in factor V, thereby altering the first cleavage site involved in the activation of factor V. The mutation has been shown to be present in patients of various ethnic origins and in 3 to 7 percent of healthy people in two white populations.

An interesting link between lipoproteins, fibrinolysis and Hcy has been postulated to involve lipoprotein (a). Lp (a) may have antifibrinolytic actions and atherothrombotic potential. Lp (a) is closely related to low-density lipoprotein but with the addition of a specific protein (apoprotein (a)) linked to apoprotein B by a disulphide bond. Concentrations of Hcy *in vitro* down to 8  $\mu$ M markedly increase the affinity of Lp (a) for plasma-modified fibrin surface<sup>84</sup>. Homocysteine-modified Lp (a) could effectively compete with plasminogen for binding sites on fibrin coated surfaces, thereby creating a more thrombotic environment. This effect needs to be examined in more detail *in vivo*.

Most studies have not reported a consistent or specific effect of Hcy on circulating coagulation factors. Therefore any effect of Hcy on thrombosis is most likely to be mediated by changes in the endothelium, which has an important role to play in coagulation/fibrinolytic systems.

#### 1.7.4 Cellular matrix effects

It is intriguing that patients with homocystinuria share some of the clinical and pathological features of Marfan syndrome. Why these two different conditions share a common phenotype remains unknown. The genetic defect in Marfan syndrome is due to a mutation in fibrillin, recently shown to be in abundance on the endothelial cell basement membrane<sup>85</sup> and may play a crucial role in endothelial cell physiology. Endothelial dysfunction has been shown to occur in Marfan syndrome<sup>86</sup>. Interestingly, fibrillin contains a number of cysteine-rich domains, which are

essential in determining the tertiary structure of fibrillin microfibrils. One can speculate that hyperhomocysteinaemia may interfere with the chemical structure of the cysteine-rich domains, impairing effective cross-linking of microfibrils. Thus a possible link between Marfan syndrome and homocystinuria may be a defect in fibrillin, genetically acquired in one and metabolically altered in the other.

Homocysteine has been shown to alter the tertiary structure of connective tissue. A reduction in cross-linking of collagen has been demonstrated in patients with homocystinuria and proposed as a possible explanation for the observed skeletal abnormalities, in particular the finding of osteoporosis.<sup>87</sup>

In summary, Hcy appears to be directly toxic to the vasculature in patients with homocystinuria and there is increasing evidence that mild hyperhomocysteinaemia may induce vascular damage. At the cellular level, the primary mechanism for this injury appears to be toxicity to the endothelium leading to disruption of normal vasomotor function and the promotion of endothelium-mediated thrombosis. At a molecular level, the mechanisms are not well understood. Promotion of free radical activity and alterations in intracellular redox status are one possibility but experimental evidence is not strong. In particular, there is evidence that Hcy does not promote oxidation of lipoproteins. Other possible effects on cellular matrix are speculative. Therefore, further studies are required directed towards elucidating the effect of Hcy on endothelial function, in particular on the effect of mild hyperhomocysteinaemia.

## 1.8 Hypotheses and aims

### Hypotheses

Experimental studies were designed to address the following hypotheses:-

*(1) Endothelial function can be measured non-invasively in the brachial artery in humans by a method utilising the phenomenon of flow-mediated vasodilatation.*

*(2) An acute elevation of plasma homocysteine concentration after an oral methionine load impairs endothelium-dependent brachial artery dilatation in humans as a consequence of an increase in oxidant stress.*

*(3) Mild hyperhomocysteinaemia is associated with endothelial dysfunction*

*(4) Folate supplementation lowers plasma homocysteine and improves endothelial function.*

*(5) At the molecular level, homocysteine reduces nitric oxide activity by a mechanism involving the production of reactive oxygen species and/or alteration in intracellular redox status.*

### Aims

The specific aims of the thesis are as follows:-

*(i) To demonstrate that brachial artery dilatation in response to hyperaemic blood flow, measured by ultrasonic vessel wall-tracking, is an endothelium-dependent mechanism. Non-invasive measurement of endothelial function is described in Chapter 2. Measurements of brachial artery vasodilatation in response to hyperaemic flow are undertaken before and after infusion of a nitric oxide synthase antagonist, N<sup>G</sup>-monomethyl-L-arginine.*

*(ii) To determine normal values and investigate the best parameter to represent flow-mediated and GTN-mediated changes in brachial artery diameter. In Chapter 2, flow-mediated and GTN-mediated brachial artery responses are measured in a large cohort of healthy subjects. Historically, changes in vessel size in response to endothelium-dependent dilatation have been published as percentage change, i.e.*

the percentage ratio of post- to baseline arterial diameter. A multivariate analysis is used to investigate this further and to test three alternative parameters, which can be used as a measure of endothelium-dependent and –independent vasodilatation

*(iii) To investigate the effect of acute hyperhomocysteinaemia on endothelial function in healthy subjects.* Plasma Hcy concentrations rise significantly following an oral methionine load. An acute elevation in Hcy would be expected to be injurious to endothelium. This is investigated in Chapter 3 using a randomised crossover study in healthy subjects. The possible protective effect of prior administration of an oral antioxidant (vitamin C) is also studied.

*(iv) To determine whether mild hyperhomocysteinaemia is associated with impaired endothelium-dependent vasodilatation.* In order to identify subjects with elevated Hcy levels, two different approaches are used to highlight various abnormalities in the transsulphuration and remethylation pathways: (a) subjects with impaired remethylation of Hcy due to low vitamin B<sub>12</sub>/ folate status are studied in Chapter 4, (b) a population study cohort consisting of 240 healthy volunteers across the range of plasma Hcy concentrations (who may have elevated levels as a consequence of genetic and/or nutritional factors) is described in Chapter 5. Endothelium-dependent and –independent brachial artery responses are compared in both groups.

*(v) To test the hypothesis that endothelial dysfunction in hyperhomocysteinaemia can be improved by lowering plasma homocysteine with vitamin supplements.* Vitamin supplementation can lower plasma Hcy concentrations even in subjects with normal homocysteine levels. Folic acid, in the absence of vitamin B<sub>12</sub> deficiency, is the simplest and most effective Hcy lowering agent. In Chapter 6, the effect of oral folic acid supplementation on flow-related endothelial function is investigated in subjects identified with mild hyperhomocysteinaemia from a large cohort of healthy volunteers in a randomised crossover study. The role of methylenetetrahydrofolate reductase genotype in modulating homocysteine concentrations and the response to folate treatment is also determined.

*(vi) To investigate the possible mechanism(s) whereby homocysteine modulates endothelium-dependent relaxation of vascular smooth muscle.* In Chapter 7, the effect of Hcy on agonist-induced, endothelium-dependent relaxation of precontracted rabbit aortic rings is investigated using a standard tissue bath preparation. The various responses to the other constituents of the transsulphuration

pathway are compared at the same concentration. The effect of prior incubation of Hcy with various antioxidants/superoxide scavengers is performed to elucidate possible pathophysiologic mechanisms.

## CHAPTER 2

### Non-Invasive Measurement Of Endothelial Function Using Ultrasonic Vessel Wall-Tracking

#### 2.1 Introduction

##### *Background*

Since the initial discovery by Furchgott in 1980 of endothelium-derived relaxing factor<sup>88</sup>, subsequently identified as nitric oxide (NO),<sup>89</sup> a number of techniques have been used to measure endothelial function in man, both using direct methods (the stimulation of NO release by receptor-mediated, endothelium-dependent agonists, e.g. acetylcholine<sup>90</sup>) and indirect methods (e.g. plasma von Willebrand factor and thrombomodulin assays<sup>91</sup>). More recently, non-invasive but direct methods have become available that utilise the phenomenon of flow-mediated vasodilatation<sup>60</sup> in conduit arteries, a mechanism mediated by nitric oxide<sup>92</sup> that enables much larger numbers of subjects to be studied compared with invasive techniques. Moreover, despite the measurement of vascular responses in an artery that is not characteristically affected by atheroma, endothelial dysfunction when present is likely to be a generalised phenomenon; it has been shown that there is close association between endothelial dysfunction in the human coronary and peripheral circulations<sup>93</sup>.

Celermajer and colleagues originally reported a well described non-invasive method of detecting endothelial dysfunction in 1992<sup>94</sup>. Flow-mediated dilatation of the brachial artery, a surrogate marker of endothelial function (induced by producing hyperaemic flow in the forearm), was shown to be impaired in children and young adults with established risk factors for atherosclerosis prior to the development of any overt clinical disease. Vasodilatation following sublingual glyceryl trinitrate (GTN), which acts on vascular smooth muscle directly, and is therefore independent of the endothelium, was unaffected in these subjects. The technique has now been utilised by a large number of investigators, validated albeit with variations in the methodology used<sup>95,96</sup>, and applied to a wide variety of diseases in which endothelial dysfunction has been demonstrated<sup>51</sup>. More importantly, the technique provides a way of investigating the effect of possible therapeutic interventions in relatively large numbers of subjects.



A drawback of the original method, however, was that brachial artery diameter was measured with standard high-resolution ultrasound equipment with a maximum theoretical resolution of 0.1 mm. In an artery with an internal diameter typically measuring between 3-4 mm, the expected vasodilatation induced by forearm hyperaemia is in the order of 0.1 - 0.2 mm, i.e. very near the maximum resolution of the equipment used. An important point in the methodology was that the cuff used to produce occlusion of blood flow to the lower arm and hand (and thus hyperaemic flow on release) was placed *downstream* of the brachial artery to avoid ischaemia of the vessel being studied. The success of the original work by Celermajer relied on large numbers of subjects (ca. n=100) in their studies, which allowed clear differences between populations to be determined.

#### *High-resolution wall-tracking*

The resolution of vascular measurements can be improved with the use of ultrasonic vessel wall-tracking, a method initially developed by Hoeks<sup>97</sup> to measure carotid artery distensibility and later adapted to measure flow-related endothelial function in the brachial artery by Ramsey and colleagues<sup>98</sup>, with a theoretical spatial resolution of  $\pm 3\mu\text{m}$ . In practice the system, which is never required to reach this specification, operates in the region of  $\pm 10\text{-}20\ \mu\text{m}$ , an approximately 100-fold improvement in resolution compared with direct measurement of internal artery diameter from the M-mode image using electronic callipers. In addition, blood flow in the brachial artery is recorded by continuous wave Doppler ultrasound to ensure that the hyperaemic stimulus to brachial artery dilatation is similar between study subjects, allowing valid comparisons to be made.

#### *Aims*

This chapter describes the methodology used to measure flow-related endothelial function and blood flow in all subsequent clinical studies. Experiments were undertaken to confirm that the method is a measure of endothelial function, demonstrated by the comparison of responses before and after infusion of the NOS-III antagonist,  $\text{N}^{\text{G}}$ -monomethyl-L-arginine (L-NMMA). Normal values for healthy subjects, repeatability data and age-related changes were also determined.

## 2.2 Subjects

Four individual groups of subjects were enrolled (see section 2.3). All volunteers were recruited from members of hospital staff. Characteristics of the study subjects are shown in Table 2.1.

### *N<sup>G</sup>-monomethyl-L-arginine (L-NMMA) study*

Five healthy subjects (mean age, 29 years; range, 21 to 49 years; 4 men) volunteered to participate in this invasive study, the infusion protocol having been approved by the Local Research Ethics Committee.

### *Repeatability Study*

Twelve subjects (mean age, 20 years; range, 21 to 40 years; 7 men) had vascular measurements performed on three separate occasions.

### *Regression modelling*

Data from 240 ‘normal’ healthy subjects recruited in a cross-sectional study to investigate the relationship between plasma homocysteine and endothelial function (see Chapter 5), were used for regression modelling analysis (mean age, 35 years; range, 19 to 69 years; 110 men).

### *Effect of age*

In order to investigate the effect of age in normal subjects without known risk factors for endothelial dysfunction, the above cohort of 240 subjects was used but 17 individuals with plasma homocysteine >15  $\mu\text{mol/L}$  were excluded, leaving  $n=223$  for subsequent analysis (mean age, 35 years; range, 19 to 69 years; 99 men).

All subjects gave informed, written consent and were non-smokers, were normotensive (blood pressure <150/90 mmHg), were not diabetic and had serum cholesterol < 6.5 mmol/L. This level of serum cholesterol was selected as the upper limit of “normal” in knowledge of levels in the community and data relating flow-mediated vasodilatation to serum cholesterol concentration<sup>99</sup>.

## 2.3 Methods

### 2.3.1 Non-invasive measurement of flow-mediated brachial artery dilatation

#### *Protocol*

The protocol was established following preliminary exploratory studies. All studies were performed in a temperature-controlled room (21-23°C) on fasting

**TABLE 2.1: Subject characteristics**

<b>Parameter</b>	<b>L-NMMA</b>	<b>Repeatability Study</b>	<b>Population Cohort</b>
No.	n=5	n=12	n=223
Age, years	29 (10)	20 (6)	35 (11)
Sex, M/F	4 / 1	7 / 5	99 / 124
Range, years	21 - 49	21 - 40	19 - 69
Total homocysteine, $\mu\text{mol/L}$	10.9 (2.9)	9.4 (2.0)	8.3 (2.5)
Total cholesterol, $\text{mmol/L}$	4.6 (0.8)	4.6 (0.5)	4.8 (0.8)
Triglyceride, $\text{mmol/L}$	0.8 (0.2)	1.0 (0.3)	1.1 (0.6)
Glucose, $\text{mmol/L}$	4.5 (0.4)	4.8 (0.5)	4.7 (0.4)

Data are mean (SD).

subjects following >15 minutes supine rest, with the left arm held outstretched on a pneumatic cushion. Caffeine-containing beverages were avoided for 12 hours before the study.

Measurements of internal brachial artery end-diastolic diameter, blood flow and blood pressure were made at the following times:-

- (i) baseline
- (ii) during increased brachial artery blood flow induced by hand hyperaemia (see below for methodology)
- (iii) after continuing return to baseline >15 minutes later
- (iv) 3 minutes after sublingual glyceryl trinitrate (GTN) spray (400µg)

The 2 interventions of increased flow and GTN were always performed in that order to avoid pharmacological carryover effects, preliminary experiments having shown that the dilator response to GTN lasts > 40 minutes.

#### *Experimental procedure and data processing*

Endothelial function was assessed by measurement of flow-mediated brachial artery dilatation as described by Celermajer<sup>94</sup>, but further adapted using ultrasonic wall-tracking.

The high-resolution wall-tracking system used in these studies consists of a specially adapted duplex colour flow ultrasound machine (Diasonics Spectra™) with a 7.5 MHz linear-phased array transducer (giving high axial resolution). A schematic representation of the equipment used is shown in Fig. 2.1. The brachial artery was identified using the ultrasound transducer. A standoff device containing ultrasound-coupling gel was placed between the transducer to prevent compression of the anterior wall of the artery. The transducer was held in a stereotactic clamp (Fig 2.2) and a two dimensional B-mode image of the brachial artery obtained.

The M-mode cursor was positioned perpendicular to the vessel (Fig 2.3 A) and the horizontal distance between the cursor and the anatomical landmark recorded using the electronic callipers. Depth and focus settings were then optimised to maximise image quality. The radio-frequent (RF) signals from the M-mode output were relayed to the wall-tracking system (Vadirec) and digitised (Fig 2.4). The

**Fig 2.1: Schematic diagram of equipment used in the measurement of forearm vascular responses**

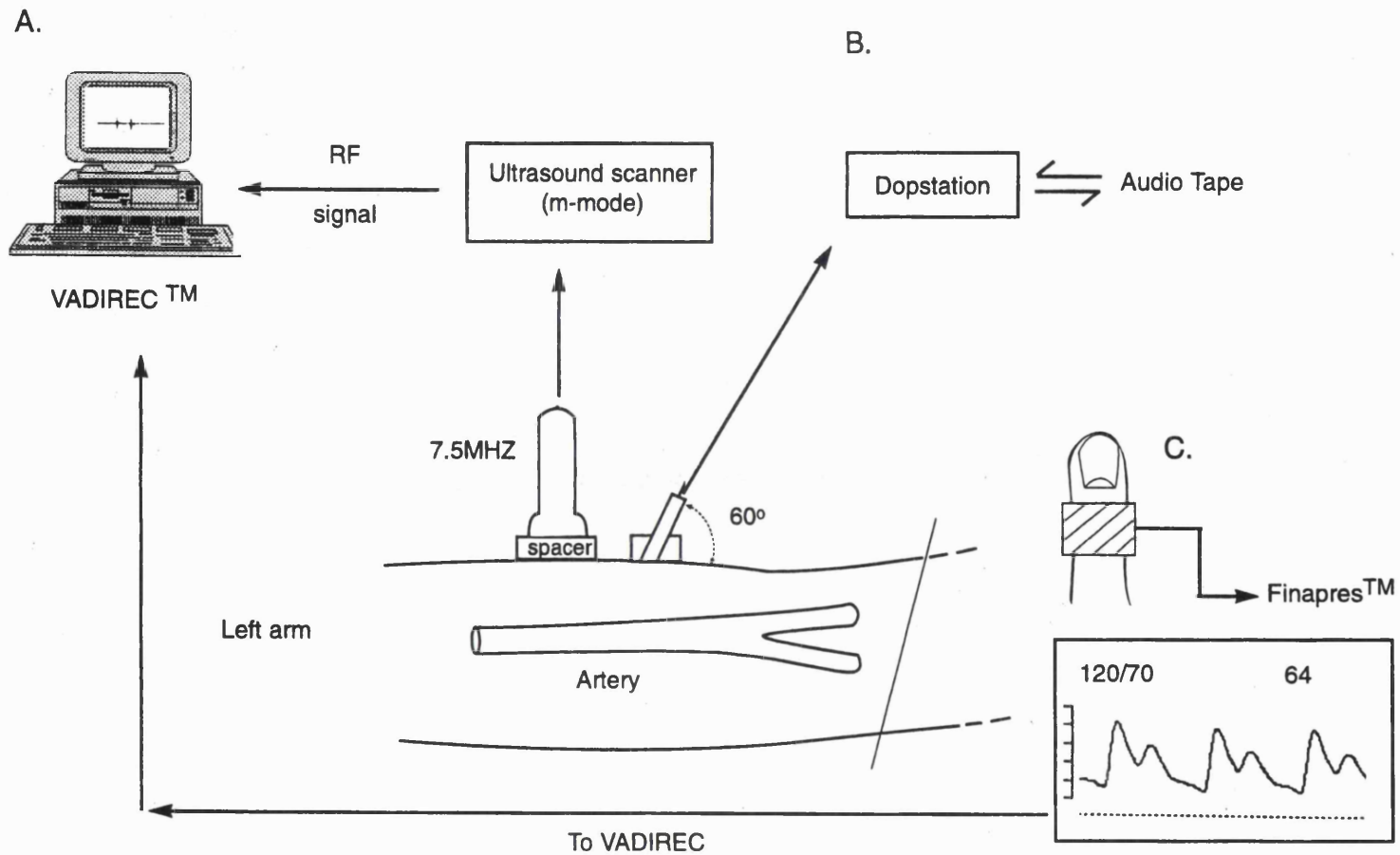
Measurements are divided into 3 different modules:-

A – measurement of brachial artery end-diastolic diameter

B – measurement of blood velocity and blood flow

C – measure of pulse and blood pressure by photoplethysmography (Finapres)

The vascular distension recorder (Vadirec) is directly connected to the Finapres to allow input of data. Blood velocity waveforms, from which blood flow is calculated, are separately recorded on magnetic audio tape using a high-performance recorder.



- A. Measurement of brachial artery end-diastolic diameter
- B. Measurement of blood velocity and blood flow
- C. Measurement of pulse and blood pressure (Photoplethysmography)

**Fig 2.2: Non-invasive hardware and set-up of ultrasound transducer with related equipment**

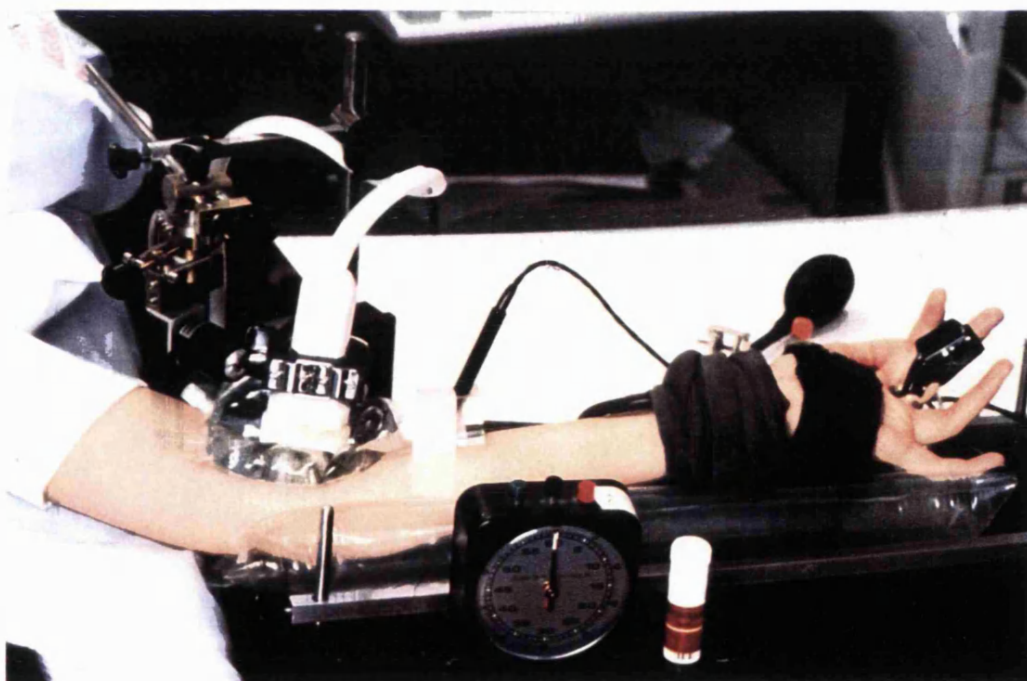
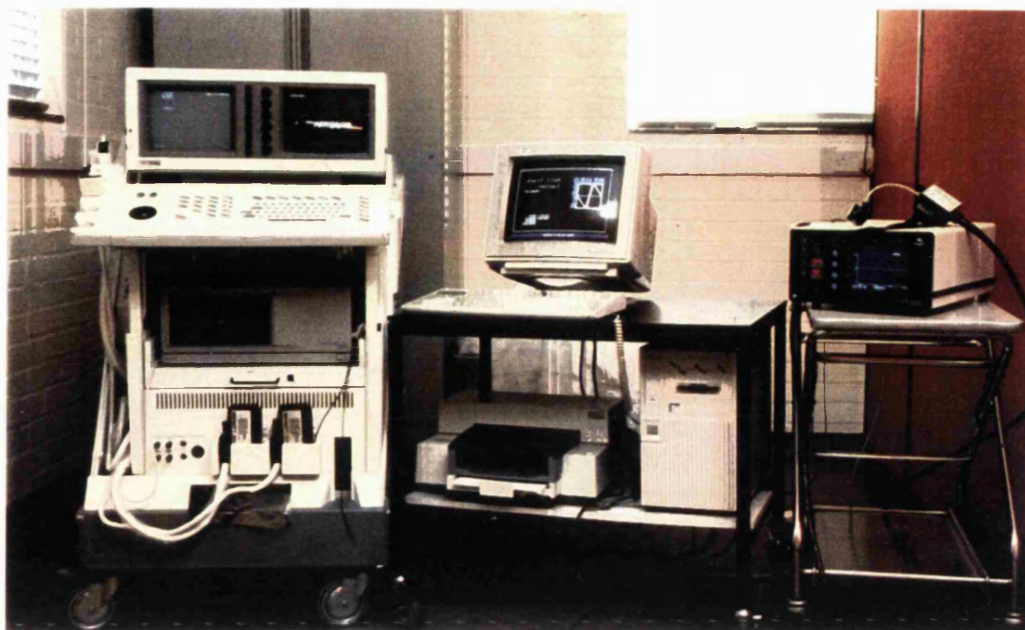
Top panel

The main hardware used consists of a dedicated high resolution ultrasound machine (Diasonics Spectra), wall-tracking computer (vascular distension recorder, Vadipec) and Finapres photoplethysmography device.

Bottom panel

The patient lies supine with the left arm outstretched on a pneumatic cushion. A sphygmomanometer cuff is placed at the wrist. A spacing device is located between the forearm and the 7.5 MHz ultrasound transducer to prevent compression of the anterior artery wall and to improve image quality. The transducer can be finely adjusted by means of a stereotactic clamp with a magnetic base. A finger cuff connected to the Finapres is attached to the ipsilateral middle finger.

An 8 MHz continuous wave Doppler probe, held in a perspex block at 60° to the forearm, is positioned to provide an optimal waveform of forearm blood velocity from which flow is later calculated using the SciMed Dopstation.



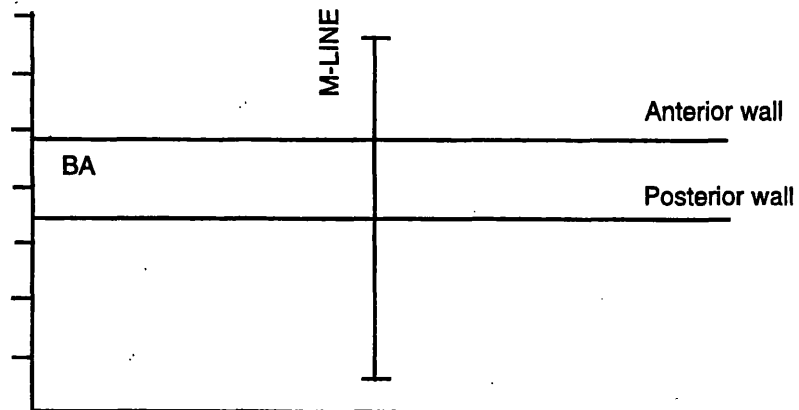


**Fig 2.3: Methodology for the determination of computer-generated waveforms and calculation of brachial artery end-diastolic diameter by the VadiREC system**

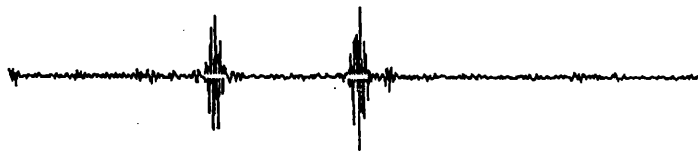
- A – The M-mode line is positioned perpendicular to the point of measurement after the anterior and posterior wall images have been satisfactorily obtained using 2D ultrasound (BA=brachial artery).
- B - Sample volume markers are placed over the deflections representing brachial artery anterior and posterior walls.
- C - Example of a displacement waveform (intravascular diameter as a function of time) generated by the wall-tracking system showing movement of anterior and posterior walls, beat to beat.
- D - Computer generated pressure and distension waveforms with calculated data for each beat presented in columns and mean values representing the following parameters:-

<i>Beat</i>	- sequence number detected heart beat
<i>Diam (mm)</i>	- minimum internal brachial artery diameter, calculated from the initial distance between markers and corrected for distension at end-diastole
<i>Dist (μm)</i>	- distension of the vessel, representing highest minus lowest value of the distension curve during one heartbeat in μm
<i>Pdia (mmHg)</i>	- minimum blood pressure
<i>pp (mmHg)</i>	- pulse pressure
<i>cc E<sup>3</sup> (mm<sup>2</sup>/Pa)</i>	- compliance coefficient
<i>dc E<sup>-3</sup> (1/KPa)</i>	- distensibility coefficient

A

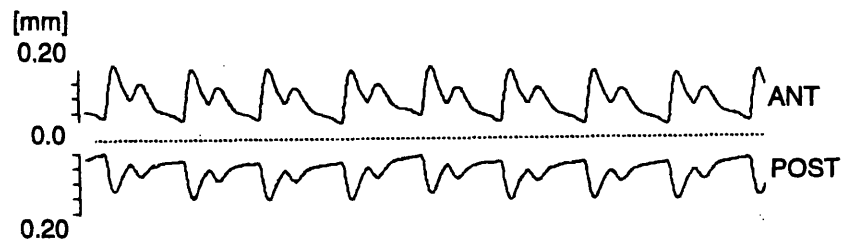


B

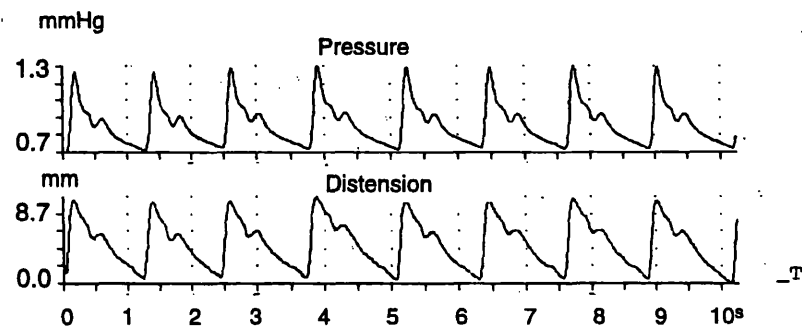


Measurement range a-> b	mm	:	10	26
Depth anterior cursor	mm	:	15	
Distance between cursors	mm	:	3	
M mode sample interval	ms	:	1	
Recording time	s	:	10	

C



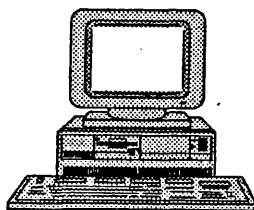
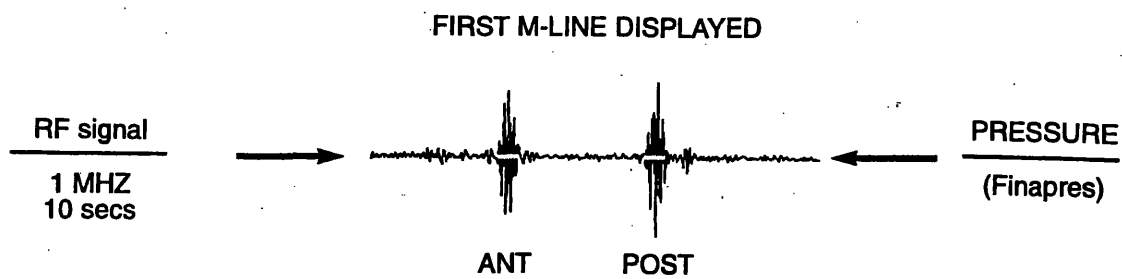
D



Beat	Diamm mm	Dist µm	Pdia mmHg	pp mmHg	cc E-3 mm2/Pa	dc E-3 1/KPa
0	3.33	72	67	55	51.9	6.0
1	3.32	76	68	54	55.8	6.4
2	3.32	75	70	55	54.1	6.2
3	3.32	80	69	57	55.7	6.4
4	3.32	78	69	57	54.2	6.3
5	3.32	78	70	55	56.2	6.5
6	3.32	79	69	57	55.0	6.3
7	3.32	77	69	57	53.5	6.2
mean	3.32	77	69	56	54.5	6.3
std	0.00	3	1	1	1.4	0.2

**Fig 2.4: Diagram demonstrating data processing steps of the wall-tracking computer from generation of sample volume markers to displacement and distension waveforms**

Data is converted from analogue to digital and held offline within an internal buffer memory on a first in first out basis. Doppler signal processing is then executed for calculation of arterial diameter between anterior and posterior wall data windows from which waveforms are generated.



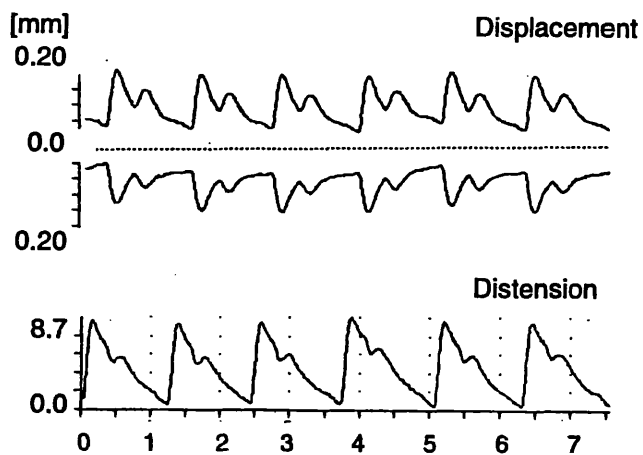
**VADIREC™**  
Wall tracking system

A → D Conversion

Internal Buffer Memory  
(FIFO)

Doppler Signal Processing  
(Executed for anterior and posterior  
data windows)

Displacement and Distension Waveforms



sampling frequency of RF signals was 1 kHz, and each total recording time was 10 seconds.

On completion of data acquisition, the first RF-signal was displayed on the computer screen. From the sample volumes, the operator marked the positions of the anterior and posterior internal artery wall (Fig 2.3 B) whose movements were 'tracked', using the stored RF signals, to give the distension waveform (intravascular diameter change as a function of time) beat-to-beat<sup>97</sup> (Fig 2.3 C). End-diastolic intravascular diameter (mm) was then calculated for each beat and the mean value displayed (Fig 2.3 D).

Preliminary studies have shown that maximum dilatation occurs up to 2 mins following cuff release (see Section 2.4.2). Internal brachial artery diameter was measured for 10-second periods at (and spanning) 1, 2, 3, 4 and 5 minutes after cuff release. The increase in diameter is influenced by basal diameter and is accordingly expressed in absolute terms (see Section 2.4.3). The peak increase in internal end-diastolic diameter, at either 1 or 2 min was used as the measure of flow-mediated dilatation.

#### *Measurement of blood flow, heart rate and blood pressure*

Blood flow was measured using an 8 MHz continuous wave Doppler probe mounted at an angle of 60° in a Perspex block and positioned over the brachial artery distal to the 7.5 MHz probe (Fig 2.1 and Fig. 2.2). The Doppler signals were analysed by a spectrum analyser (SciMed Dopstation™) and stored on metal audiotape using a high performance recorder (Nakamichi B-100E). Brachial artery blood flow was derived from the mean blood velocity (corrected for Doppler angle). Heart rate and blood pressure were recorded by photoplethysmography (Finapres™) from a finger cuff on the middle finger of the ipsilateral arm.

#### *Hand hyperaemia*

Increased brachial artery blood flow was induced by hyperaemia of the hand. After taking baseline measurements, a paediatric sphygmomanometer cuff was inflated at the wrist (thus avoiding ischaemia of the brachial artery itself) to supra-systolic pressure for 5 minutes and then abruptly released. The duration of cuff inflation was chosen to produce maximal vasodilatation<sup>100</sup>. Blood flow was monitored continuously for 15 seconds before until 5 minutes after cuff release.

Recording on tape was done over 15 second periods starting at cuff release (hyperaemic flow) (Fig 2.5) and at 1 min intervals thereafter to correspond with diameter measurements. Flow at each time point was derived from the mean velocity time integral corrected for Doppler angle and internal brachial artery diameter. Increase in blood flow is expressed as a simple increment from the preceding basal level.

The time-course of changes in blood flow following cuff release is shown in Fig. 2.6 (mean values for subjects in the repeatability study, n=12).

### 2.3.2 Intra-arterial infusion of L-NMMA

#### *Protocol*

In order to demonstrate that flow-mediated dilatation using this method is predominantly an endothelium-dependent mechanism, brachial artery dilatation in response to hyperaemic blood flow was measured before and after infusion of the NOS-III antagonist, N<sup>G</sup>-monomethyl-L-arginine (L-NMMA) in 5 adult volunteers.

Subjects lay with their non-dominant arm outstretched on a pneumatic cushion (left arm in all 5 subjects). Using 1% lignocaine local anaesthesia, a 24G cannula (Venisystems) was inserted percutaneously into the brachial artery 2-5 cm proximal to the antecubital fossa. Brachial artery end-diastolic diameter, blood flow and blood pressure were measured non-invasively distal to the puncture site in the manner described above, during the following sequence of infusions:

- (i) 0.9% saline (with added Heparin 1U/mL)  
Baseline measurement of flow-mediated brachial artery dilatation.
- (ii) L-NMMA (Clinalfa AG) infused at 3 mg/min (16µmol/min) for 20 minutes. Measurement of brachial artery dilatation made thereafter.

The drug solution was freshly prepared using an aseptic technique in a sterile environment and was administered using a P4000 IVAC infusion pump. The rate of arterial infusion of 0.9% saline was maintained at 0.5 mL/min throughout the study. Following completion of the infusion regimen and vascular measurements, the

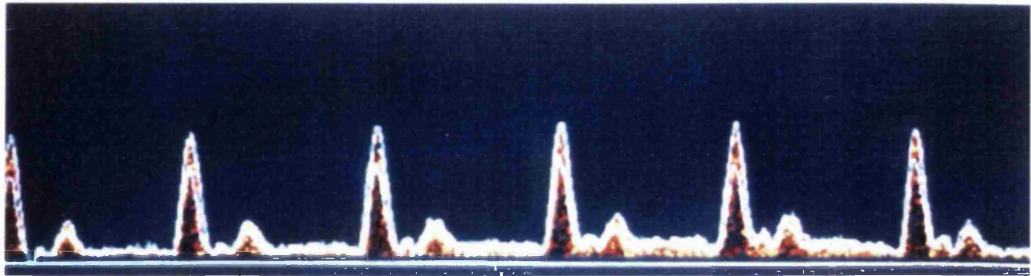
### **Fig 2.5: Blood velocity waveforms**

Waveforms are generated using an 8 MHz continuous wave Doppler probe connected to a SciMed Dopstation and recorded on metal audio tape.

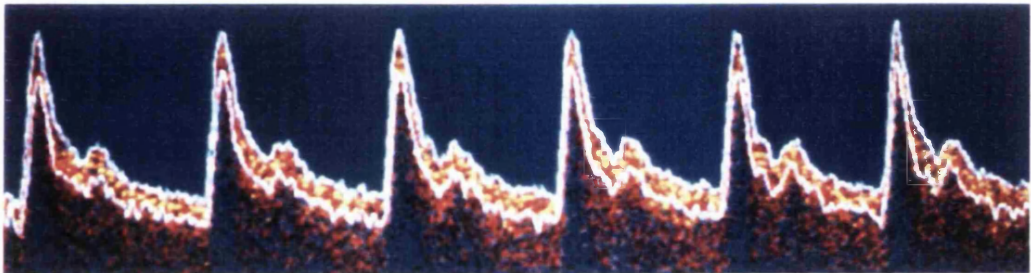
Changes in blood velocity induced by hand hyperaemia are illustrated overleaf:-

- A – basal: after 15 minutes supine rest
- B – hand hyperaemia: maximum increase in blood velocity occurs within the first 15 seconds after wrist cuff release
- C – After completion of the study and via play back on audio tape, SciMed Dopstation software allows automatic identification of end-diastole with respective positioning of cursor lines. The mean time-velocity integral for an average of 6 to 8 beats per screen is calculated and corrected for Doppler angle and brachial artery diameter to determine blood flow. This is repeated for 3 screens at each time point and mean flow recorded.

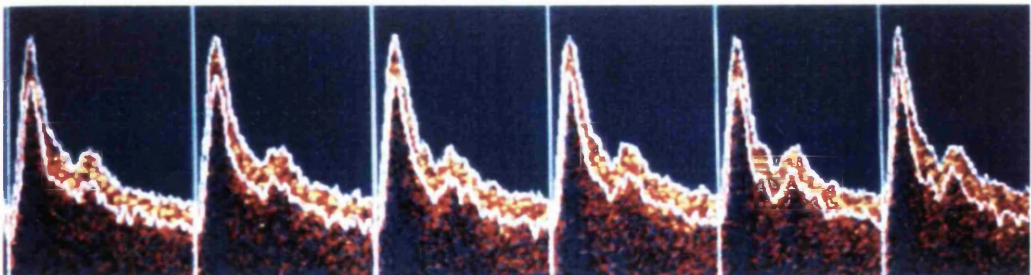
A



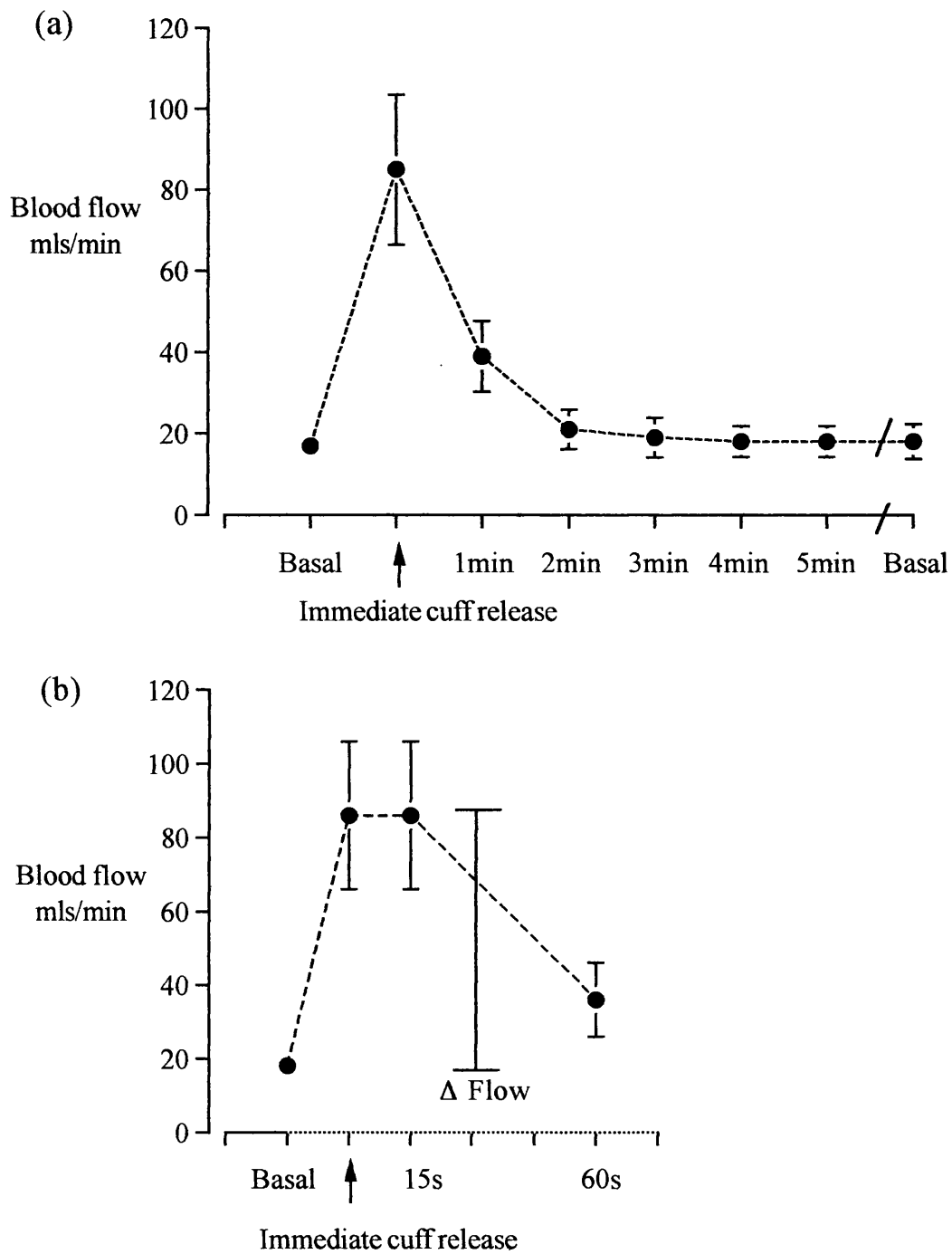
B



C







**Fig. 2.6 : Brachial artery blood flow in response to hand hyperaemia**

(a) Time-course: Graph showing change in blood flow from basal conditions up to 5 minutes after wrist-cuff release and then after return of haemodynamic variables to baseline. Data are mean  $\pm$  SEM, n=12.

(b) Duration of peak flow: Peak hyperaemic blood flow occurred in the first 15 seconds [ $\Delta$  flow = peak flow - basal].

cannula was removed and gentle pressure applied over the brachial artery for 15 minutes to ensure haemostasis.

### 2.3.3 Statistical analysis (carried out with the assistance of Dr R Newcombe, Dept. of Medical Statistics and Dr J Wilson, Dept. of Pharmacology)

Data are presented as mean  $\pm$  SD unless stated otherwise.

#### Regression modelling

All variables were continuous except sex, which was binary. Regression models were examined in regard to changes in hyperaemia-induced and GTN-induced brachial artery diameter in order to determine the ‘best method’ of representing endothelium-dependent and –independent responses respectively. Although most investigators have used percentage change historically after Celermajer’s original paper describing the technique<sup>94</sup>, data may not necessarily be best expressed using it. Percentage change is the ratio of post- to pre-intervention values and is strongly influenced by basal internal brachial artery diameter, age and sex. Three other possible variables were examined that could alternatively be used to characterise flow-mediated or GTN-mediated responses.

The four possible options were (i) a simple absolute increment in diameter, (ii) a ratio or percentage change measure, (iii) an absolute increase in cross-sectional area (imputed as if the vessel is cylindrical [i.e.  $\pi d^2/4$ ], and (iv) change in log diameter (which is the same as the log of the ratio of post- to pre-intervention diameters). Preferably, the same measure would be used for both flow-mediated and GTN-mediated responses.

Using the cohort of 240 subjects (see below), a correlation matrix using non-parametric (Spearman) rank correlations was derived for each option with the corresponding (untransformed) basal diameter value and with age and sex. This was carried out for flow-mediated and GTN-mediated responses individually. A good method of adjustment was defined as an index that had little correlation with the basal diameter value (a rank correlation close to zero) and ideally no relation to sex or age.

#### Coefficients of variation

Repeatability of haemodynamic data and vascular responses was determined by comparing data recorded on 3 separate visits (visit 1 and 2: within day

and visit 3: between day) with calculation of the respective coefficients of variation for each measurement. Analysis was performed by a nested analysis of variance.

#### Effect of age

Age-related changes are presented as simple scatter plots.

## 2.4 Results

### 2.4.1 Effect of N<sup>G</sup>-monomethyl-L-arginine (L-NMMA)

Haemodynamic data pre- and post- L-NMMA is shown in Table 2.2. After infusion of control (normal saline 0.9%), basal end-diastolic diameter was  $3.58 \pm 0.09$  mm. Basal blood flow was  $27 \pm 35$  mL/min. Following hand hyperaemia, mean flow-mediated brachial artery dilatation was  $+0.14 \pm 0.09$  mm (i.e.  $+4.9 \pm 4.6\%$ ,  $n=5$ ).

Infusion of L-NMMA for 20 minutes led to a small but non-significant decrease in both basal diameter ( $3.45 \pm 0.81$  mm) and basal blood flow ( $15 \pm 19$  mL/min). There was an increase in systolic blood pressure (from  $122 \pm 13$  to  $134 \pm 14$  mmHg,  $P=0.062$ ).

L-NMMA significantly inhibited hyperaemia-induced vasodilatation  $-0.04 \pm 0.11$  mm,  $P=0.033$  vs. control (i.e.  $-0.5 \pm 4.0\%$ ,  $P=0.022$ ) (Fig. 2.7) without significantly affecting hyperaemic blood flow.

### 2.4.2 Normal values and repeatability

Mean flow-mediated brachial artery dilatation measured in all 12 subjects on 3 separate occasions was  $+0.11 \pm 0.06$  mm (i.e.  $+3.74 \pm 2.21\%$ ), the maximum increase in arterial end-diastolic diameter occurring within the first 2 minutes after cuff release (Fig. 2.8). Peak increase in hyperaemic blood flow from baseline was  $+72 \pm 43$  mL/min.

The coefficient of variation for basal arterial diameter measurements (within day) in the 12 subjects made approximately 4 to 6 hours apart was 1.3%. Between day variability for the same subjects was 7.0%. This compared favourably with the variability of haemodynamic variables measured by photoplethysmography (Table 2.3).

There was greater biological variation in repeated flow-mediated responses. The maximum increase in diameter after cuff release varied slightly between

**TABLE 2.2: L-NMMA study: haemodynamic data**

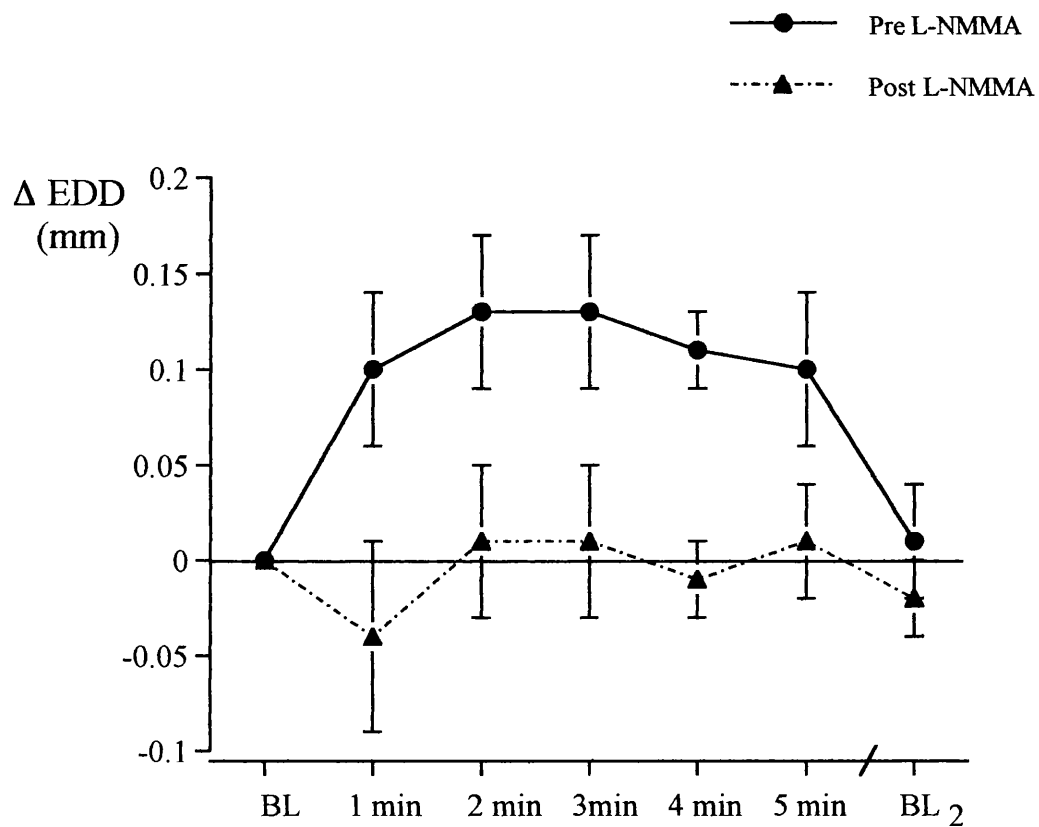
Parameter	L-NMMA		P value
	Pre	Post	
Heart rate, min <sup>-1</sup>	62 (9)	62 (9)	0.838
SBP, mmHg	122 (13)	134 (14)	0.062
DBP, mmHg	76 (15)	77 (11)	0.762
Basal end-diastolic diameter (EDD), mm	3.58 (0.09)	3.45 (0.81)	0.209
<b><u>Blood Flow</u></b>			
Basal, mls.min <sup>-1</sup>	27 (35)	15 (19)	0.250†
Reactive hyperaemia, mls.min <sup>-1</sup>	216 (142)	184 (165)	0.625†
<b><u>Flow-mediated dilatation</u></b>			
Δ EDD max, mm*	+0.14 (0.09)	-0.04 (0.11)	0.033**
Δ EDD max, %	+4.9 (4.6)	-0.5 (4.0)	0.022**

Data are mean (SD).

\*Δ EDD max is maximum change in brachial end-diastolic diameter over measurement period.

Comparison by paired t test except where shown †Wilcoxon-paired test.

\*\* Significant reduction in flow-mediated dilatation after L-NMMA.



**Fig. 2.7: Flow-mediated vasodilatation pre- and post- L-NMMA**

Graphs showing change in brachial artery end-diastolic diameter (EDD) induced by hand hyperaemia before and after infusion of the nitric oxide synthase antagonist, N<sup>G</sup>-monomethyl-L-arginine (L-NMMA). Data are shown for changes in EDD as simple increment from baseline. Data are mean  $\pm$  SD, n=5.

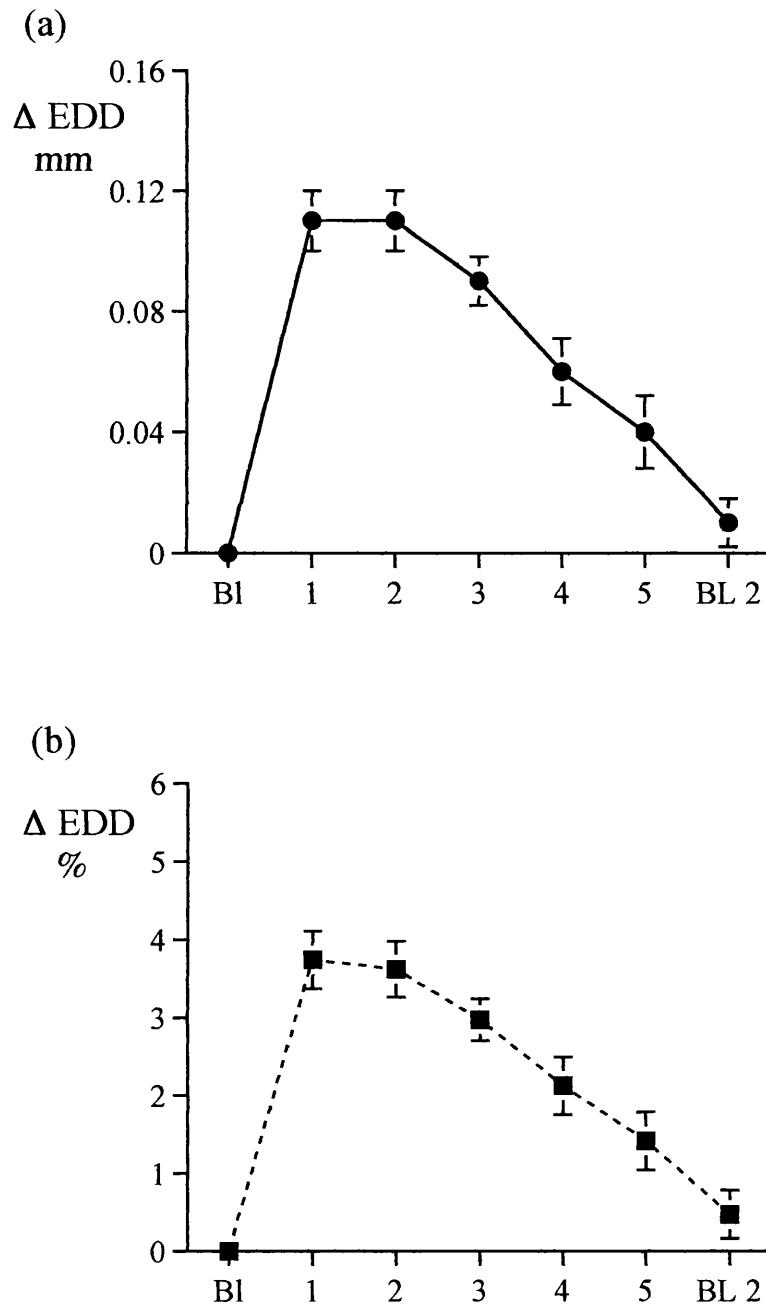
**TABLE 2.3: Repeatability study**

Haemodynamic data (n=12) for each visit and coefficients of variation comparing values between visit 1 and 2 (same day) and between visit 1 and 3 (different day).

Parameter	Day 1		Day 7	CV	
	AM Visit 1	PM Visit 2	AM Visit 3	Same Day Visit 1 & 2	Between Day Visit 1 & 3
<b><u>Basal</u></b>					
HR, min <sup>-1</sup>	64 (10)	62 (9)	60 (8)	4.2	8.2
SBP, mmHg	117 (13)	117 (12)	110 (11)	1.7	5.9
DBP, mmHg	62 (9)	63 (7)	60 (8)	3.8	9.9
EDD, mm	2.97 (0.47)	2.95 (0.44)	3.01 (0.50)	1.3	7.0
<b><u>Hyperaemia</u></b>					
Δ EDD max, Δmm	0.13 (0.05)	0.12 (0.05)	0.15 (0.05)	17.2	26.1
Δ EDD max, Δ %	4.3 (1.9)	4.3 (1.9)	5.1 (1.0)	17.7	27.2

Data are mean (SD). EDD = brachial artery end-diastolic diameter.

CV=coefficient of variation (%)



**Fig. 2.8 : Flow-mediated dilatation - time course**

Graphs showing changes in brachial artery end-diastolic diameter (EDD), induced by hand hyperaemia, at various time points after wrist-cuff release for (a) a simple increment, mm and (b) percentage change. 12 subjects were studied on three occasions and the above mean  $\pm$ SEM values are derived from all 36 scans performed. Peak dilatation occurs in the first 2 minutes.

measurements leading to a higher coefficient of variation compared with basal arterial diameter. However, all subjects showed a similar response profile with time on every occasion. Subjects with impaired responses demonstrate a '*flat*' response to flow similar to that seen following the infusion of L-NMMA. The mean coefficient of variation for within day measurements of increases in arterial diameter (mm) induced by hand hyperaemia was 17.2%. Between day variability showed more variation with a CV of 26.1%.

Despite these relatively higher CVs compared with haemodynamic and basal diameter measurements, appropriately designed blinded studies using placebo-controlled, crossover methods and sufficient numbers of subjects will allow the relevant hypotheses and therapeutic interventions to be tested using this technique.

#### **2.4.3 Regression models for flow-mediated and GTN-mediated brachial artery dilatation**

The primary consideration for a good method of adjustment was to define an index that has little correlation with the baseline value, certainly a rank correlation close to zero. It also simplifies matters if there is no relation to age or sex. It is hoped that the distributional form of the resulting index will be close to Gaussian. Table 2.4 draws together the relevant correlations for each specified parameter. The relationship to baseline diameter (or area) is shown in Fig. 2.9.

Examining these correlations together with the scatter plots, it is clear that ratio and log scales over-adjust for the baseline diameter value, both for the flow-mediated response and grossly so for the GTN-mediated response. There is also considerable sex-dependence. Both absolute and area increments work well for flow-mediated and GTN-mediated responses alike and there is little to choose between them.

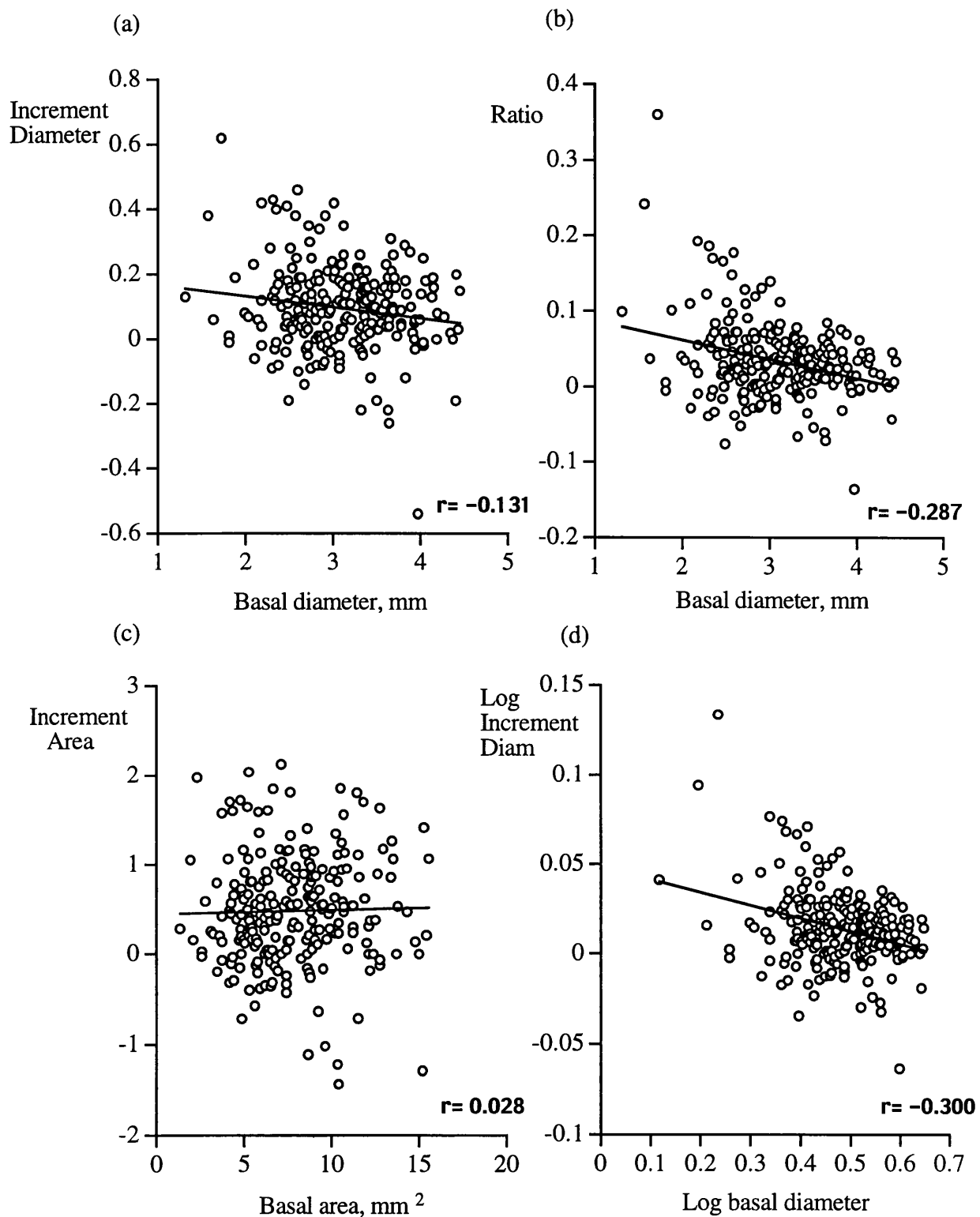
For the GTN response, all four outcome indices tend to be skew; the absolute increment is less skew than the area increment and would appear to be the best choice.

Flow-mediated and GTN-mediated responses will therefore be expressed as a simple absolute increment in brachial artery end-diastolic diameter and will be used throughout the following experimental chapters. However, values for percentage change will also be quoted to aid comparison with published data.



**TABLE 2.4: Correlation data for regression modelling**

<b>Parameter</b>	<b>Basal Diameter</b>	<b>Age</b>	<b>Sex</b>
<b><u>Hyperaemia</u></b>			
Increment	-0.131	-0.138	+0.079
<i>Rank</i>	<i>-0.039</i>	<i>-0.098</i>	<i>+0.023</i>
Ratio	-0.287	-0.173	+0.175
<i>Rank</i>	<i>-0.175</i>	<i>-0.151</i>	<i>+0.121</i>
Area	+0.028	-0.082	-0.026
<i>Rank</i>	<i>+0.097</i>	<i>-0.036</i>	<i>-0.081</i>
Log	-0.300	-0.176	+0.169
<b><u>GTN</u></b>			
Increment	-0.185	-0.083	+0.064
<i>Rank</i>	<i>-0.220</i>	<i>-0.135</i>	<i>+0.094</i>
Ratio	-0.585	-0.230	+0.342
<i>Rank</i>	<i>-0.580</i>	<i>-0.252</i>	<i>+0.365</i>
Area	+0.217	+0.085	-0.228
<i>Rank</i>	<i>+0.212</i>	<i>+0.037</i>	<i>-0.218</i>
Log	-0.605	-0.231	+0.347



**Fig 2.9: Scatter graphs demonstrating relationship between different models used to express endothelium-dependent flow-mediated vasodilatation and basal brachial artery diameter or cross-sectional area.**

- (a) simple absolute increment in diameter
- (b) ratio or percentage change measure
- (c) absolute increase in cross-sectional area
- (d) change in log diameter

#### **2.4.4 Effect of age**

Flow-related endothelial function declines with age and in women there appears to be a marked change at the time of the menopause suggesting an important protective role of endogenous oestrogens<sup>101</sup>.

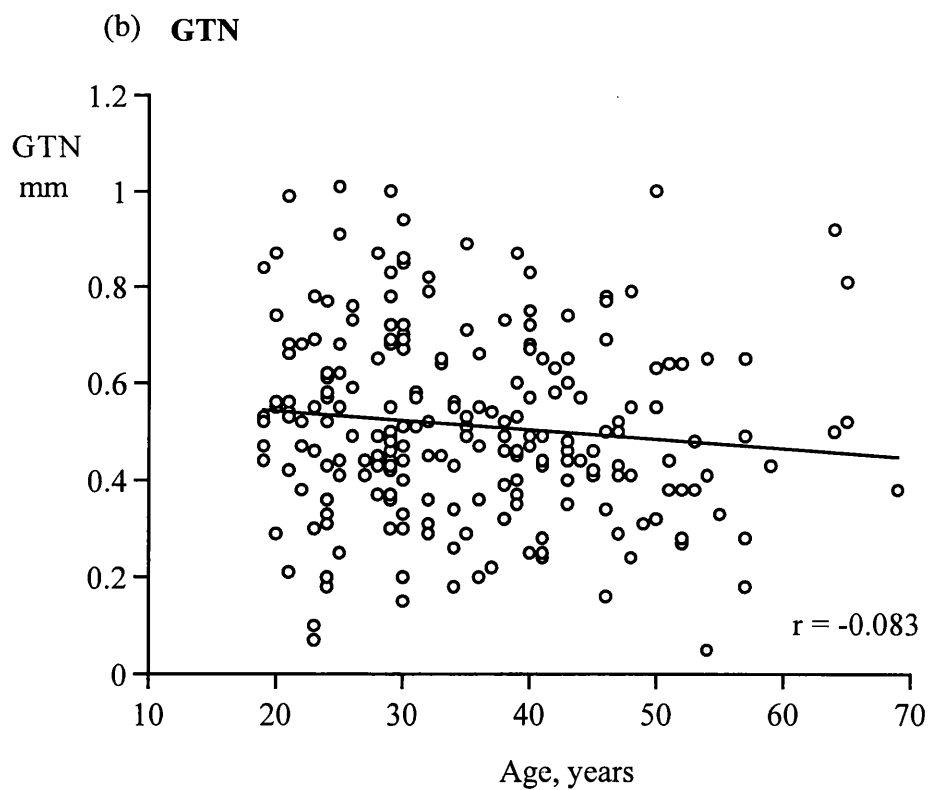
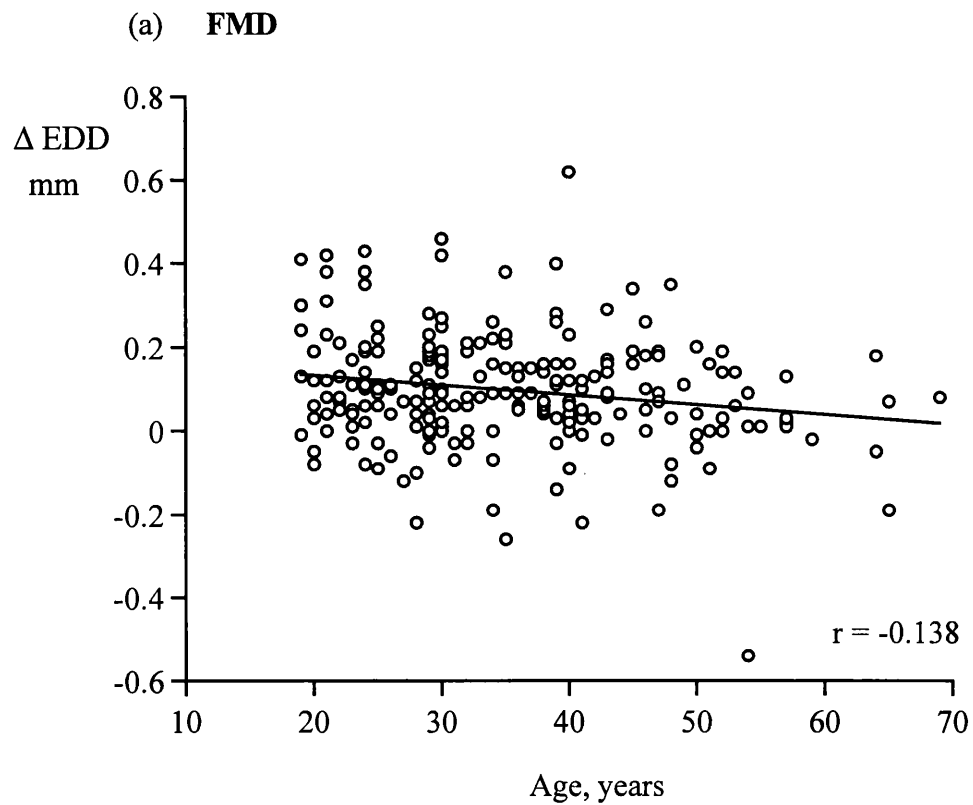
Fig. 2.10 illustrates the distribution of flow-mediated and GTN-mediated brachial artery dilatation as a function of age in the local study population.

There was a marked diversity of responses for both measurements. Despite the exclusion of known risk factors for endothelial function, a number of individuals demonstrated flow-mediated vasoconstriction or minimal vasodilatation suggesting an adverse effect of other as yet unknown causes for impaired vasomotor function, including possible genetic factors that are as yet not well understood.

#### **2.4.5 Estimation of sample size and power calculations**

In subsequent chapters, including the methionine and folate intervention studies, subject recruitment and sample size were determined by practical constraints rather than specific power calculations.

A general estimate, however, can be derived from the repeatability data. A total of 18 or more patients in a crossover interventional study would provide at least 80 % power of detecting a 0.03 mm change in end-diastolic brachial artery diameter, with a significance level of 0.05 (two-tailed).



**Fig 2.10: Effect of age on flow-mediated and GTN-mediated brachial artery dilatation**

## 2.5 Discussion

Compared with previously published data<sup>94,100,102</sup>, normal mean values for flow-mediated dilatation in healthy subjects with no known risk factors for endothelial dysfunction are lower. This may be a result of two important distinctions in the methodology used.

Firstly, differences may arise reflecting the method of inducing hyperaemic blood flow. A number of investigators (particularly researchers in the USA) place the cuff above the antecubital fossa<sup>96</sup>. This increases maximum hyperaemic flow and hence greater vasodilatation but by so doing induces ischaemia in the brachial artery itself, which may have important implications when interpreting the results. In placing the cuff below the arterial segment being studied, ischaemia of the brachial artery is avoided and results are more meaningful, as other contributors to vasodilatation during ischaemia (e.g. adenosine) are minimised.

Secondly, there are important distinctions between wall-tracking and 2D-arterial measurement. The improved resolution of measurements using wall-tracking methods may also paradoxically explain differences in the accuracy and repeatability of flow-mediated responses compared to published values<sup>95</sup> beyond biological variation. As discussed previously (section 2.3), ultrasonic wall tracking leads to an approximate 100-fold improvement in the resolution of brachial artery diameter measurement. In the original method by Celermajer<sup>94</sup>, electronic callipers were used on B-mode ultrasound images with a theoretical maximum resolution of 0.1mm. In a 4 mm artery, the smallest possible detectable change in arterial diameter would be +0.1mm, i.e. 4.1 mm, corresponding to a percentage change of 2.5%. Any larger degree of dilatation would be in increments of similar magnitude (i.e. 2.5%, 5%, 7.5%, 10% etc). For a 3mm vessel, increments would be even larger at 3.3%. For any given cohort of patients, especially if numbers are small, which is generally the case for repeatability studies, the variation in arterial diameter on repeat measurement is clearly going to be less than a method with a 100-fold improvement in resolution. Wall tracking will therefore produce a potentially larger measurable variation in vascular responses, dependent in part on detectable biological variation.

The degree of brachial artery dilatation observed in response to (i) hyperaemic flow and (ii) sublingual nitrate, is dependent on the duration of wrist cuff occlusion and the dose of nitrate given. In order to induce maximal vessel dilatation, at least 4.5 mins of cuff occlusion is needed<sup>100</sup>. In this study, a period of 5 mins was used throughout and

similar increases in maximal hyperaemic blood flow were also observed to those previously published<sup>100</sup>.

A sublingual GTN dose of 300µg was chosen to measure endothelium-independent responses as used in Celermajer's original methodology<sup>94</sup>. Maximal vasodilatation has been shown to occur with doses of 200µg isosorbide dinitrate (ISDN) or above, with dilatation of similar magnitude to flow-mediated values after 100µg ISDN<sup>100</sup>. A possible criticism of the present protocol is that a 100µg dose could have also been included allowing an additional direct comparison at similar values of dilatation.

Finally, the presentation of changes in vascular diameter as a simple increment has certain advantages over the published method of percentage change being far less dependent on baseline diameter, age and sex. It will be used throughout the following Chapters.

## **CHAPTER 3**

### **Hyperhomocysteinaemia And Endothelial Function After An Oral Methionine Load**

#### **3.1 Introduction**

An oral methionine-loading test historically has been used to identify heterozygotes for cystathionine  $\beta$ -synthase deficiency that tend to show an exaggerated rise in plasma homocysteine after oral ingestion of methionine (usually 0.1g/kg)<sup>13</sup>. Methionine loading may also identify those subjects who have normal fasting plasma homocysteine concentrations but who have impaired homocysteine metabolism<sup>103</sup>, either reduced capacity for transsulphuration or remethylation, the latter in association with genetic variants (MTHFR genotype) and/or suboptimal vitamin B<sub>12</sub>/folate status. Moreover, a methionine load can be used experimentally to induce an acute elevation in homocysteine in any subject.

A role for homocysteine in mediating vascular injury is supported by animal studies where dietary modification in monkeys by methionine supplementation and folate restriction, which raises homocysteine levels to those that are comparable to mild hyperhomocysteinaemia in humans, has been shown to produce changes in vascular reactivity<sup>63</sup>. This primate model has demonstrated impaired vascular function in association with chronically elevated plasma homocysteine that is more likely to be of physiological relevance to man, in contrast to previous studies described in Chapter 1 where the concentrations of homocysteine have been high and of little physiological importance.

Mild to moderate elevations of plasma homocysteine would therefore be expected to be acutely injurious to endothelium in man. The objective of the series of experiments in this chapter was to investigate the effect of an oral methionine load on plasma homocysteine together with flow-related endothelial function in healthy subjects and to observe the time course of any such effects. Homocysteine may in part cause endothelial injury by promoting oxidant stress, generating reactive oxygen species as discussed previously (Chapter 1.7), an effect that may be prevented by prior administration of antioxidants, such as Vitamin C. Modulation of the effect of methionine loading on vascular responses by prior oral administration

of ascorbic acid was therefore also studied. The number of subjects involved was small and the data must therefore be regarded as preliminary.

### 3.2 Subjects

#### *Crossover study (Fig. 3.1)*

Twenty-eight members of hospital staff were invited to enter the study but three volunteers later declined to participate after randomisation and allocation to treatment. One subject developed a severe headache after sublingual glyceryl trinitrate (GTN) and withdrew after completing their first visit. Twenty-four subjects (mean age, 32 years; range, 21 to 46 years; 14 men) completed the protocol.

#### **Time-course study**

##### *(a) Methionine*

Ten subjects took part in this study of which 5 had also taken part in a crossover study on a separate occasion (at least 7 days apart).

##### *(b) Methionine and vitamin C*

Five subjects who participated in both the crossover and methionine time-course study attended on a final visit during which oral vitamin C was administered prior to the methionine load (see below).

All were non-smokers, normotensive (blood pressure <150/90 mmHg), had serum cholesterol <6.5 mmol/L and were not taking vitamin supplements. Baseline characteristics are shown in Table 3.1. All subjects gave informed, written consent, and the local Research Ethics Committee approved the study protocol.

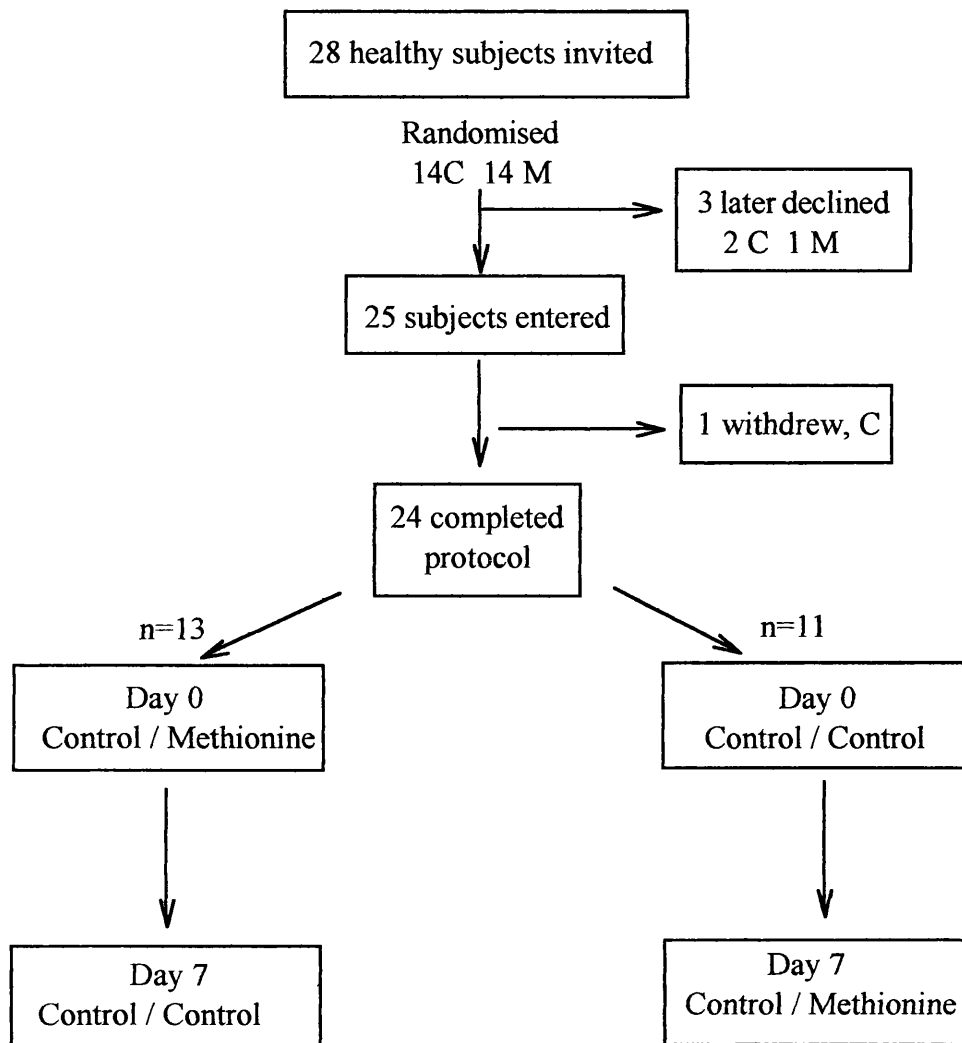
### 3.3 Methods

#### 3.3.1 Study design

##### *Randomised crossover study of an oral methionine load*

Twenty-four subjects attended after an overnight fast on 2 separate days, one week apart. Subjects were randomised to receive oral L-methionine (0.1g/kg, Scientific Hospital Supplies) on either day 0 or day 7 as a crossover study. Randomisation to methionine on day 0 or day 7 was performed by a research nurse





**Fig 3.1: Outline of recruitment and crossover study protocol**

28 volunteers agreed to participate and randomised to receive methionine on day 0 or day 7. Three subjects later declined to participate when called to attend (2 of which had been allocated control [C], 1 allocated methionine [M] on day 0). Of 25 subjects that commenced the study, 1 withdrew after developing a severe headache following sublingual GTN.

**TABLE 3.1: Subject characteristics: methionine study**

<b>Parameter</b>	<b>Methionine</b>		<b>Methionine +Vitamin C</b>
	<b>Crossover</b>	<b>Time course</b>	<b>Time course</b>
No.	n=24	n=10	n=5
Age, y	32 (7)	29 (6)	29 (7)
Sex, M/F	14 / 10	8 / 2	3 / 2
Body mass index, kg/m <sup>2</sup>	23 (2)	23 (3)	21 (1)
Total homocysteine, µmol/L	7.5 (2.2)	7.2 (1.4)	7.7 (1.4)
Folate, µg/L	6.9 (2.3)	7.5 (3.0)	9.3 (2.6)
Vitamin B12, ng/L	447 (107)	505 (90)	436 (49)
Total cholesterol, mmol/L	4.7 (0.8)	4.5 (0.5)	4.6 (0.7)
Triglyceride, mmol/L	0.9 (0.4)	1.1 (0.4)	0.9 (0.1)
Glucose, mmol/L	4.8 (0.4)	4.5 (0.3)	4.5 (0.1)

Data are mean (SD).

who administered the oral methionine load in a separate room from the vascular laboratory to ensure that the investigators performing the vascular measurements remained blinded throughout the study. Due to the distinctive taste of methionine, subjects knew which day they had received methionine but were specifically instructed not to inform the ultrasound operators. At baseline, flow-mediated (endothelium-dependent) and GTN-mediated (endothelium-independent) brachial artery dilatation were measured and venous blood was sampled for total plasma homocysteine (with additional sampling for total cholesterol, triglyceride, glucose, folate and B<sub>12</sub> on their first visit). Subjects then received oral methionine or no medication (control), and measurements were repeated 4 hours later.

### ***Time course study***

#### ***(a) Methionine***

To further examine the relationship between changes in plasma methionine, homocysteine and flow-mediated brachial artery dilatation, 10 volunteers (mean age, 29 years; range, 21 to 40 years; 8 men) received an oral methionine load (0.1g/kg) and flow-mediated brachial artery dilatation, plasma methionine, total plasma homocysteine and cysteine were measured at baseline and at 1, 2, 3, 4 and 8 hours. This was an open (non-blinded) study. In those subjects who had previously participated in the crossover study (5 of the 10 subjects), these measurements were performed on a separate visit at least 7 days later.

#### ***(b) Methionine and Vitamin C***

The above protocol was repeated in 5 volunteers (mean age, 29 years; range 21 to 40 years; 3 men) but on this occasion 2g oral vitamin C (effervescent tablets dissolved in water), a dose previously reported to reverse endothelial dysfunction in patients with coronary artery disease<sup>104</sup>, was administered after the baseline scan but 30 min before the oral methionine load (0.1g/kg).

### **3.3.2 Measurement of endothelial function**

Endothelial function was measured according to the methods described in Chapter 2.

### 3.3.3 Blood samples and assays

Venous blood was sampled into tubes containing EDTA (for homocysteine), lithium-heparin (for methionine, cysteine and ascorbic acid), SST (gel and clot activator) (for lipids, B<sub>12</sub> and folate) and fluoride-oxalate (for glucose). Samples for homocysteine were immediately placed on ice and plasma separated within 30 minutes by centrifugation. Plasma for ascorbic acid was diluted (1 in 10) with 0.5M metaphosphoric acid. Plasma for methionine and cysteine assay was deproteinised immediately with 10% 5-sulphosalicyclic acid. Samples were stored at -70°C until analysis.

*Total plasma homocysteine.* Samples were analysed within the same batch by HPLC using SBD-F (ammonium 7-fluoro-2-oxa-1,3-diazole-4-sulphonate) derivatisation (within batch precision, 2.2%)<sup>105</sup>.

*Methionine and cysteine.* Samples were measured by ion-exchange amino acid chromatography (Biotronik LC 5001) (performed by Dr D Bradley, Newborn Screening Biochemistry Unit, UWCM).

*Folate and vitamin B<sub>12</sub>.* Serum folate and vitamin B<sub>12</sub> were measured by protein binding assays using an Abbott IMX analyser. (performed by Mr R Ellis, Department of Haematology, UWCM).

*Ascorbic acid (vitamin C).* Plasma ascorbic acid was determined by HPLC with electrochemical detection (performed by Dr. IS Young, Department of Clinical Biochemistry, The Queen's University of Belfast).

*Nitrotyrosine.* Detection of protein-nitrotyrosine (phenylalanine) residues was performed by a competitive ELISA method<sup>106</sup> (performed by Prof. KR Bruckdorfer, Department of Biochemistry, Royal Free Hospital School of Medicine, London).

### 3.3.4 Statistical analysis (carried out with the assistance of Dr. R Newcombe)

Data are presented in the text, tables and figures as group mean  $\pm$  SD unless stated otherwise. Changes in haemodynamic data, brachial artery flow-mediated and

GTN-mediated dilatation from baseline to 4 hours during the methionine period were compared to those occurring during the control period using a paired analysis based on that of the two-period crossover trial<sup>107</sup>. Thus the difference between changes occurring in first and second periods was compared between the two treatment order groups by an unpaired t-test, which obviates confounding with period differences introduced by unequal numbers in the two groups, which occurred by chance. In the time course experiments, a Wilcoxon paired test was used to compare changes in methionine, homocysteine, cysteine, ascorbic acid and flow-mediated dilatation at each of the five time points relative to baseline.

### **3.4 Results**

#### **3.4.1 Crossover study (Table 3.2)**

Fasting plasma total homocysteine and basal vascular measurements were similar on each of the 2 study days and there was no evidence of an order effect. Administration of the oral methionine load increased total homocysteine from  $7.9 \pm 2.0$   $\mu\text{mol/L}$  at baseline to  $23.1 \pm 5.4$   $\mu\text{mol/L}$  at 4 hours ( $P < 0.0001$ ). Flow-mediated brachial artery dilatation decreased from  $0.12 \pm 0.09$  to  $0.06 \pm 0.09$  mm ( $P = 0.045$ ), i.e.  $4.0 \pm 3.2\%$  to  $1.7 \pm 3.3\%$  (Fig 3.2), despite similar hyperaemic blood flow ( $67 \pm 66$  vs.  $78 \pm 81$  mL/min). GTN-induced dilatation was similar at baseline and at 4 hours ( $0.48 \pm 0.17$  vs.  $0.40 \pm 0.11$  mm,  $P = 0.32$ , i.e.  $14.7 \pm 5.7$  vs.  $12.6 \pm 5.5\%$ ) (Fig. 3.3). There was no significant correlation between change in flow-mediated dilatation and basal, post-load or increase in plasma homocysteine. Five subjects reported mild nausea after oral methionine which was most pronounced in the first hour after administration.

#### **3.4.2 Time-course study**

##### **3.4.2.1 Methionine**

In the group of 10 subjects who participated in the time-course study, plasma methionine increased from  $27 \pm 5$   $\mu\text{mol/L}$  at baseline to a maximum of  $714 \pm 155$   $\mu\text{mol/L}$  at 1 hour and declined thereafter but remained above baseline in all subjects at 8 hours ( $P = 0.002$ ) (Figure 3.4). Plasma total homocysteine increased from  $7.2 \pm 1.4$   $\mu\text{mol/L}$  at baseline to  $23.5 \pm 5.2$   $\mu\text{mol/L}$  at 4 hours ( $P < 0.001$ ) and remained elevated at  $25.9 \pm 7.0$   $\mu\text{mol/L}$  at 8 hours ( $P = 0.002$  vs. baseline). Flow-mediated brachial artery

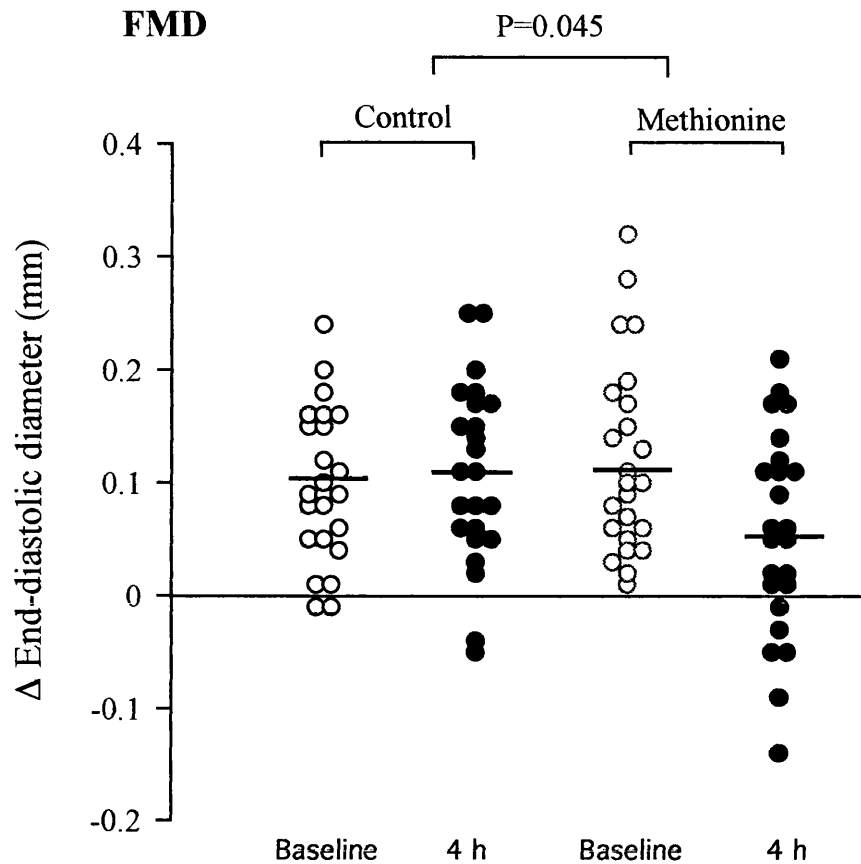
**TABLE 3.2 Methionine study: haemodynamic data and changes in total plasma homocysteine concentrations**

	<b>Control</b>		<b>Methionine</b>	
	<b>Baseline</b>	<b>4 h</b>	<b>Baseline</b>	<b>4 h</b>
Total homocysteine, $\mu\text{mol.L}^{-1}$	7.3 $\pm$ 2.2	7.9 $\pm$ 2.2	7.9 $\pm$ 2.0	23.1 $\pm$ 5.4*
<b><u>Basal</u></b>				
Brachial artery EDD, mm	3.12 $\pm$ 0.61	3.21 $\pm$ 0.60	3.18 $\pm$ 0.57	3.26 $\pm$ 0.64
Blood flow, mL .min $^{-1}$	22 $\pm$ 24	31 $\pm$ 26	27 $\pm$ 27	29 $\pm$ 29
Heart rate, min $^{-1}$	63 $\pm$ 11	65 $\pm$ 11	64 $\pm$ 12	66 $\pm$ 12
Systolic blood pressure, mmHg	121 $\pm$ 21	113 $\pm$ 21	118 $\pm$ 18	114 $\pm$ 20
Diastolic blood pressure, mmHg	67 $\pm$ 10	63 $\pm$ 14	65 $\pm$ 8	61 $\pm$ 9
<b><u>Flow-mediated dilatation</u></b>				
$\Delta$ Brachial artery EDD, mm	+0.10 $\pm$ 0.07	+0.11 $\pm$ 0.08	+0.12 $\pm$ 0.09	+0.06 $\pm$ 0.09†
%	+3.5 $\pm$ 2.9	+3.6 $\pm$ 2.8	+4.0 $\pm$ 3.2	+1.7 $\pm$ 3.3†
$\Delta$ Blood flow, mL.min $^{-1}$	+56 $\pm$ 35	+61 $\pm$ 47	+67 $\pm$ 66	+78 $\pm$ 81
<b><u>Glyceryl trinitrate (400<math>\mu</math>g)</u></b>				
$\Delta$ Brachial artery EDD, mm	+0.47 $\pm$ 0.15	+0.45 $\pm$ 0.20	+0.48 $\pm$ 0.17	+0.40 $\pm$ 0.11
%	+15.4 $\pm$ 5.9	+14.9 $\pm$ 7.9	+14.7 $\pm$ 5.7	+12.6 $\pm$ 5.5
$\Delta$ Blood flow, mL.min $^{-1}$	-1 $\pm$ 16	+4 $\pm$ 13	0 $\pm$ 8	+1 $\pm$ 10

Data are mean  $\pm$  SD.

EDD = end-diastolic diameter

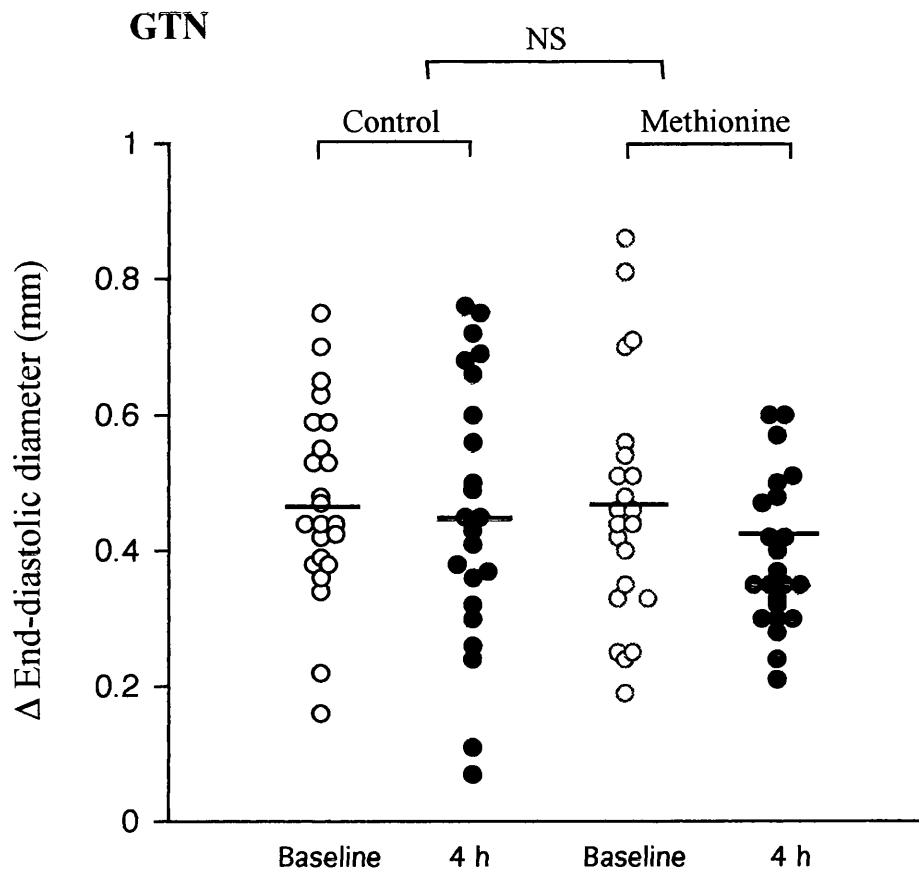
\* P<0.0001 vs methionine at baseline and control; †P=0.045 vs control.



**Fig 3.2: Endothelium-dependent flow-mediated vasodilatation following oral methionine**

Changes in flow-mediated brachial artery dilatation expressed as absolute change in diameter (mm) from basal measurement.

Flow-mediated vasodilatation was impaired at 4 hrs after oral methionine compared with control (no methionine), n=24.

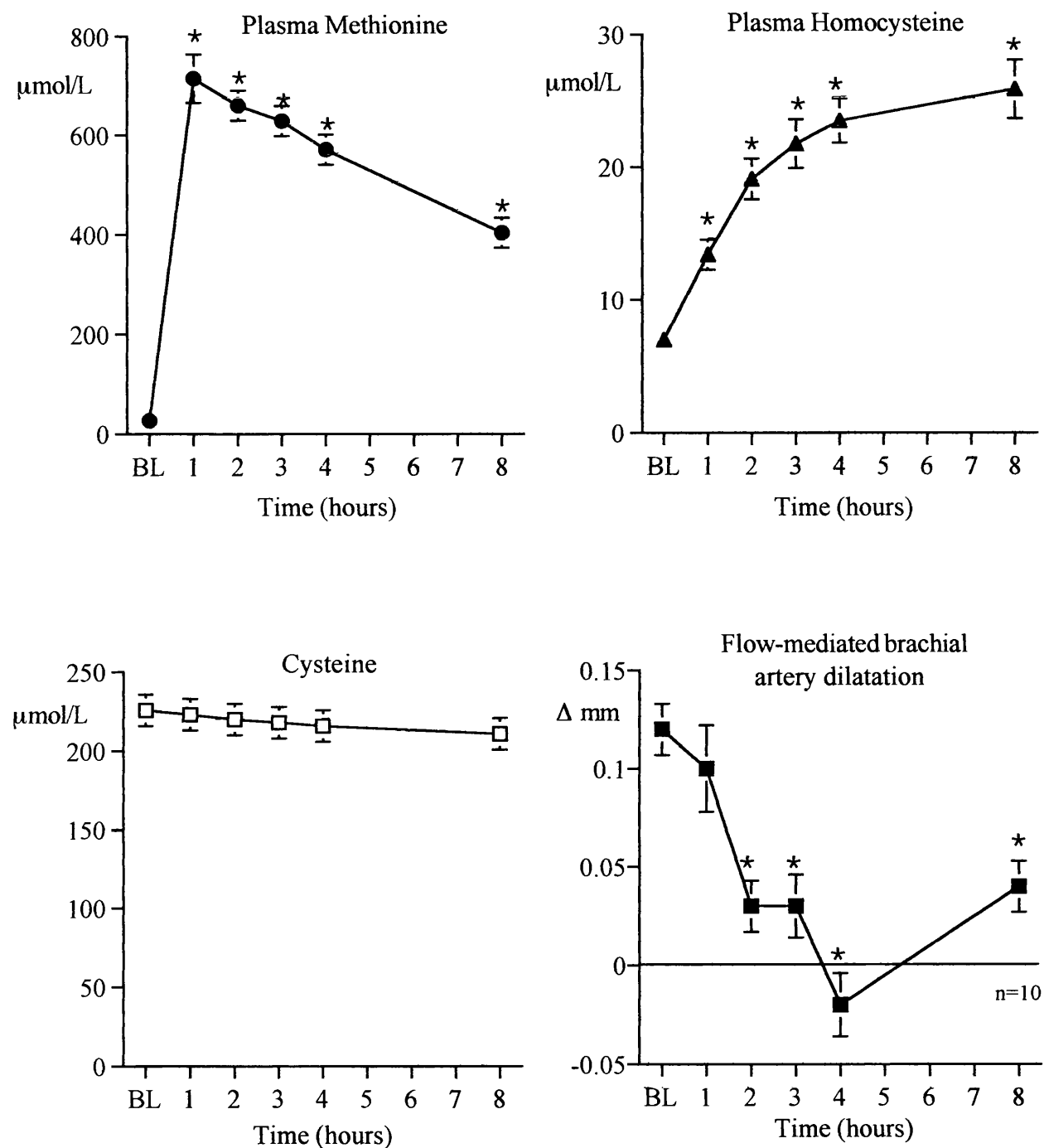


**Fig 3.3: Endothelium-independent vasodilatation following oral methionine**

Changes in GTN-mediated brachial artery dilatation expressed as absolute change in diameter (mm) from basal measurement.

GTN-mediated vasodilatation was unchanged at 4 hrs after oral methionine compared with control (no methionine), n=24.





**Fig 3.4: Time course experiment -methionine**

Changes in plasma methionine, total homocysteine, cysteine and flow-mediated brachial artery dilatation with time.

Data are mean (SEM), (\* P<0.002 vs baseline [BL], n=10).

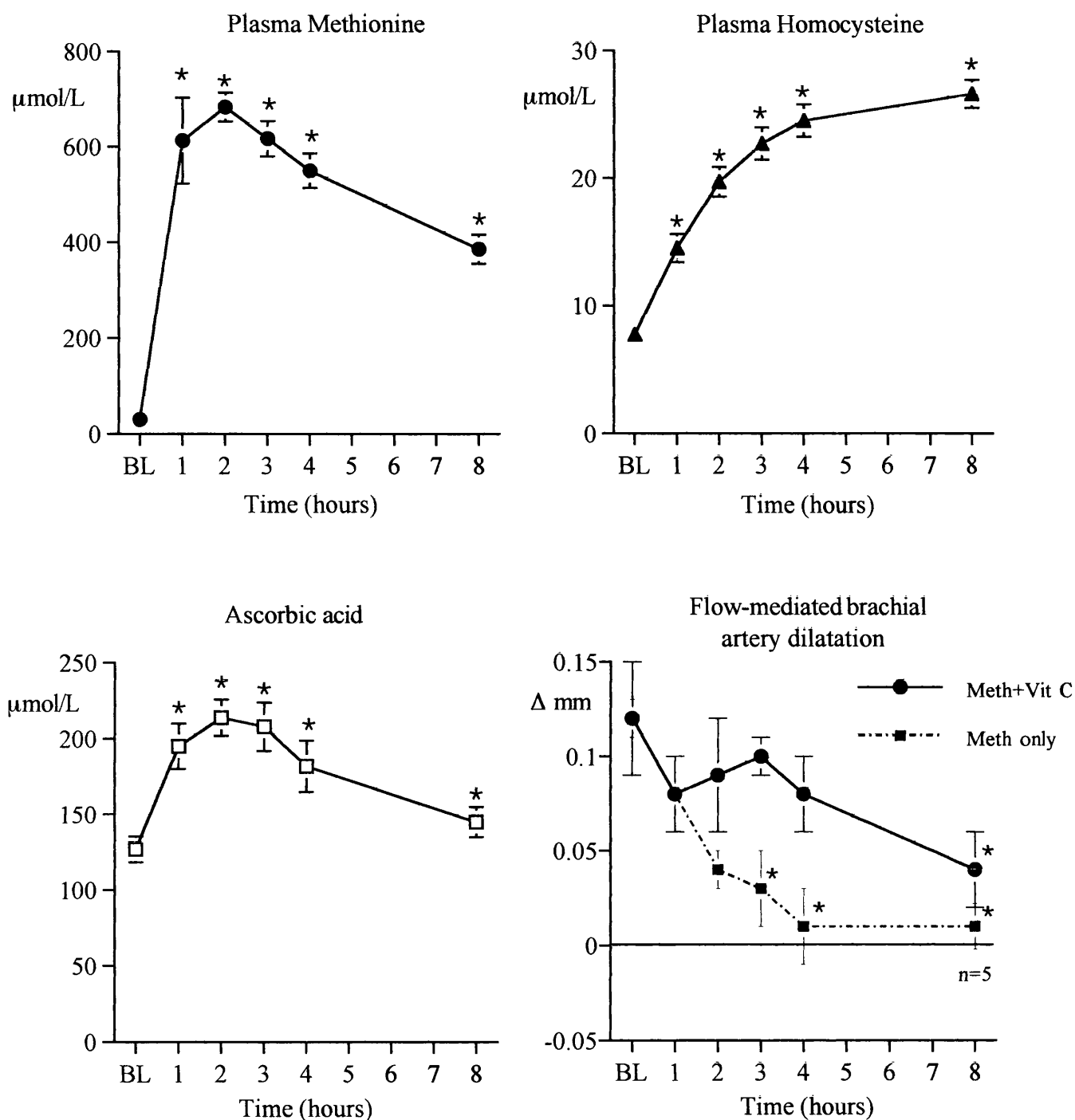
dilatation decreased in all subjects ( $P=0.002$ ) from  $0.12\pm0.04$  mm at baseline and remained impaired up to 8 hours. There was a trend to decreasing plasma cysteine concentrations with time.

#### 3.4.2.2 *Methionine and vitamin C*

Increases in methionine and homocysteine were similar to those following methionine alone (methionine:  $31\pm6$   $\mu\text{mol/L}$  at baseline and  $613\pm202$   $\mu\text{mol/L}$  at 1 hour; Hcy:  $7.7\pm1.5$   $\mu\text{mol/L}$  at baseline and  $24.5\pm2.9$   $\mu\text{mol/L}$  at 4 hours,  $n=5$ ). Plasma ascorbic acid increased from 127  $\mu\text{mol/L}$  at baseline to a maximum of 214  $\mu\text{mol/L}$  at 2 hours ( $p<0.001$ ) [i.e.  $+\Delta 87\mu\text{mol/L}$ ,  $+\Delta 68\%$ ], and remained  $> +\Delta 40\%$  at 4 hours post methionine load. Levels slowly returned towards baseline at 8 hours (Fig. 3.5).

There was no significant increase in plasma nitrotyrosine levels after methionine (Fig. 3.6), although there was a rise at 4 hours observed in 2 individual subjects.

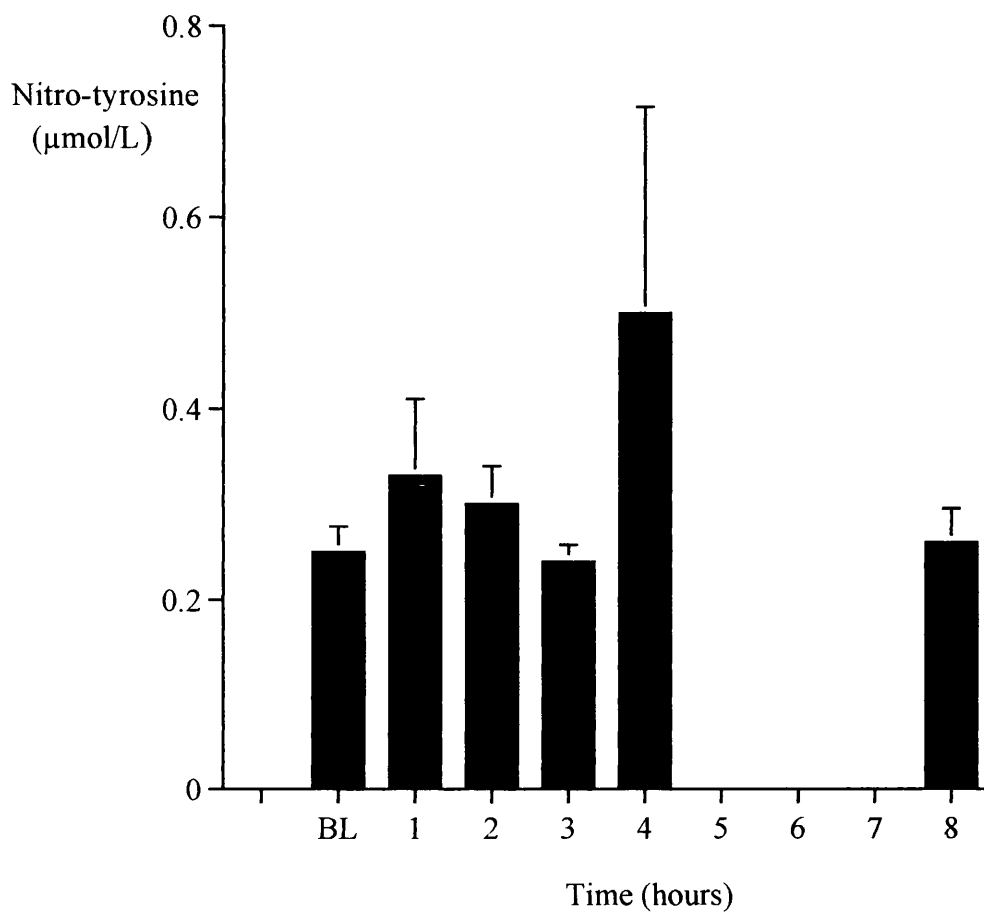
Changes in flow-mediated brachial artery dilatation for methionine alone and for methionine + vitamin C are shown for comparison in Fig 3.5 (bottom right panel,  $n=5$ ). Pre-treatment with 2g oral vitamin C attenuated the maximum decrease in flow-mediated dilatation previously observed at 4 hours with methionine alone. Flow-mediated responses, however, were significantly impaired at 8 hours.



**Fig 3.5: Time course experiment: methionine + vitamin C**

Changes in plasma methionine, total homocysteine, ascorbic acid and flow-mediated brachial artery dilatation with time (n=5).

Pre-administration of 2g ascorbic acid orally prior to the methionine load attenuated the decrease in flow-mediated dilatation seen after methionine alone (bottom right panel) in those 5 subjects who took part in both time course studies. Data are mean (SEM), (\* $P < 0.002$  vs baseline [BL]). Ascorbic acid normal range = 22-110  $\mu\text{mol/L}$ .



**Fig 3.6: Plasma nitro-tyrosine concentrations post-methionine load**

Data are mean  $\pm$  SEM, n=5

### 3.5 Discussion

The principal finding is that an oral methionine load, which produces an increase in plasma homocysteine, acutely impairs flow-mediated brachial artery dilatation in healthy subjects. The time-course experiments demonstrate a temporal relationship between a rise in homocysteine and changes in flow-related endothelial function. The data are consistent with the hypothesis that homocysteine is a risk factor for vascular disease by a mechanism which involves injury to endothelium. However, a cause and effect relationship has not been established by these findings.

Other possible interpretations include a direct effect of methionine or an alteration in methylation reactions, which are dependent upon methionine-homocysteine interconversion. Inspection of the time course data is relevant to this point. The impairment of endothelial function from 0 to 4 hours mirrored in all subjects the increase in plasma homocysteine, which is consistent with a direct toxic effect of homocysteine. The tendency for flow-mediated responses, however, to improve at 8 hours when methionine is falling, but homocysteine continues to rise is more indicative of a direct methionine effect. Different experimental approaches will be required to distinguish these possibilities.

The absence of any carryover effect in those subjects who received methionine on day 0 suggests that the effect on endothelium-dependent dilatation is reversible within seven days. This is consistent with the return of plasma homocysteine to baseline at approximately twenty-four hours following an oral methionine load.

A potential drawback of the crossover study was that a matched placebo was not possible due to the distinctive taste of methionine. The study did however incorporate a randomised crossover design and particular attention was paid to ensure that the observers performing vascular measurements were unaware of the order of methionine administration. The time-course studies were non-blinded and observer bias in measuring flow-mediated dilatation could not be excluded from potentially influencing the results. However, the effect of methionine loading was repeatable. In the five subjects who received methionine three times, similar increases in homocysteine were measured at four hours in association with similar decreases in flow-mediated vasodilatation on all occasions.

As discussed in section 1.7.2, vascular levels of nitric oxide are controlled not simply by its rate of production, but also by its rate of inactivation, in particular

by superoxide anions. Since both nitric oxide ( $\text{NO}^\bullet$ ) and superoxide ( $\text{O}_2^{\bullet-}$ ) are radicals, they undergo an extremely rapid reaction with one another leading to the formation of the peroxynitrite anion ( $\text{ONOO}^-$ )<sup>73,74</sup>. There is considerable interest in the possibility that formation of peroxynitrite is a process, which attenuates the action of NO and may lead to pathological changes. Indeed, a decrease in NO bioavailability due to peroxynitrite production has been proposed as one possible mechanism for homocysteine-induced endothelial injury<sup>66,70</sup>. The chemistry of peroxynitrite is very diverse and it will readily modify proteins by nitration or nitrosation of specific amino acids<sup>108</sup>. Free tyrosine is nitrated to 3-nitrotyrosine as are some of the tyrosine residues of proteins, such as phenylalanine, measurement of which allows indirect quantification of peroxynitrite formation<sup>106</sup>. In this study subgroup with small numbers, nitrotyrosine levels were not significantly elevated following a methionine load although a rise was observed in 2 subjects at 4 hours when homocysteine levels are maximal. No firm conclusions however can be drawn regarding changes in peroxynitrite and further studies with larger numbers of subjects are required.

In the 5 subjects where oral vitamin C was administered prior to methionine, the effect on flow-related endothelial function was attenuated over the first 4 hours. Flow-mediated responses did however decrease significantly at 8 hours. An increase in oxidative stress is a common feature of many of the risk factors of atherosclerosis, including smoking, diabetes and hypercholesterolaemia<sup>109</sup>. Vitamin C is the main water-soluble antioxidant in human plasma and is often used as an antioxidant although its precise mechanism underlying any beneficial effect on vascular function is not entirely clear, although it is capable of scavenging superoxide anions and sparing other endogenous antioxidants from consumption<sup>110,111</sup>. The experiments described here suggest a possible role for oxidative stress in mediating endothelial dysfunction in association with hyperhomocysteinaemia after an oral methionine load and would warrant further investigation in a larger study.

This study did not address the mechanism whereby vitamin C may exert any protective effect on the endothelium. One possibility is that vitamin C plays a central role in the regulation of intracellular redox state and improves endothelial function by sparing intracellular glutathione from oxidative degradation<sup>112</sup>. Glutathione is an important source of intracellular reduced thiols and can be degraded by oxidation to

glutathione disulfide<sup>113</sup>. Depletion of intracellular reduced thiols impairs NO synthase activity in cultured endothelial cells<sup>114</sup>. Moreover, reduced thiols can react with NO to form S-nitrosothiol species, which may result in stabilisation and increased bioavailability of NO<sup>61</sup>.

Methionine is an amino acid present in dietary animal protein. The issue then arises as to whether post-prandial changes in methionine are likely to be of significance in man. In a typical western diet, post-prandial increases in plasma methionine and homocysteine are usually much smaller than induced here, the latter in the order of 1-2 $\mu$ mol/L and therefore may only have a minor effect on vascular reactivity. Further studies will be needed to investigate this aspect, in particular to determine any dose-response effect of methionine and thus homocysteine concentrations on flow-related endothelial function.

The findings of this study are relevant to the general population. Mild to moderate elevations in plasma homocysteine, of a similar magnitude to post-load levels, may commonly occur as a result of inherited enzyme variants<sup>26</sup> and/or suboptimal vitamin status<sup>30</sup>, or in association with disease states such as renal failure<sup>42</sup>. Subjects homozygous for the thermolabile variant of methylene tetrahydrofolate reductase, the frequency of which is approximately 12% in healthy subjects in our region (Clark-unpublished observations), are prone to develop raised homocysteine concentrations, which may be comparable to those seen here. It therefore is plausible to propose that endothelial dysfunction arising from mild to moderate hyperhomocysteinaemia could contribute to the atherogenic process in such individuals.

## CHAPTER 4

### Vitamin Status As A Determinant of Plasma Homocysteine And Endothelial Function

#### 4.1 Introduction

Plasma homocysteine levels are inversely related to folate and vitamin B<sub>12</sub> concentrations<sup>30</sup>, both vitamins being essential requirements for remethylation of homocysteine to methionine. Patients with low or low-normal levels of serum folate and/or vitamin B<sub>12</sub> may have elevated plasma total homocysteine concentrations and would thus be an easily identifiable group of subjects in which to investigate the effect of mild hyperhomocysteinaemia on endothelial function.

In this chapter, flow-related endothelium-dependent responses are measured in a small cohort of subjects with hyperhomocysteinaemia due to low vitamin B<sub>12</sub>/folate status. Subjects with known risk factors for endothelial dysfunction were excluded. In contrast to the previous methionine loading studies, these patients have ‘chronic’ hyperhomocysteinaemia with exposure of the vasculature to abnormal levels over a prolonged period of time (months or years). This study could therefore be considered to be more physiologically relevant to the general population.

This chapter also includes a description of changes observed in a homocystinuric female whose homocysteine levels are elevated due to both a defect in cystathionine  $\beta$ -synthase and suboptimal folate/vitamin B<sub>12</sub> status. Changes in flow-related endothelial function were measured before and after vitamin supplementation.

#### 4.2. Subjects (Table 4.1)

##### *Homocystinuric patient*

A 21-year-old female patient with homozygous homocystinuria agreed to be studied. She had been diagnosed in her late teens with cystathionine  $\beta$ -synthase (CBS) deficiency at Sheffield Children’s Hospital and was under regular review in the Department of Paediatric Chemical Pathology, although control of homocysteine levels had been suboptimal. She was known to have some residual CBS activity.



**Table 4.1 Subject characteristics: low B<sub>12</sub>/folate / homocystinuria**

<b>Parameter</b>	<b>Low B<sub>12</sub>/Folate Status</b>		<b>Homocystinuria</b>
	<b>Cases</b>	<b>Controls</b>	
No.	n=10	n=10	n=1
Age, y	45 (13)	45 (13)	21
Sex, M/F	3 / 7	3 / 7	0/1
Haemoglobin, g/dL	13.4 (1.2)	12.9 (1.4)	12.1
Folate, µg/L	5.6 (2.6)	9.2 (3.5)	2.5
Vitamin B12, ng/L	174 (65)	414 (241)	120
Total cholesterol, mmol/L	5.4 (0.6)	5.1 (1.1)	4.4
Triglyceride, mmol/L	1.3 (0.7)	0.9 (0.3)	1.0
Glucose, mmol/L	4.4 (0.5)	4.5 (0.3)	4.5
Creatinine, µmol/L	92 (13)	91 (13)	85

Data are mean (SD)

Normal ranges= Vitamin B12: 160 - 750 ng/L ; Folate: 1.6 - 6.0 µg/L

#### *Patients with low or low-normal vitamin B<sub>12</sub>/folate status*

Ten subjects (mean age, 45±13 years; range, 24 to 64 years; 3 men) with low or low-normal vitamin B<sub>12</sub> levels (serum B<sub>12</sub><200 ng/L; normal range, 160 to 750 ng/L) and/or folate (serum folate<2.0 µg/L; normal range, 1.6 to 6.0 µg/L) were recruited via the Haematology Department at the University Hospital of Wales.

Results of vitamin B<sub>12</sub> and folate levels (routine clinical requests by their medical practitioners) were screened from all patients aged 18-65 years over a six-month period. Forty suitable subjects were identified for inclusion of which 10 agreed to participate. All patients were identified on the basis of their low vitamin B<sub>12</sub> level, which was the most frequently abnormal parameter. None of the patients were identified with a low folate concentration as the only abnormal parameter (Table 4.2).

#### *Controls*

Ten age and sex-matched healthy controls were selected at random (by a research nurse who was not involved with vascular measurements), from a database of over 200 healthy subjects who had previously undergone measurement of flow-related endothelial function as part of another study (see Chapter 5). All controls had serum B<sub>12</sub>>200 ng/L and folate>3.0 µg/L.

Subjects were excluded if they were currently taking vitamin supplements or had received vitamin B<sub>12</sub> injections prior to entering the study. All subjects were well, not clinically anaemic (haemoglobin =11g/dL), normotensive (BP<160/90 mmHg), not diabetic, serum cholesterol <6.5 mmol/L, were non-smokers and were not taking any drugs that would potentially interfere with the results (e.g. non-steroidal anti-inflammatory drugs, oral nitrates or steroids).

### **4.3 Methods**

#### **4.3.1 Clinical assessment**

History and physical examination confirmed that all subjects were well, were not taking drug therapy that would interfere with vascular measurements. Subjects were selected to exclude known causes of endothelial dysfunction (as listed above). However, one subject with a diagnosis of ischaemic heart disease was included as

**Table 4.2: Individual patient data and clinical details at recruitment prior to study entry**

<b>ID</b>	<b>Age</b>	<b>Sex</b>	<b>Clinical reason for blood request</b>	<b>Hb g/dL</b>	<b>MCV</b>	<b>Vitamin B12 ng/L</b>	<b>Folate µg/L</b>
1	60	F	Macrocytosis	13.5	100.7	190	2.9
2	36	F	Asthma ? anaemia	12.5	92.4	103	3.3
3	24	F	Macrocytosis	11.2	102.4	134	4.1
4	39	F	Macrocytosis	13.1	103.5	115	2.3
5	57	F	Oral ulceration	13.3	98.7	118	2.9
6	43	F	Irritable bowel syndrome	14.2	102.1	154	4.1
7	52	M	Osteoarthritis ? aneamia	14.6	86.9	117	7.2
8	46	F	Pernicious anaemia	12.7	101.6	77	2.0
9	64	M	Ischaemic heart disease ? anaemia	13.1	85.8	188	5.6
10	28	M	Oral ulceration	15.3	90.6	182	5.2

other conventional risk factors for atheroma were absent (oral nitrates and calcium antagonists were stopped 48 hours prior to vascular measurements).

#### **4.3.2 Blood samples and assays**

Fasting venous blood samples were taken for measurement of serum folate, vitamin B<sub>12</sub>, plasma homocysteine, lipids, glucose, creatinine and haemoglobin, prior to vascular measurements. Homocysteine samples were immediately placed on ice and plasma separated within 30 minutes by centrifugation. All samples were stored at -70°C until analysis. Individual assays were performed as described in Chapter 3.3.3.

#### **4.3.3 Protocol**

##### *Homocystinuric patient*

This subject attended after an overnight fast on Day 1. Venous blood was sampled as described above and vascular measurements recorded. This procedure was repeated approximately 3 months later (Day +82) after treatment with vitamin supplements, including betaine, and dietary methionine restriction (vitamin B<sub>6</sub>, 450mg tid; betaine, 2g tid; and folic acid 10mg bid). High dose vitamin B<sub>6</sub> has been shown to be effective in some subjects with some residual cystathionine  $\beta$ -synthase activity (pyridoxine responsiveness)<sup>5</sup>. Vitamin B<sub>12</sub> was not administered initially by the Sheffield clinicians, but later added after this study was completed.

##### *Low vitamin B<sub>12</sub>/folate status versus controls*

Subjects attended after an overnight fast on one occasion only. This was an open (non-blinded) study.

#### **4.3.4 Measurement of endothelial function**

Performed as described in Chapter 2.

#### **4.3.5 Statistical analysis**

Group data for patient characteristics are presented as mean  $\pm$  SD and compared using Student's unpaired t-test. Blood levels and brachial artery diameter data were also compared using Student's unpaired t-test but data for blood flow were shown not to be normally distributed in this group of subjects and were accordingly

compared by Mann-Whitney test,  $p < 0.05$  being regarded as significant. A total of 10 subjects in each group would provide 80 % power of detecting a 0.04 mm change in end-diastolic brachial artery diameter with a significance level of 0.05.

#### 4.4 Results

Characteristics of the study population are shown in Table 4.1. Cases and controls appeared well matched except in relation to vitamin B<sub>12</sub> and folate levels.

##### 4.4.1 Measurements in blood

###### *Homocystinuric patient*

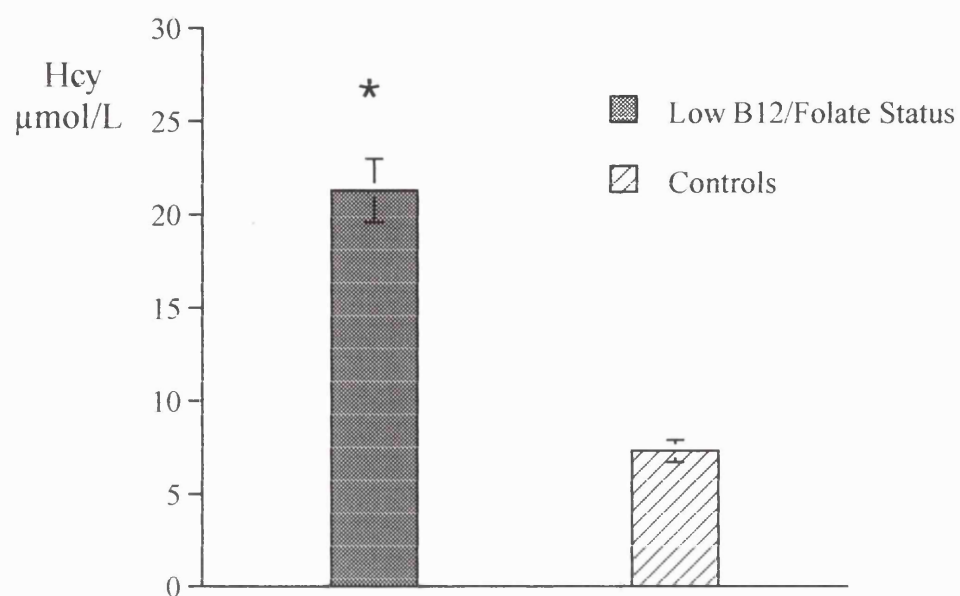
Serum vitamin B<sub>12</sub> concentration was low (120 ng/L) and folate concentration (2.5 µg/L) was at the lower end of the normal range. Plasma total homocysteine was markedly elevated at 112 µmol/L for the baseline visit.

Following vitamin and betaine supplementation, plasma homocysteine decreased to 25 µmol/L by 3 months (i.e.  $\Delta$  -78%). Plasma folate increased to >20 µg/L but vitamin B<sub>12</sub>, total cholesterol and creatinine remained essentially unchanged (131 ng/L; 5.1 mmol/L; 89 µmol/L, respectively).

###### *Low vitamin B<sub>12</sub>/folate status versus controls*

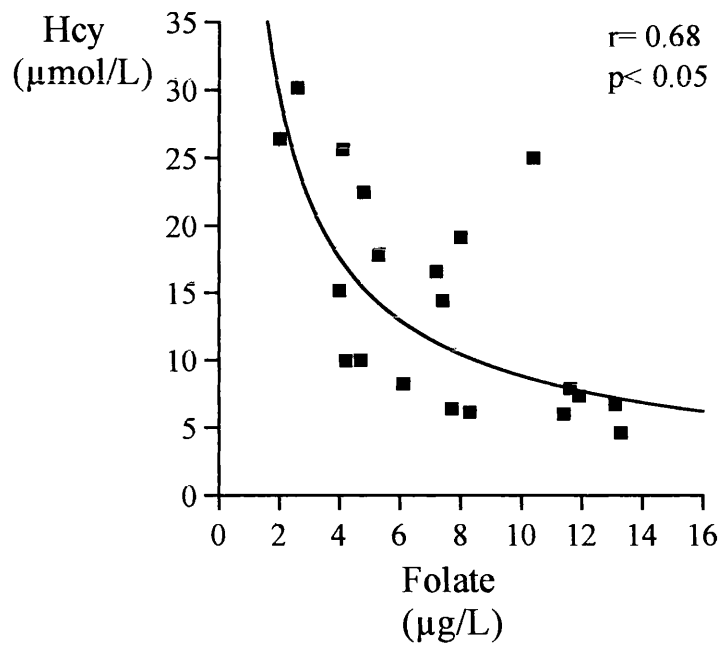
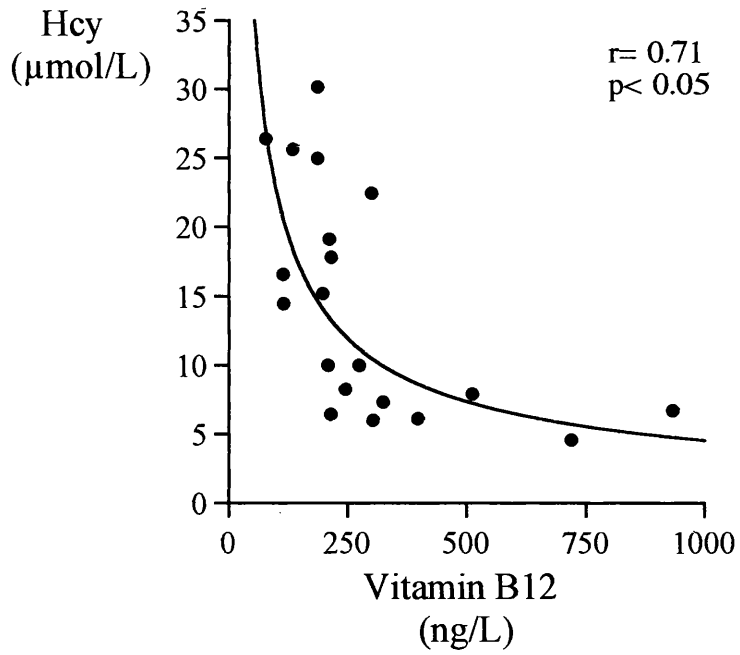
Mean serum vitamin B<sub>12</sub> was  $174 \pm 65$  ng/L in patients compared with  $414 \pm 241$  ng/L in controls. Although all patients were selected primarily on the basis of their vitamin B<sub>12</sub> level, folate concentrations were also significantly lower in cases compared with control subjects ( $5.6 \pm 2.6$  versus  $9.2 \pm 3.5$  µg/L,  $P < 0.001$ , respectively) (Fig. 4.1), but within the normal range.

For all 20 subjects there was a significant inverse relationship between serum vitamin B<sub>12</sub> and plasma homocysteine, and similarly between serum folate and homocysteine (Fig. 4.2).



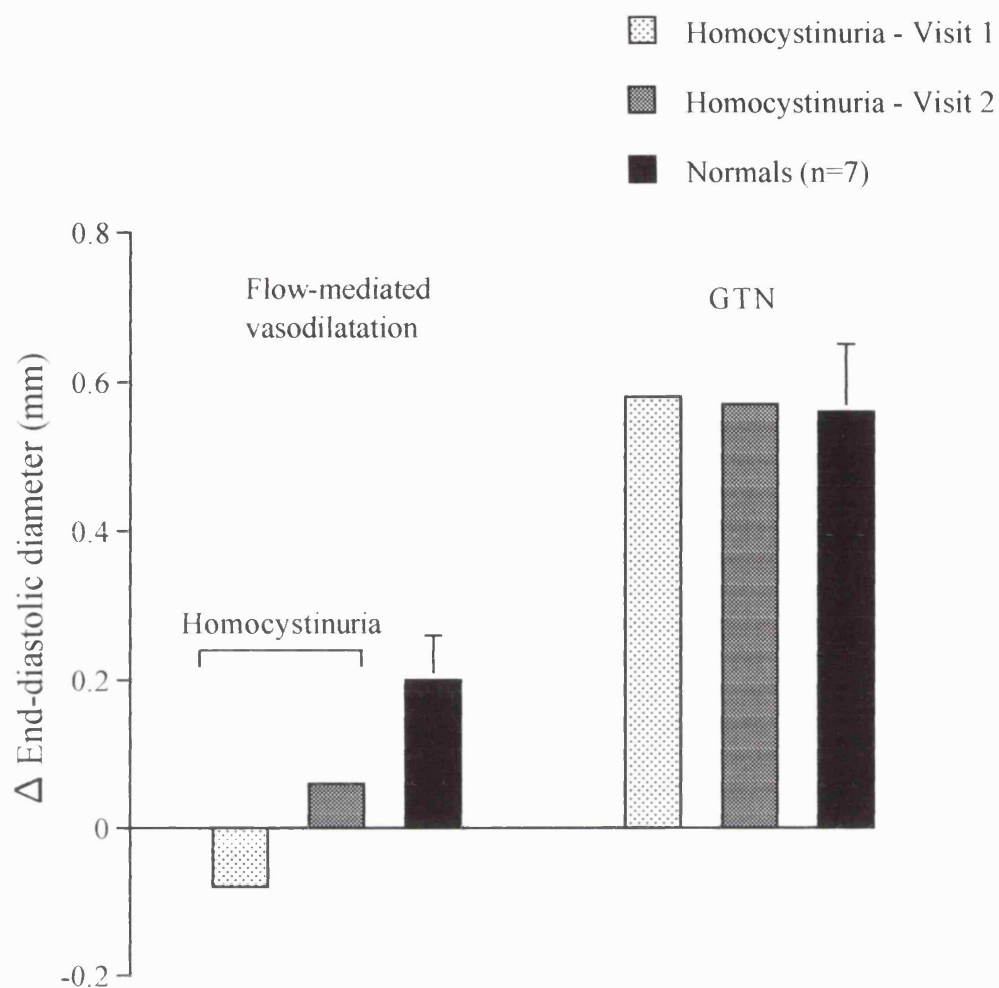
**Fig 4.1: Plasma total homocysteine in cases and controls**

Low B<sub>12</sub>/folate status subjects had significantly higher Hcy levels compared with controls (\*P<0.001).



**Fig 4.2: Scattergrams showing relationship between total plasma homocysteine and serum vitamin B<sub>12</sub> (top panel) and serum folate (bottom panel)**

There was a significant inverse relationship between vit B<sub>12</sub> and Hcy [ $\text{Hcy} = 551.6 \times \text{B}_{12}^{-0.69}$ ] and similarly for folate [ $\text{Hcy} = 49.8 \times \text{folate}^{-0.75}$ ].



**Fig 4.3 : Flow-mediated and GTN-mediated responses in homocystinuria**

Comparison of endothelium-dependent and -independent vasodilatation at visit 1 and following 3 months oral supplements (vitamin B6, folate and betaine), visit 2. Normal responses for age and sex-matched normal subjects is also shown (n=7, from "normal" database - see Chapter 5).



#### 4.4.2 Brachial artery study

##### 4.4.2.1 Homocystinuric patient

At visit 1, flow-mediated *vasoconstriction* of the brachial artery was observed. Brachial artery end-diastolic diameter decreased from 2.58 mm to 2.50 mm following cuff release (i.e.  $\Delta -0.08$  mm,  $\Delta -3.1\%$ ) despite an increase in hyperaemic blood flow (+44 mL/min) which was similar to that obtained in normal subjects (Fig 4.3). GTN-mediated vasodilatation however was not impaired (2.51mm to 3.09 mm, i.e.  $\Delta +0.58$  mm,  $\Delta +21\%$ ).

At visit 2, following 3 months treatment with vitamin supplements and betaine, flow-mediated *vasodilatation* was demonstrated ( $\Delta +0.06$  mm,  $\Delta +2.1\%$ ) but not to the level that would be expected in age and sex-matched subjects (approx.  $\Delta +0.20$  mm,  $\Delta +6.0\%$ ,  $n=7$ ) (Fig 4.3) with results obtained from the database of study volunteers in the normal population (see Chapter 5.2). GTN -mediated dilatation was unchanged by vitamin supplementation (Table 4.3).

##### 4.4.2.2 Low vitamin B<sub>12</sub>/folate status versus controls

Flow-mediated endothelium-dependent vasodilatation was significantly lower in cases compared with controls ( $-0.02 \pm 0.12$  vs.  $+0.09 \pm 0.08$  mm, respectively  $P < 0.001$ ) (i.e.  $-0.8 \pm 3.4$  vs.  $+3.7 \pm 3.3\%$ ,  $P < 0.001$ ). There was no difference in the endothelial-independent response to GTN ( $+0.54 \pm 0.15$  vs.  $+0.54 \pm 0.13$  mm, NS) (i.e.  $+17.1 \pm 7.9$  vs.  $+18.3 \pm 5.2\%$ , NS) (Fig. 4.4 & Table 4.4).

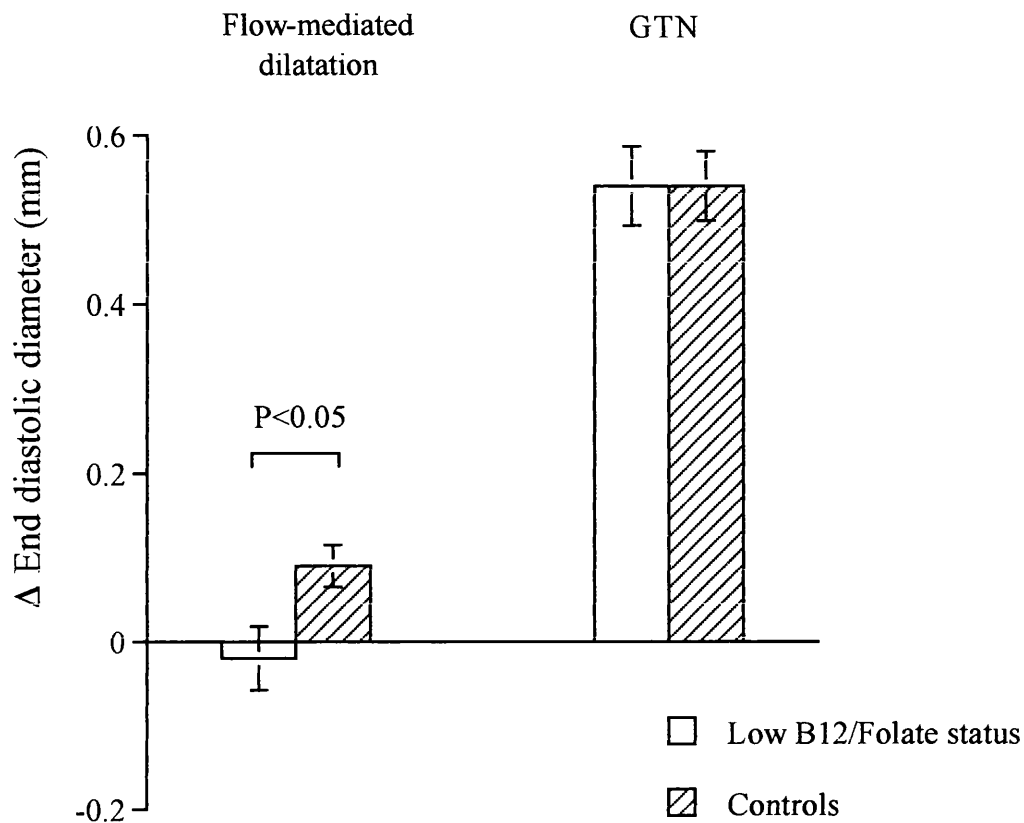
Hyperaemic flow, the stimulus for nitric oxide release, increased similarly during hand hyperaemia in both groups ( $+55 \pm 37$  vs.  $+56 \pm 46$  mL/min, for cases and controls respectively, NS) and also similarly following sublingual GTN (Fig 4.5).

There was an inverse relationship between flow-mediated brachial artery dilatation and plasma homocysteine but not to the point of statistical significance in these 20 subjects ( $r = -0.42$ ,  $P = 0.067$ , Fig. 4.6).

**Table 4.3 Homocystinuria: haemodynamic data**

Basal haemodynamic data, changes in brachial artery end-diastolic diameter (EDD), and blood flow during flow-mediated dilatation and after GTN for visit 1 and visit 2.

	Visit 1	Visit 2
	Baseline	Treatment
<b><u>Basal</u></b>		
Brachial artery EDD, mm	2.58	2.81
Blood flow, mL .min <sup>-1</sup>	12	17
Heart rate, min <sup>-1</sup>	83	82
Systolic blood pressure, mmHg	131	135
Diastolic blood pressure, mmHg	69	68
<b><u>Flow-mediated dilatation</u></b>		
Δ Brachial artery EDD, mm	-0.08	+0.06
%	-3.1	+2.1
Δ Blood flow, mL.min <sup>-1</sup>	+44	+32
<b><u>Glyceryl trinitrate (400μg)</u></b>		
Δ Brachial artery EDD, mm	+0.58	+0.57
%	+23.1	+20.3
Δ Blood flow, mL.min <sup>-1</sup>	+4	+4



**Fig. 4.4: Flow-mediated and GTN-mediated vasodilatation: low B12/folate versus controls**

Flow-mediated, endothelium-dependent responses were significantly impaired in cases compared with controls. Endothelium-independent responses after GTN were similar in both groups.

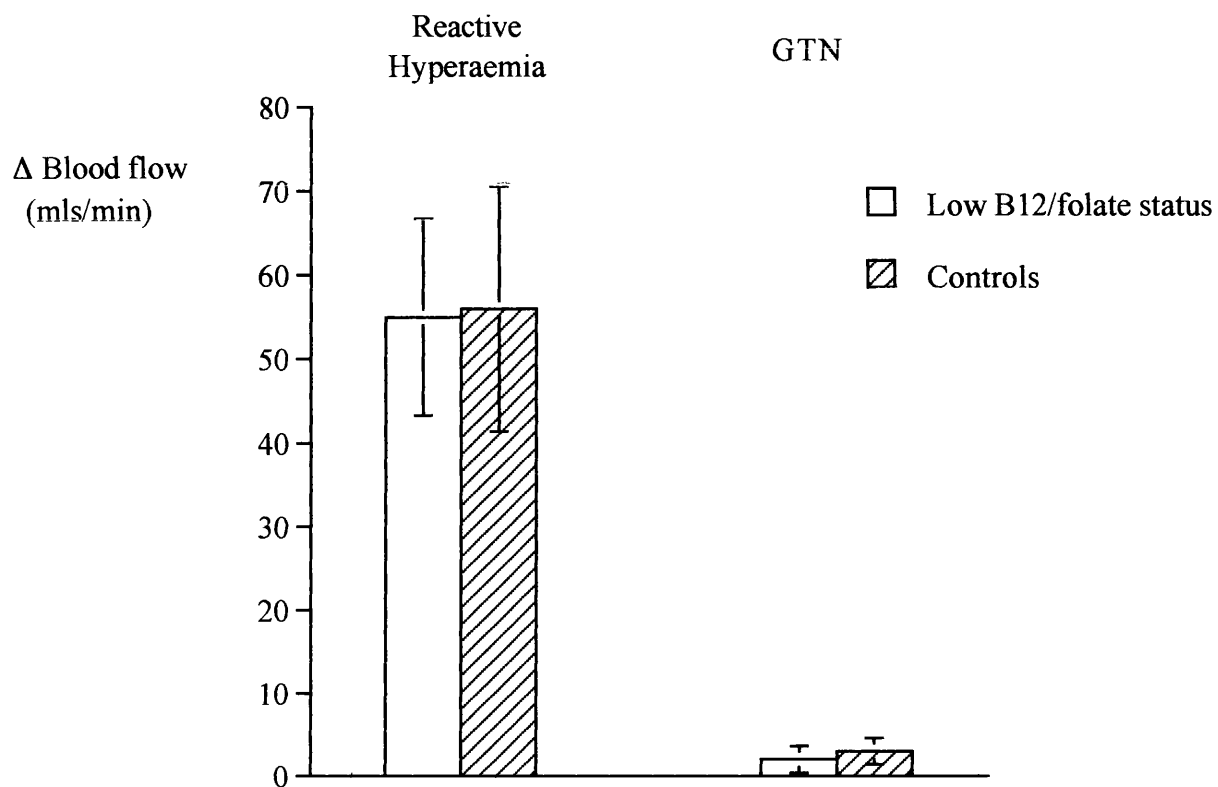
**Table 4.4 Low vitamin B<sub>12</sub> / folate status vs controls : haemodynamic data**

Basal haemodynamic data, changes in brachial artery end-diastolic diameter (EDD), and blood flow during flow-mediated dilatation and after GTN.

	<b>Low B12/Folate Status</b>	<b>Controls</b>
<b><u>Basal</u></b>		
Brachial artery EDD, mm	3.41±0.92	3.01±0.59
Blood flow, mL .min <sup>-1</sup>	10±7	14±16
Heart rate, min <sup>-1</sup>	76±7	75±6
Systolic blood pressure, mmHg	126±15	120±17
Diastolic blood pressure, mmHg	68±14	64±8
<b><u>Flow-mediated dilatation</u></b>		
Δ Brachial artery EDD, mm	-0.02±0.12*	+0.09±0.08
%	-0.8±3.4*	+3.7±3.3
Δ Blood flow, mL.min <sup>-1</sup>	+55±37	+56±46
<b><u>Glyceryl trinitrate (400μg)</u></b>		
Δ Brachial artery EDD, mm	+0.54±0.15	+0.54±0.13
%	+17.1±7.9	+18.3±5.2
Δ Blood flow, mL.min <sup>-1</sup>	+2±5	+3±5

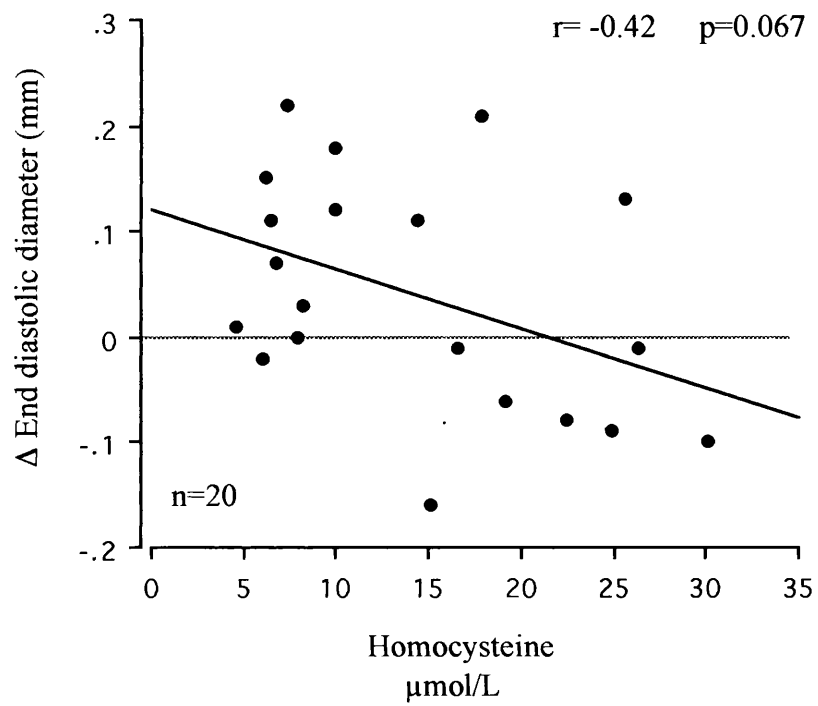
Data are mean ± SD

\* P<0.001 vs controls



**Fig 4.5: Changes in blood flow**

Similar increases in both hyperaemic blood flow and following sublingual GTN were observed in cases and control subjects.



**Fig 4.6: Relationship between plasma homocysteine and flow-mediated brachial artery dilatation in low B12/folate cases and control subjects**

#### 4.5 Discussion

The study of subjects with low B<sub>12</sub>/folate status and secondary hyperhomocysteinaemia demonstrates that this metabolic disturbance was associated with impaired flow-related endothelial function. However, these data need to be interpreted cautiously before concluding that the relationship is one of cause and effect. Numbers are small and the correlation between plasma homocysteine and flow-mediated dilatation was not significant with the small number of subjects, but there nevertheless appeared to be an association.

Control subjects were age and sex-matched but it is possible that the patients may have differed in some other respect, e.g. exercised less frequently, greater passive smoke exposure, or other as yet unknown causes of endothelial dysfunction may have been present. Another potential drawback of the study was that the operator performing vascular measurements was aware of the status of the low B<sub>12</sub>/folate patients and despite all care taken with the recording of data, bias may have influenced the results.

The vascular response to vitamin treatment in these patients was not studied and would be of interest. Improvement of flow-mediated responses in association with lowering of homocysteine by vitamin supplementation would have supported a cause and effect relationship. Unfortunately, vitamin replacement therapy was commenced in some patients by their physicians before all 10 volunteers had completed the study, removing the opportunity to carry out an appropriately designed intervention phase with the same treatment regimen in all subjects.

An opportunity to observe the response to treatment was however available for the single homocystinuric subject recruited to the study. After a combination of vitamin B<sub>6</sub>, betaine and high dose folic acid, a response to treatment was indeed observed at 3 months with hyperaemia-induced brachial artery vasodilatation seen instead of vasoconstriction. Interpretation of this response in a single subject cannot of course be extrapolated to the population as a whole but it provides some preliminary support for a beneficial effect of vitamin supplementation on endothelial function in such individuals with a high risk of vascular complications.

The proposal that chronic B<sub>12</sub> deficiency, as would occur in pernicious anaemia, would be a risk factor for atherosclerosis is a novel concept and warrants further investigation with larger numbers of patients.

## **CHAPTER 5**

### **Relationship Between Plasma Homocysteine And Endothelial Function In The General Population: A Cross-Sectional Study**

#### **5.1 Introduction**

Elevated plasma homocysteine levels occur commonly in the general population as described previously. Both case-control and prospective studies have demonstrated an association between elevated homocysteine concentrations, vascular disease<sup>44</sup> and venous thrombosis<sup>115</sup>.

In this chapter, a cross-sectional study was undertaken to investigate the determinants of plasma homocysteine and to examine the relationship between homocysteine and endothelial function. A representative cohort of the healthy local population was needed and volunteers attending blood transfusion collection centres and members of hospital staff were thought to be the best source of potential subjects.

Further to the observation of impaired endothelium-dependent responses following an oral methionine load and in patients with low B<sub>12</sub>/folate status, this study therefore aimed to determine if plasma total homocysteine was a predictor of flow-mediated, endothelium-dependent responses using a multivariate analysis in a relatively large group of healthy subjects, after exclusion of known causes of endothelial dysfunction.

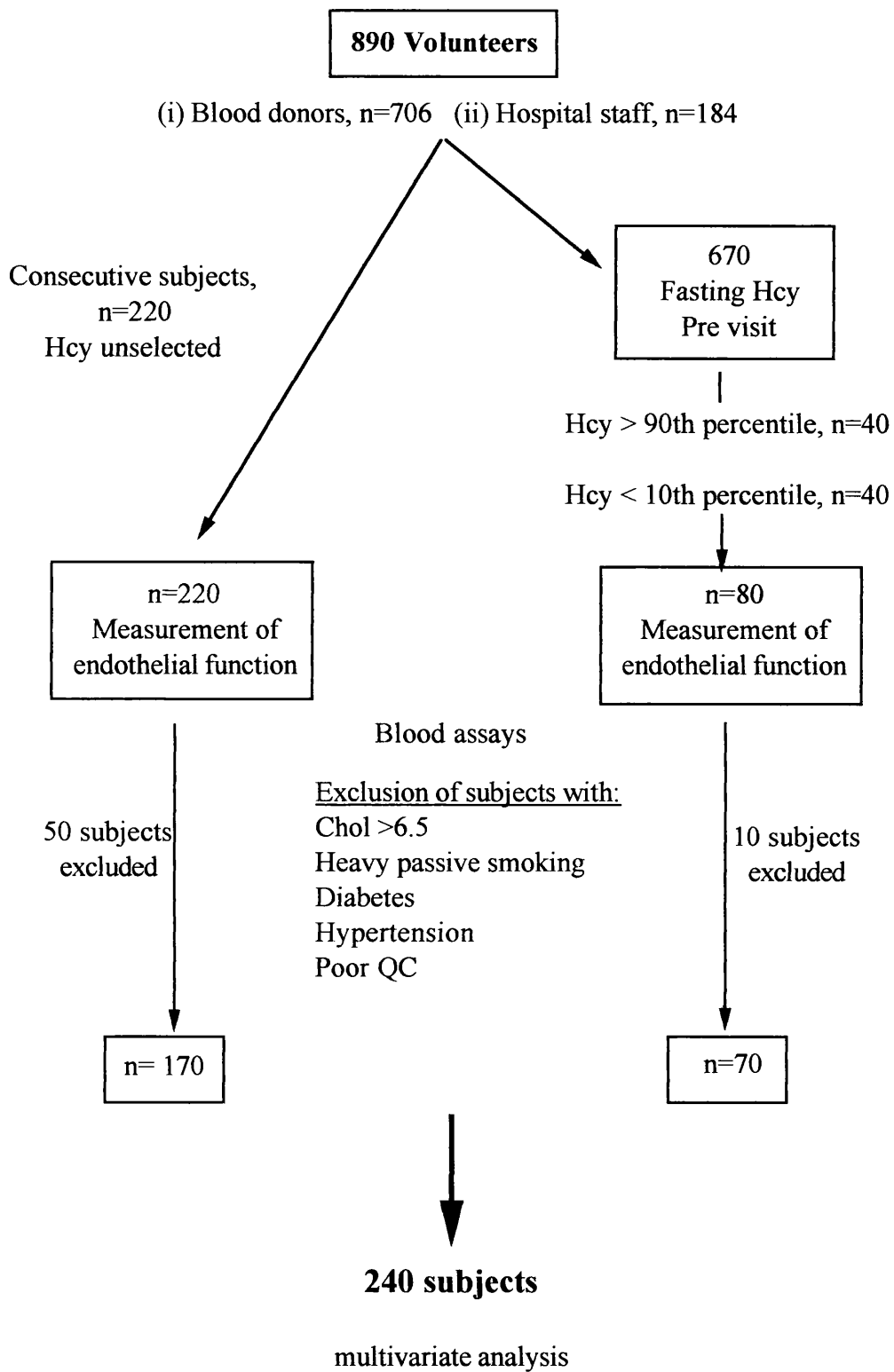
#### **5.2 Subjects**

Subjects between 18 and 65 years of age were recruited from the following 2 groups (see Fig 5.1):-

(i) the Welsh Blood Transfusion Service kindly agreed for volunteers attending routine blood donation sessions to be interviewed following donation. Samples were not however allowed to be collected at the time of venesection. Non-smoking volunteers were then personally invited to attend on a separate visit (see below) to participate in the study (n=706), and

(ii) healthy volunteers were recruited from members of hospital staff (n=184).





**Fig 5.1: Outline of subject recruitment and selection protocol**

All subjects gave written informed consent to the study protocol, which was approved by the Local Research Ethics Committee.

### 5.3 Methods

#### 5.3.1 Study design

Subjects were entered into the study protocol according to the following selection criteria:-

##### Homocysteine-unselected (n=220)

The first 220 consecutive subjects recruited from either of the above groups attended after an overnight fast for measurement of plasma total homocysteine, folate, vitamin B12, lipids, glucose and creatinine. Immediately following blood sampling, measurement of flow-related endothelial function was performed.

##### Homocysteine-selected (n=80)

In order to ensure the selection of subjects across the range of homocysteine concentrations, the remaining 670 volunteers attended after an overnight fast for blood sampling only. EDTA blood was placed on ice and plasma separated within 1 hour and stored at  $-70^{\circ}\text{C}$  until analysis. Plasma homocysteine was measured in batches (n=20 subjects per batch x 33; n=10 subjects x 1).

Those subjects within each batch with plasma homocysteine  $>90^{\text{th}}$  percentile or  $<10^{\text{th}}$  percentile (i.e. 20% of subjects within each batch) were then invited to attend for measurement of flow-related endothelial function as performed in the homocysteine-unselected group. Eighty of the selected individuals agreed to attend on a second visit (Hcy $>90^{\text{th}}$  percentile, n=20; Hcy $<10^{\text{th}}$  percentile, n=20). Venous blood for homocysteine assay was taken again immediately before vascular measurements together with folate, vitamin B<sub>12</sub>, lipids, glucose and creatinine concentrations.

##### Exclusions

Subjects were selected to exclude known causes of endothelial dysfunction and were therefore omitted from subsequent analysis if any of the following were identified:-

(i) total cholesterol  $>6.5\text{mmol/L}$ ; (ii) fasting glucose  $>7.8\text{ mmol/L}$ ; (iii) blood pressure  $>160/90\text{ mmHg}$  at rest, on  $>3$  repeat measurements during the study (iv)

exposure to heavy passive smoking (spouse or partner active smoker in their household).

Of the 300 subjects who underwent measurement of vascular responses, 60 were excluded from subsequent analysis. Fifty of the 220 homocysteine-unselected subjects were later excluded (26 Chol>6.5; 1 Gluc>7.8; 2 hypertensive; 10 exposed to heavy passive smoking; 11 poor quality vascular recordings despite repeat measurement) and 10 of the homocysteine-selected group (10 Chol>6.5), leaving 240 subjects who met the entry criteria.

### **5.3.2 Measurement of endothelial function**

Non-invasive measurement was performed as described in Chapter 2. Operators performing the vascular measurements were not aware of the subject's homocysteine concentration, but homocysteine-selected subjects were studied as a group after the unselected cohort.

### **5.3.3 Blood samples and assays**

Fasting venous blood samples were taken for measurement of plasma homocysteine, serum folate, vitamin B<sub>12</sub>, lipids, glucose, and creatinine prior to vascular measurements. Blood for homocysteine assay was immediately placed on ice and plasma separated within 30 minutes by centrifugation. All samples were stored at -70°C until analysis. Individual assays were performed as described in Chapter 3.3.3.

### **5.3.4 Statistical analysis** (carried out with the assistance of Dr. R Newcombe, Dept. of Medical Statistics, UWCM)

Data are presented as mean  $\pm$  SD unless stated otherwise. Data for homocysteine, vitamin B<sub>12</sub>, triglyceride, and %flow-mediated dilatation were improved by log transformation. All variables were continuous except sex, which was binary. Analyses used linear or non-parametric (Spearman Rank) regression as appropriate and subsequently a multivariate analysis was performed where endothelium-dependent and independent vascular responses were modelled on total plasma homocysteine, age, sex, B<sub>12</sub>, folate, creatinine, cholesterol, triglyceride, and glucose.

Historically, data for flow-mediated dilatation has been presented as percentage change. As discussed in Chapter 2, a simple increment in brachial artery end-diastolic diameter to represent flow-mediated and GTN-mediated vasodilatation was shown to be a better measure being less dependent on baseline diameter, age and sex. Data were analysed using both methods for comparison. Multivariate analyses were performed according to a number of different regression models. Analysis of covariance was then used to determine respective P values for individual factors.

The regression equation for % flow-mediated dilatation required age and sex in the model together with homocysteine (regression equation: %FMD =  $1.99 - 0.00034 \text{ age} + 0.00769 \text{ sex} + 0.0212 \log \text{Hcy}$ ). Using a simple increment, the model for flow-mediated dilatation involved only age and homocysteine as predictors (regression equation: FMD ( $\Delta$ EDD mm) =  $0.0420 - 0.00179 \text{ age} + 0.127 \log \text{Hcy}$ ). The model for GTN-mediated responses involved creatinine alone, age and homocysteine were not predictive (regression equation: GTN ( $\Delta$ GTN mm) =  $0.706 - 0.00207 \text{ Creat}$ ), see below.

A total of 240 subjects would allow a minimum correlation coefficient,  $r$ , of 0.18 to be detected with 80 % power and a significance level of 0.05.

## 5.4 Results

Characteristics of the study population are shown in Table 5.1. Age at entry ranged from 19 to 59 years and there were slightly more females than males in the cohort of 240 subjects.

### 5.4.1 Plasma homocysteine distributions

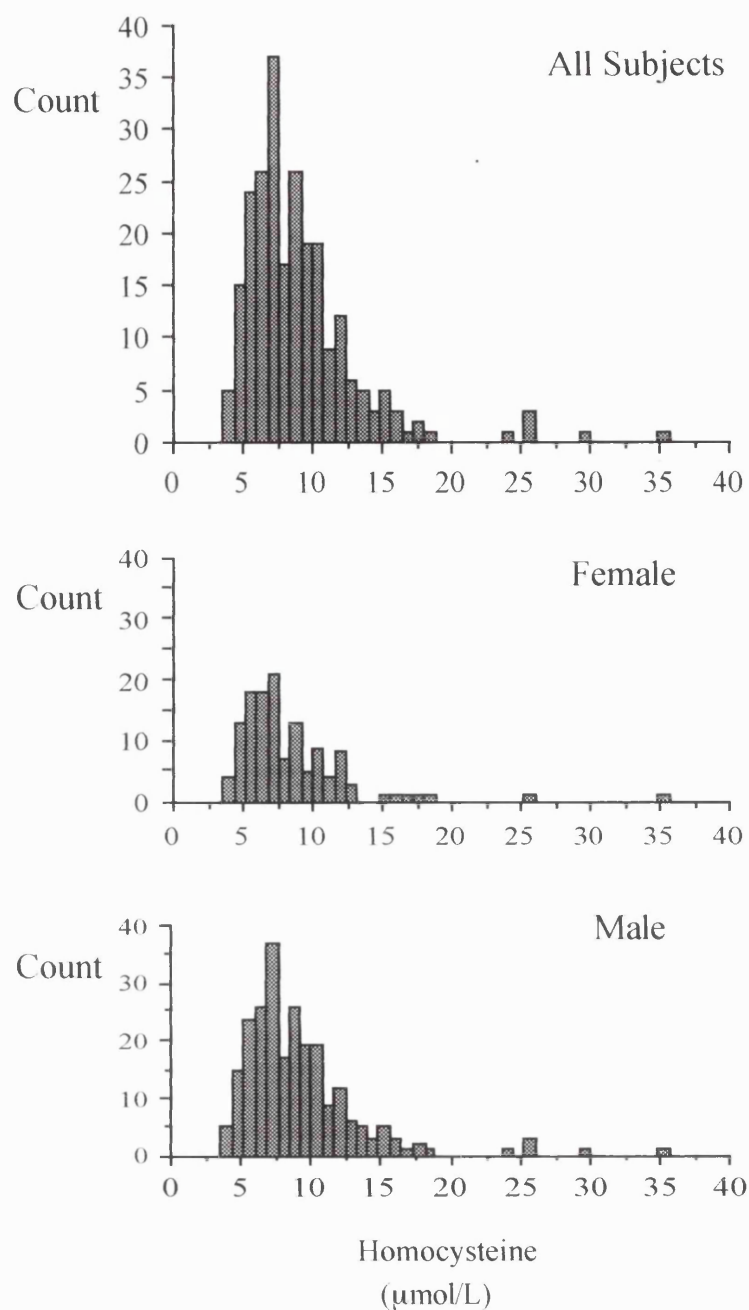
The distribution of fasting plasma total homocysteine concentrations for all ( $n=240$ ) subjects is shown in Fig.5.2. Plasma total homocysteine ranged from 3.6 to 35.7  $\mu\text{mol/L}$ , median 8.3 $\mu\text{mol/L}$ . The distribution is skewed to the right.

The respective distributions for male ( $n=110$ ) and female ( $n=130$ ) subjects are also shown in Fig 5.2. Male subjects showed a tendency to slightly higher values than females (median: 9.3 vs. 7.4  $\mu\text{mol/L}$ , respectively) with more male subjects having homocysteine concentrations above the normal range (male,  $n=10$  vs. female,  $n=6$ ;  $\text{Hcy} > 15 \mu\text{mol/L}$ ).

**Table 5.1 Subject characteristics: cross-sectional study**

<b>Parameter</b>	
No.	n=240
Age, years	35 (11)
<i>Range, years</i>	19 - 59
Sex, M/F	110 / 130
<i>Total homocysteine, <math>\mu\text{mol/L}</math> (median)</i>	8.3
<i>Range, <math>\mu\text{mol/L}</math></i>	3.6 - 35.7
Folate, $\mu\text{g/L}$	8.1 (2.8)
Vitamin B12, ng/L	381 (158)
Total cholesterol, mmol/L	4.8 (0.8)
Triglyceride, mmol/L	1.1 (0.6)
Glucose, mmol/L	4.7 (0.4)
Creatinine, $\mu\text{mol/L}$	90 (13)

Data are mean (SD), unless otherwise stated.



**Fig 5.2: Plasma total homocysteine distributions**

Histograms showing the range of homocysteine concentrations for all subjects (top panel, n=240); for females (middle panel, n=130) and for males (bottom panel, n=110). More male subjects had homocysteine levels above the normal range of 7-15 μmol/L compared with females.

#### **5.4.2 Determinants of plasma homocysteine concentration: sex, folate, vitamin B<sub>12</sub> and creatinine (Table 5.2)**

Data for homocysteine, vitamin B<sub>12</sub> and triglyceride were log transformed. Plasma homocysteine was inversely correlated with serum folate ( $r = -0.53$ ,  $P < 0.0001$ ) and serum vitamin B<sub>12</sub> ( $-0.37$ ,  $P < 0.0001$ ) (Fig 5.3 and Fig 5.4). Creatinine was also correlated with plasma homocysteine ( $r = +0.35$ ,  $P < 0.0001$ ). As noted in the histograms for male and female subjects, gender was also associated with homocysteine levels. There was no correlation between age, serum lipids or glucose.

#### **5.4.3 Relationship between plasma homocysteine and flow-related endothelial function**

Flow-mediated and GTN-mediated responses are shown in Fig 5.5 and Fig 5.6, respectively. Data are expressed as both (i) a ratio calculated as percentage change from baseline and (ii) as a simple absolute increment in brachial artery diameter.

For both flow-mediated and GTN-mediated brachial artery dilatation, there was a marked diversity in values measured. Flow-mediated, endothelium-dependent dilatation was markedly impaired in many subjects with homocysteine values at the lower end of normal range, often demonstrating flow-mediated vasoconstriction. This occurred despite exclusion of subjects with known risk factors for endothelial dysfunction.

Multivariate analysis, using a simple increment in end-diastolic diameter (mm) to express flow-mediated changes, demonstrated a weak positive correlation between plasma homocysteine and flow-mediated brachial artery dilatation, but in the opposite direction to that expected ( $r = +0.127$ ,  $P = 0.014$ ). No significant correlation was observed using percentage change before or after log transformation of the data.

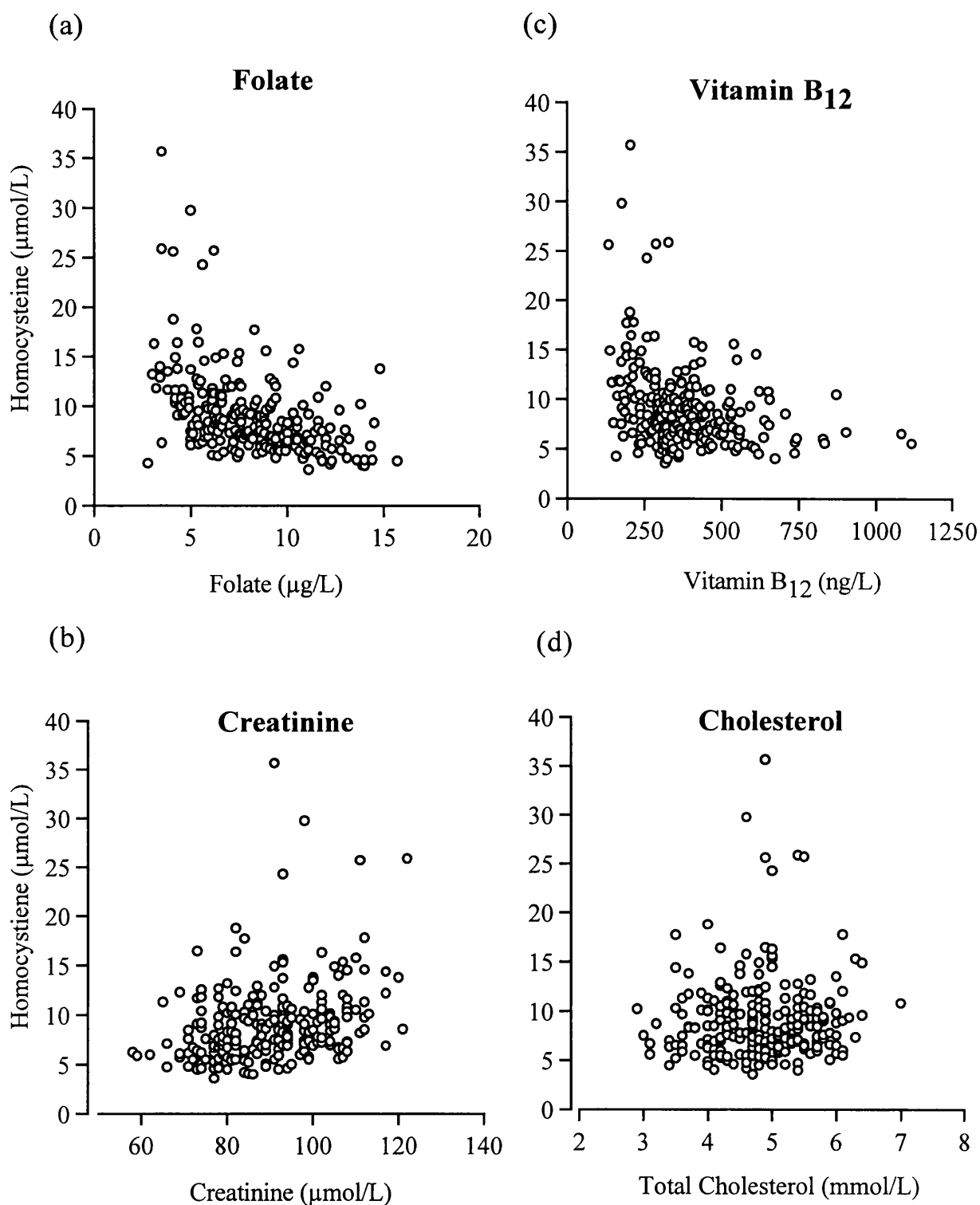
Homocysteine was not a predictor of GTN-mediated vasodilatation ( $r = +0.034$ ,  $P = 0.145$ ) for either method. Creatinine was the only variable on multivariate analysis to show a weak inverse correlation with GTN-mediated responses when expressed as a simple increment in brachial artery diameter ( $r = -0.13$ ,  $P = 0.05$ ).

**Table 5.2: Determinants of plasma homocysteine\* concentration**

<b>Parameter</b>	<b>Correlation coefficient r</b>	<b>P value</b>
Age	0.087	0.18
Sex	-0.276	<0.0001
Folate	-0.531	<0.0001
Vitamin B <sub>12</sub> *	-0.374	<0.0001
Total cholesterol	0.080	0.22
Triglyceride*	0.090	0.17
Glucose	0.069	0.30
Creatinine	0.349	<0.0001

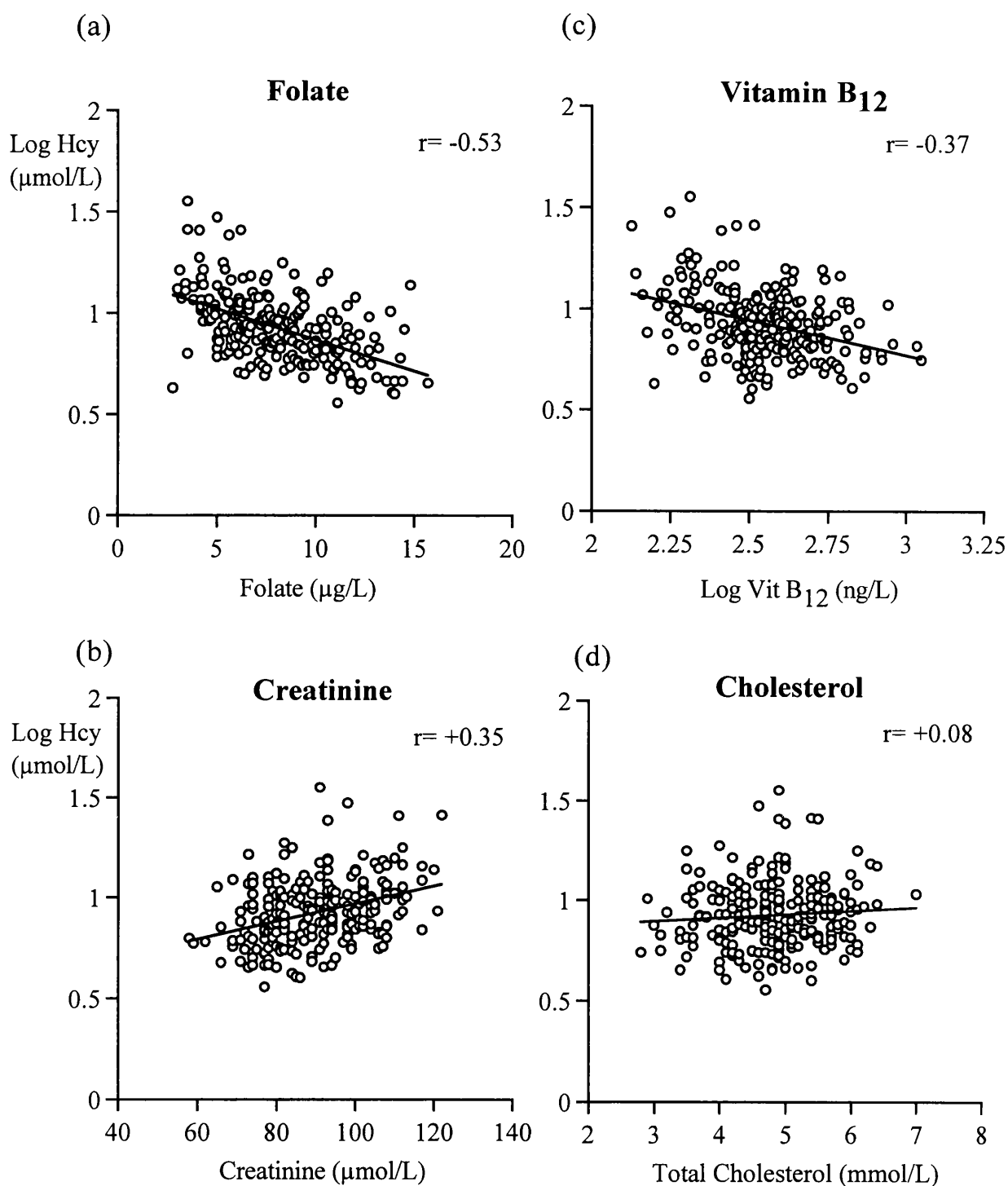
\*Plasma homocysteine, vitamin B<sub>12</sub> and triglyceride data have been log-transformed.





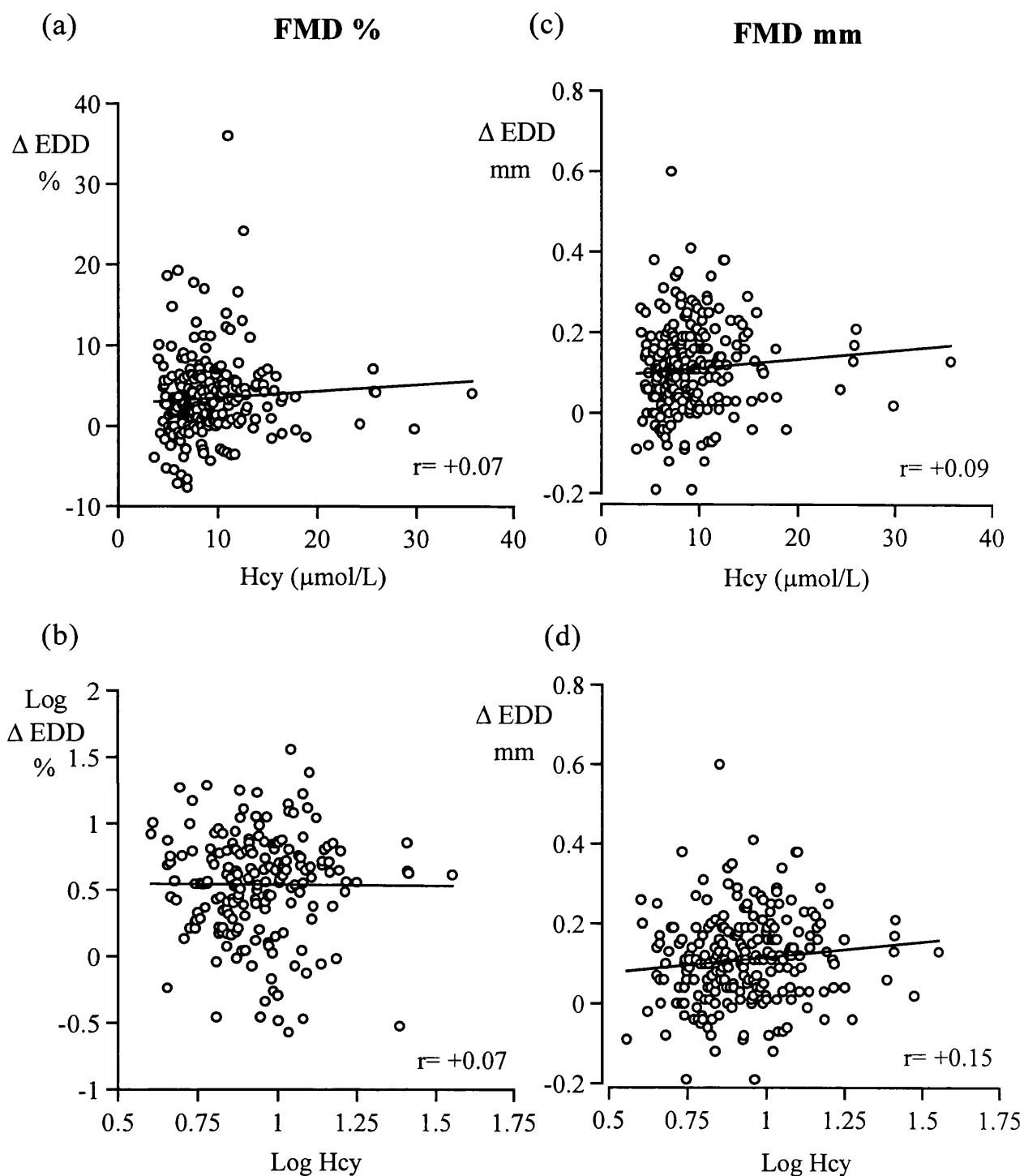
**Fig 5.3: Scatter plots of relationship between plasma homocysteine and vitamin levels, creatinine and cholesterol concentrations**

Data are shown without log transformation of homocysteine.



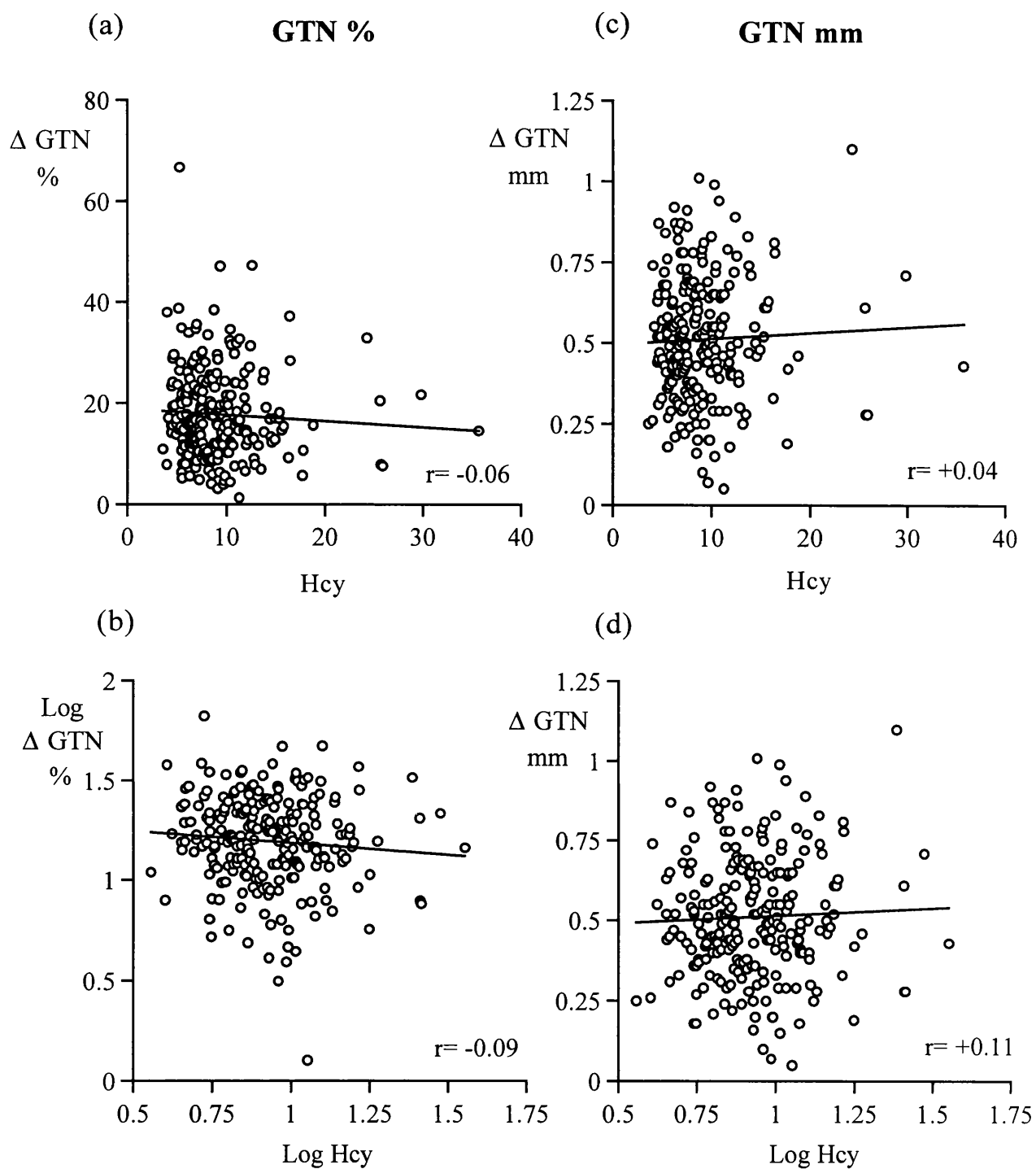
**Fig 5.4: Log transformed scatter plots for total plasma homocysteine**  
Data for vitamin B<sub>12</sub> was also improved by log transformation.

Scatter plots are shown with their respective correlation coefficients for the relationships between log total homocysteine concentration and folate, log vit B<sub>12</sub>, creatinine and cholesterol. Folate was most strongly inversely correlated with homocysteine. There was no correlation with total cholesterol.



**Fig 5.5: Relationship between flow-mediated vasodilatation (FMD) and plasma homocysteine concentration (pre- and post-log transformation)**

The variables homocysteine and percentage change in end-diastolic diameter ( $\Delta \text{EDD \%}$ ) have been log transformed to improve data. Scatter plots are shown with their respective correlation coefficients. There was a weak correlation between homocysteine and absolute change in end-diastolic diameter (EDD mm), but opposite to that expected ( $P=0.026$ ).



**Fig 5.6: Relationship between GTN-mediated vasodilatation and plasma homocysteine concentration (pre- and post-log transformation)**

The variables homocysteine and percentage change in end-diastolic diameter ( $\Delta \text{EDD \%}$ ) have been log transformed to improve data. Scatter plots are shown with their respective correlation coefficients. No significant correlation was observed.

## 5.5 Discussion

The data in this cross-sectional study do not support an association between elevated plasma tHcy and endothelial dysfunction. Indeed, a weak positive correlation between plasma tHcy and FMD was demonstrated. This result was unexpected and is in contrast to two recently published studies<sup>116,117</sup> demonstrating an inverse association, the data following induction of acute hyperhomocysteinaemia after an oral methionine load (Chapter 3) and the observation of impaired endothelial function in patients with low vitamin B<sub>12</sub>/folate status (Chapter 4).

There are a number of possible explanations as to why an association between tHcy and endothelial dysfunction may not have been demonstrated in this study. A large number of subjects with low tHcy concentrations have unexpectedly abnormal flow-mediated responses, some of which demonstrate flow-mediated vasoconstriction, despite exclusion of the known risk factors for endothelial dysfunction. The distribution of flow-mediated values in Fig. 5.5 shows this clearly. Excluding operator error and chance, one plausible explanation may be the presence of other as yet unknown risk factors. Individual genotype may have an important independent influence; for example, a positive family history of coronary artery disease has been shown to be associated with endothelial dysfunction<sup>118</sup>. There is some preliminary evidence to suggest that Angiotensin converting enzyme genotype may be another factor affecting vasodilator function and the insertion/deletion (I/D) polymorphism of the ACE gene has been shown to be associated with abnormal vascular reactivity in the coronary circulation<sup>119</sup>. It is likely that other genetic determinants may become apparent as more potential genes are investigated.

Environmental factors may also be important. Passive smoking has been shown to induce endothelial dysfunction<sup>120</sup> and is very difficult to quantify and exclude. A history of exposure was taken from each patient and subjects excluded if their partner or spouse smoked regularly within their household. However, it is possible that some subjects may have had significant exposure socially or in their workplace, which could have influenced the results. A more robust method would have been to measure plasma or salivary cotinine and to exclude those subjects with raised levels. However, this may have markedly reduced the number of subjects eligible for entry.

A methionine loading test was not included as part of the study protocol. Subjects with normal fasting homocysteine concentrations, who may potentially develop hyperhomocysteinaemia and subsequent vascular disease due to impaired homocysteine metabolism, would not be correctly identified. This may have accounted for a proportion of subjects with normal fasting homocysteine<sup>16</sup>. The loading test was not included due to the unpalatable taste of methionine which may have restricted recruitment, a doubling of homocysteine assays needed and the increased workload required with the large number of subjects studied.

Due to the large number of vascular measurements performed, more than one operator was needed to complete the study (a vascular technician performed approximately 80 of the scans). Although strenuous efforts were made to ensure precise measurements and quality control, inter- and intra-operator variation may have led to differences in measuring responses.

Cholesterol is a well established risk factor for atherosclerosis and endothelial injury and was measured in all subjects. Despite multivariate analysis, no correlation between cholesterol and flow-mediated endothelium-dependent dilatation was demonstrated. This further suggests that a number of factors may be involved to obscure any positive associations and that a much larger cohort of subjects across the range of homocysteine and cholesterol concentrations may have been necessary.

An interventional approach aimed at lowering plasma homocysteine would improve the likelihood of demonstrating any association. In this regard, serum folate was the variable most strongly inversely correlated with homocysteine, followed by vitamin B<sub>12</sub> and creatinine. Oral folate supplementation would therefore be the simplest method of reducing plasma homocysteine concentrations.

In summary, no association was found between plasma tHcy and endothelial dysfunction. However, a number of factors here may have obscured a positive association between Hcy and endothelial dysfunction and no firm conclusions can be drawn. A much larger cohort of subjects across a wider range of tHcy concentrations, with inclusion of a methionine-loading test, would have improved the study but this was beyond the scope of investigator time and resources. A clear correlation has been confirmed between plasma tHcy and folate, vitamin B<sub>12</sub> and creatinine. Oral folate supplementation would appear to be the simplest method of lowering Hcy levels in the absence of vitamin B<sub>12</sub> deficiency.

## CHAPTER 6

### Effect Of Oral Folic Acid Supplementation On Endothelial Function In Mild Hyperhomocysteinaemia

#### 6.1 Introduction

Homocysteine is a modifiable risk factor for vascular disease and can be lowered by oral vitamin supplementation<sup>121</sup>, a combination of folic acid, B<sub>6</sub> and B<sub>12</sub> has been shown to be effective even in the presence of “normal” blood concentrations of these vitamins<sup>36</sup>. In the absence of vitamin B<sub>12</sub> deficiency, effective reduction in homocysteine can be achieved with folate supplementation alone, and as demonstrated in the previous chapter, folate is inversely correlated with plasma homocysteine and is potentially the simplest and most effective supplement for an interventional study.

The potential benefit of homocysteine lowering is exemplified when vascular complications in homocystinuria are reduced when homocysteine is lowered in those individuals with a pyridoxine (vitamin B<sub>6</sub>)-sensitive enzyme defect<sup>5</sup>. Moreover, in regard to possible mechanisms, a reduction in plasma homocysteine may augment endothelial function in these subjects as was demonstrated by the preliminary study in Chapter 4, where flow-mediated responses in a homocystinuric patient improved following treatment.

Vitamin supplementation may have wider public health implications than previously thought. A link between vitamin intake and vascular disease has been highlighted by recent studies demonstrating an association between low levels of circulating folate and B<sub>6</sub> and an increased risk of vascular disease<sup>39,40</sup>. Low levels of vitamin B<sub>6</sub> were, however, independent of any effect on homocysteine<sup>40</sup>. Vitamin supplements may therefore be important in the primary and secondary prevention of vascular disease in susceptible individuals.

However, in mild hyperhomocysteinaemia, despite persuasive evidence, a cause and effect relationship has not been fully established. Large randomised studies are now underway to investigate the effect of lowering homocysteine on cardiovascular morbidity and mortality.<sup>122</sup> As endothelial dysfunction is an early event in the atherosclerotic process,<sup>54</sup> and may serve as a surrogate for cardiovascular risk, improvement of endothelial dysfunction in man by lowering

plasma homocysteine would provide preliminary evidence for a potential preventative and therapeutic role for folates in hyperhomocysteinaemia.

In this chapter, a double-blind randomised crossover trial is described where the effect of oral folic acid supplements on endothelial function is investigated in healthy subjects with homocysteine levels in the upper part of the normal range.

## 6.2 Subjects (see also Chapter 5.2)

Plasma total homocysteine was measured in the cohort of 890 volunteers recruited from (i) blood donors attending Welsh Blood Transfusion Service collection centres (n=706), and (ii) members of hospital staff (n=184).

Volunteers attended after an overnight fast and venous blood for homocysteine, folate, vitamin B<sub>12</sub>, lipids, glucose, creatinine and von Willebrand factor was sampled from the antecubital vein. All subjects denied smoking or heavy passive smoking, were normotensive (BP<150/90 mmHg), not diabetic and were not taking any medication or vitamin supplements.

We selected those subjects with plasma homocysteine >13 µmol/L on their screening visit, a level reported to be associated with an increased risk of cardiovascular disease in previous epidemiological studies<sup>46</sup>. Thirty one (3.5%) of the cohort were thus identified, of which six had serum cholesterol >6.5 mmol/L and were therefore excluded in order to select subjects with no known cause of endothelial dysfunction. The remaining twenty-five volunteers were invited to enter the study, of which twenty agreed to participate (mean plasma homocysteine 18.3±6.1 µmol/L, range 13.2 - 35.7 µmol/L, n=20). All subjects were screened for vitamin B<sub>12</sub> deficiency (B<sub>12</sub><160 ng/dL) but none detected. One subject withdrew before commencing the study after randomisation and one further subject withdrew on completion of the placebo phase having been commenced on beta-blocker treatment for stress-related anxiety by his general practitioner.

All subjects gave informed written consent to the study, which was approved by the local Research Ethics Committee.



## 6.3 Methods

### 6.3.1 Study design (outlined in Fig. 6.1)

This study was of double-blind placebo-controlled crossover design, comprising two six-week treatment periods with oral folic acid or matching placebo in random order, with a six-week interval between treatments. Subjects received 5mg oral folic acid once daily (one capsule per day) with food, or the equivalent matched placebo (lactose) capsules.

Each patient was studied at the same time of day at the start of the study (week 0), and at 6 week intervals thereafter (i.e. at week 6, week 12 and week 18), providing data at baseline, after 6 weeks of folic acid or placebo, after the intervening “washout” period, and after 6 weeks placebo or folic acid.

### 6.3.2 Measurement of endothelial function

Non-invasive measurement of flow-mediated, endothelium-dependent and -independent vasodilatation was performed as described in Chapter 2.

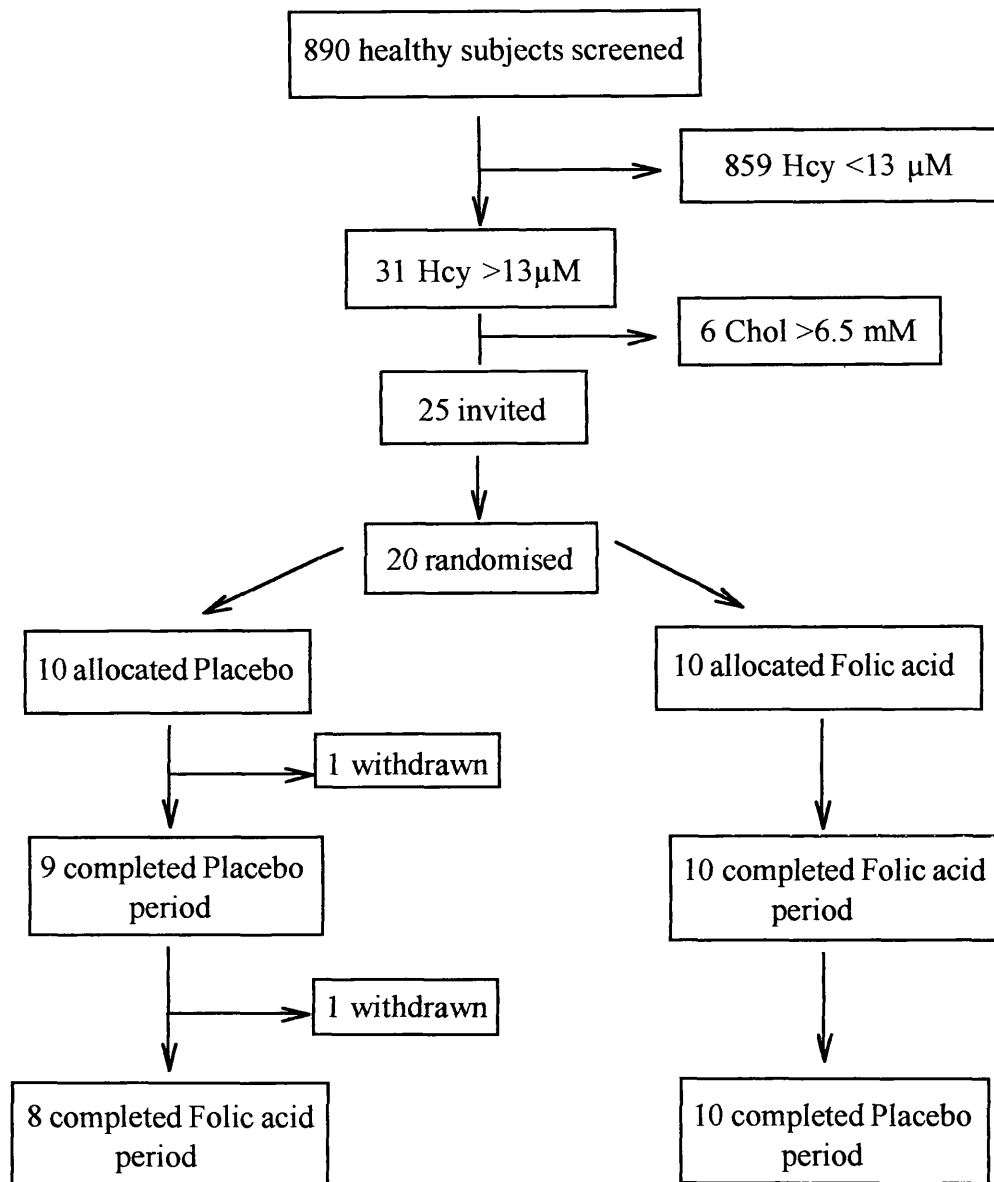
### 6.3.3 Blood samples and assays

Venous blood was sampled into tubes containing EDTA (for homocysteine, genotyping and von Willebrand factor antigen [vWF-Ag]), SST (serum separation tube) (for measurement of folate, vitamin B<sub>12</sub>, lipids and creatinine) and fluoride-oxalate (for glucose). Homocysteine samples were immediately placed on ice and plasma separated within 30 minutes by centrifugation. Samples were stored at -70°C until analysis.

*Total plasma homocysteine.* Samples from each patient (visits 1 to 4) were analysed within the same batch by HPLC (within batch precision, 2.2%)<sup>105</sup>.

*Methylenetetrahydrofolate reductase (MTHFR) genotyping.* The C677T MTHFR polymorphism (thermolabile variant) was determined by heteroduplex technology on DNA extracted from EDTA blood<sup>28</sup> (performed by Z Clark, Dept. of Biochemistry)

*Folate and vitamin B<sub>12</sub>.* Serum folate and vitamin B<sub>12</sub> were measured by protein binding assays using an Abbott IMX analyser.



**Fig 6.1: Folate intervention study: outline of recruitment and crossover study protocol**

*von Willebrand factor antigen*. Plasma vWF-Ag was measured by ELISA based on the method of Cejka<sup>131</sup>, with coating antibody A0082 (1/2000 dilution), conjugating antibody P0226 (1/3000 dilution) from DAKO, Denmark and substrate K-Blue from Bionostics Ltd, UK (performed by Dr J Giddings, Dept. of Haematology, UWCM).

#### 6.3.4 Statistical analysis (carried out with the assistance of Dr R Newcombe, Dept. of Medical Statistics, UWCM)

Data are reported in the text and tables as group mean  $\pm$  SD unless otherwise stated. Changes in blood levels of homocysteine, vitamins, cholesterol and creatinine, haemodynamic data, brachial artery flow-mediated and GTN-mediated dilatation for each treatment period were compared using a paired analysis similar to that of the two-period crossover trial<sup>107</sup>. Thus the difference between changes occurring in folate and placebo periods were compared between the two treatment order groups by unpaired t-test. This obviates confounding with period differences introduced by unequal numbers in the two groups (this occurred by chance as a result of two subjects withdrawing from the study). Linear regression was used to determine the relationship between plasma homocysteine at baseline and vitamin, lipid and creatinine concentrations. A non-parametric Spearman Rank correlation was used to determine the relationship between changes in flow-mediated dilatation and changes in plasma folate and homocysteine before and after treatment.

### 6.4 Results

Characteristics of the study population are shown in Table 6.1.

#### 6.4.1 Measurements in blood

Mean plasma homocysteine before treatment (at visit 1) was  $14.9 \pm 7.4$   $\mu\text{mol/L}$ . There was a significant inverse correlation between plasma homocysteine and serum folate ( $r = -0.53$ ,  $P = 0.02$ ) but not vitamin B<sub>12</sub>, creatinine or cholesterol concentration.

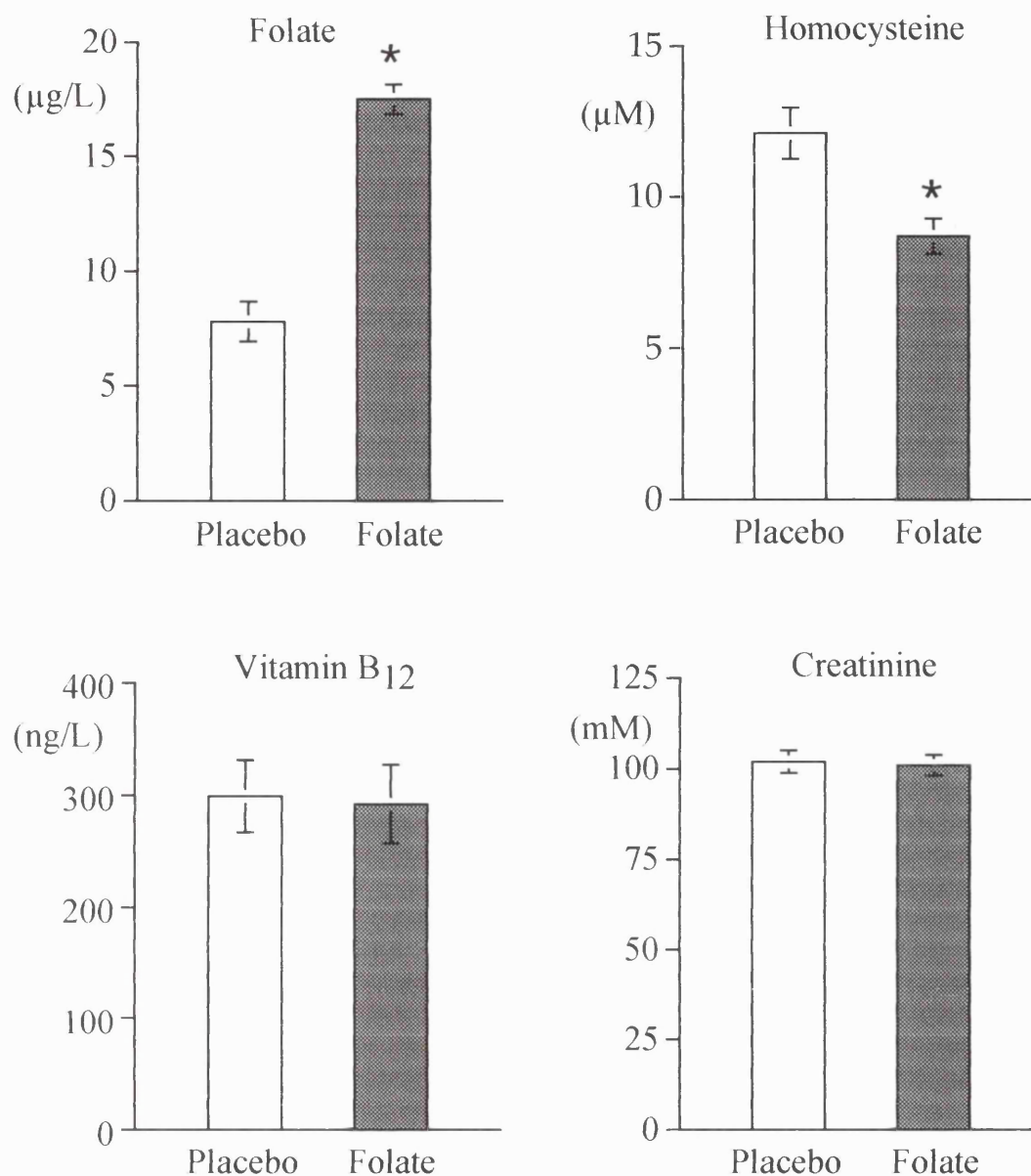
Changes in serum folate, plasma total homocysteine, serum vitamin B<sub>12</sub> and serum creatinine after placebo and folate are shown in Figure 6.2. There was no change in serum vitamin B<sub>12</sub>, creatinine or cholesterol concentration following treatment with placebo compared with treatment with folic acid (Vit B<sub>12</sub>:  $299 \pm 137$

**Table 6.1 Subject characteristics: folate intervention study**

<b>Parameter</b>	<b>Mean (SD)</b>
No.	n=18
Age, y	35 (6)
Sex, M/F	12 / 6
Body mass index, kg/m <sup>2</sup>	26 (4)
Total homocysteine, µmol/L	14.9 (7.4)
Folate, µg/L <sup>†</sup>	6.1 (2.7)
Vitamin B12, ng/L	297 (127)
Total cholesterol, mmol/L	5.4 (0.8)
Triglyceride, mmol/L	1.2 (0.4)
vWF-Ag, IU/L	112 (31)
Glucose, mmol/L	4.8 (0.5)
Creatinine, µmol/L	102 (12)

Data are mean (SD)

<sup>†</sup>Serum folate was inversely correlated with plasma homocysteine (r=-0.53, p=0.02).



**Fig 6.2: Measurements in blood following 6 weeks placebo and folate:**

Folate levels increased significantly and were associated with a corresponding decrease in plasma total homocysteine.

Data are mean ± SEM, n=18, \* p<0.01

There was no change in vitamin B<sub>12</sub> or creatinine concentration.

vs.  $292 \pm 149$   $\mu\text{mol/L}$ ; Creat:  $102 \pm 13$  vs.  $101 \pm 12$   $\mu\text{mol/L}$ ; Chol:  $5.3 \pm 0.8$  vs.  $5.4 \pm 0.6$   $\text{mmol/L}$ ).

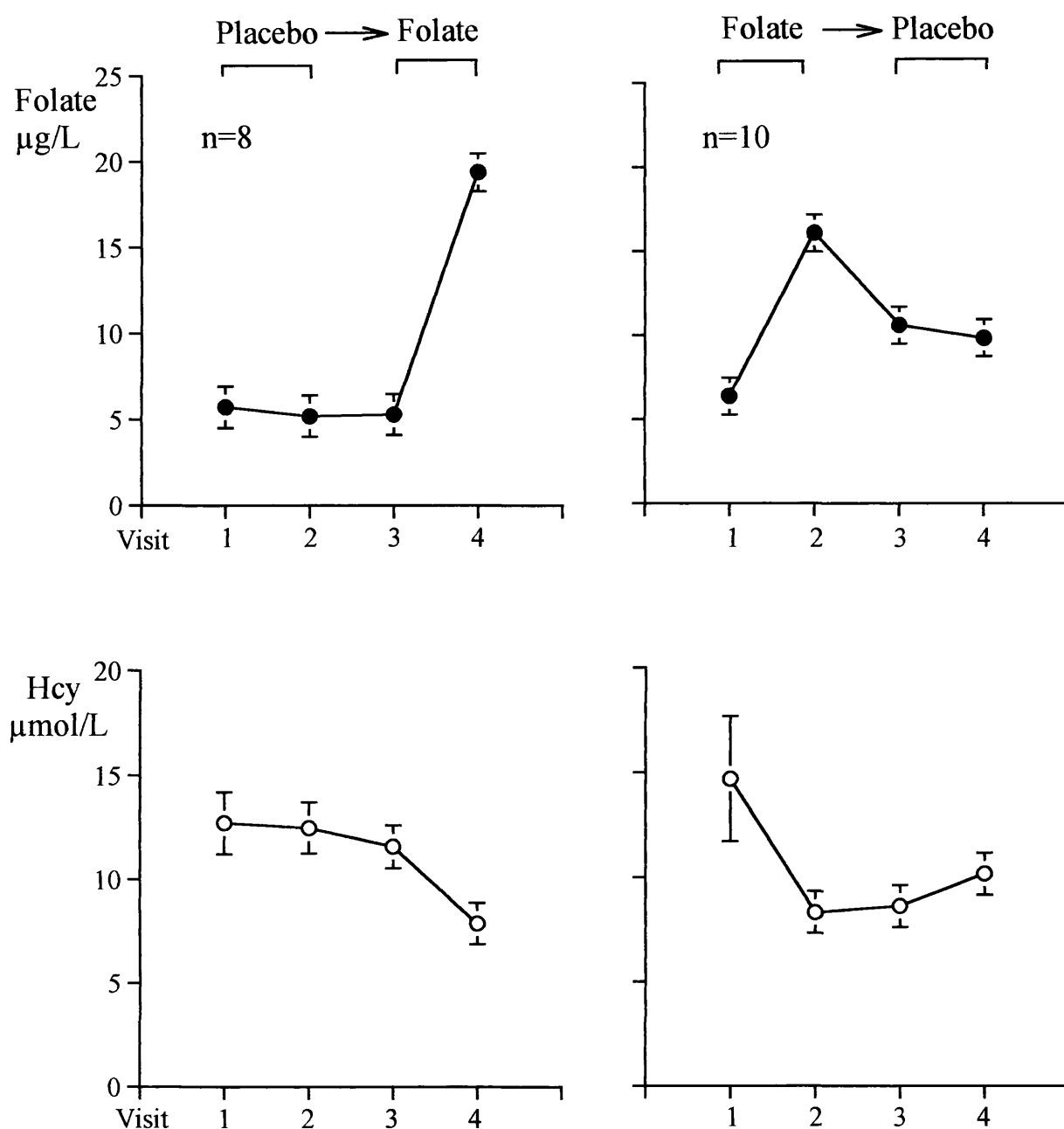
Following six weeks folate supplementation, serum folate increased to  $17.5 \pm 2.8$   $\mu\text{g/L}$  compared with  $7.8 \pm 3.7$   $\mu\text{g/L}$  after placebo, with a corresponding decrease in plasma homocysteine to  $8.7 \pm 2.5$   $\mu\text{mol/L}$  compared with  $12.1 \pm 3.6$   $\mu\text{mol/L}$  after placebo ( $p < 0.01$ ). There was a carry-over effect between treatments such that subjects receiving placebo 6 weeks after stopping folic acid showed a residual effect of the first treatment. In the 8 patients in whom the placebo period preceded the folic acid treatment period, mean serum folate was  $19.4$   $\mu\text{g/L}$  on folic acid vs.  $5.5$   $\mu\text{g/L}$  on placebo ( $\Delta +253\%$ ), while in the 10 patients in whom the folic acid treatment period preceded the placebo period, serum folate was  $16.1$   $\mu\text{g/L}$  on folic acid vs.  $9.8$   $\mu\text{g/L}$  on placebo ( $\Delta +64\%$ ) (Fig. 6.3).

There was a small decrease in von Willebrand factor antigen levels (an indirect marker of endothelial function) following folate treatment but not to the point of statistical significance ( $116 \pm 35$  [plac] vs.  $105 \pm 33$   $\text{iu/L}$  [folate],  $P = 0.33$ ).

#### **6.4.2 Brachial artery study**

Folic acid improved flow-mediated dilatation to  $+0.08 \pm 0.05$   $\text{mm}$  compared with  $+0.04 \pm 0.04$   $\text{mm}$  on placebo ( $P = 0.015$ ) (Table 6.2), although a carryover effect was again observed in the ten subjects in whom the folic acid treatment period preceded the placebo period (Fig. 6.4). Changes in flow-mediated dilatation did not correlate with either changes in plasma homocysteine or changes in serum folate concentration. GTN-induced dilatation was unaltered by folate supplements (Table 6.3).

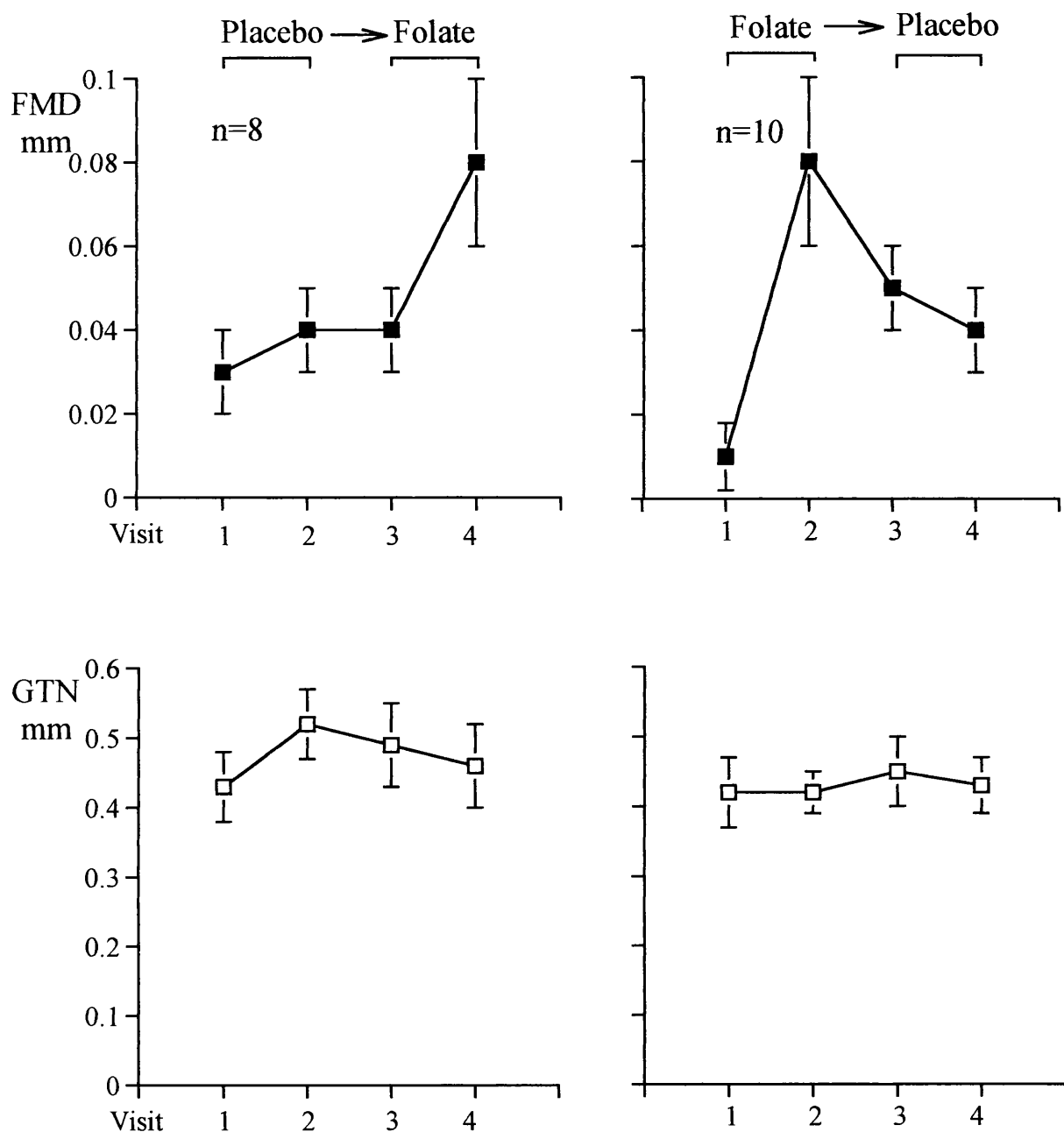
There were no differences between placebo and folate for basal end-diastolic diameter or haemodynamic parameters at each visit.



**Fig 6.3: Effect of oral folic acid on plasma total homocysteine concentration**

Graphs showing changes at visits 1 to 4 in serum folate [top panel (●)] and plasma homocysteine (Hcy) [lower panel (o)] for subjects (n=8) in whom placebo preceded folate treatment (left side) and subjects (n=10) in whom folate preceded placebo (right side). Each of the four visits is represented by the periods marked on the x-axis. Data are mean  $\pm$  SE.

A carryover effect was observed in the latter group of 10 subjects.



**Fig 6.4: Effect of folic acid on flow-mediated and GTN-mediated vasodilatation**

Graphs showing changes at visits 1 to 4 in flow-mediated brachial artery dilatation (FMD) [top panel (■)] and sublingual glyceryl tinitrate (GTN) [lower panel (□)] for subjects (n=8) in whom placebo preceded folate treatment (left side) and subjects (n=10) in whom folate preceded placebo (right side). Each of the four visits is represented by the periods marked on the x-axis. Data are mean  $\pm$  SE.



**Table 6.2:****Haemodynamic parameters at baseline (visit 1) and following placebo and folic acid: basal values and hyperaemia**

		<b>Baseline</b>	<b>Placebo</b>	<b>Folic Acid</b>
<b><u>Basal</u></b>				
Heart rate	(min-1)	59 (12)	60 (10)	59 (10)
Systolic BP	(mmHg)	119 (14)	123 (15)	122 (18)
Diastolic BP	(mmHg)	68 (11)	68 (10)	67 (6)
Blood flow	(mls/min)	24 (17)	26 (21)	21 (14)
End-diastolic diameter, EDD (mm)		3.30 (0.53)	3.32 (0.52)	3.28 (0.55)
<b><u>Hand Hyperaemia (FMD)</u></b>				
Heart rate	(min-1)	59 (10)	61 (10)	58 (9)
Systolic BP	(mmHg)	112 (14)	117 (18)	114 (17)
Diastolic BP	(mmHg)	65 (13)	65 (8)	63 (7)
$\Delta$ Blood flow	( $\Delta$ mls/min)	+50 (42)	+60 (53)	+50 (30)
$\Delta$ EDD all subjects, n=18	( $\Delta$ mm)	+0.02 (0.04)	+0.04 (0.04)	+0.08 (0.05)*
	( $\Delta\%$ )	+0.5 (0.3)	+1.1 (0.3)	+2.5 (0.4)
<i>MTHFR T/T homozygotes, n=6</i>		+0.03 (0.04)	+0.06 (0.06)	+0.11 (0.06)
<i>C/T heterozygotes, n=5</i>		+0.01 (0.04)	+0.01 (0.03)	+0.06 (0.04)
<i>C/C wild type, n=7</i>		+0.02 (0.04)	+0.04 (0.03)	+0.06 (0.04)

MTHFR = methylenetetrahydrofolate reductase genotype

\* p&lt;0.01 cf. placebo

**Table 6.3:**

**Haemodynamic parameters at baseline (visit 1) and following placebo and folic acid: values for GTN**

		<b>Baseline</b>	<b>Placebo</b>	<b>Folic Acid</b>
<b><u>Glyceryl trinitrate (400µg)</u></b>				
Heart rate	(min-1)	62 (9)	66 (10)	63 (10)
Systolic BP	(mmHg)	131 (12)	132 (16)	132 (16)
Diastolic BP	(mmHg)	73 (10)	73 (12)	72 (6)
Δ Blood flow	(Δ mls/min)	+1 (6)	+2 (7)	+2 (9)
Δ EDD all subjects, n=18	(Δmm)	+0.43 (0.15)	+0.47 (0.14)	+0.44 (0.13)
	(Δ%)	+13.8 (1.8)	+14.7 (1.4)	+13.9 (1.3)
<i>MTHFR T/T homozygotes, n=6</i>		+0.40 (0.11)	+0.53 (0.22)	+0.53 (0.14)
<i>C/T heterozygotes, n=5</i>		+0.42 (0.13)	+0.46 (0.08)	+0.44 (0.06)
<i>C/C wild type, n=7</i>		+0.45 (0.20)	+0.41 (0.08)	+0.36 (0.13)

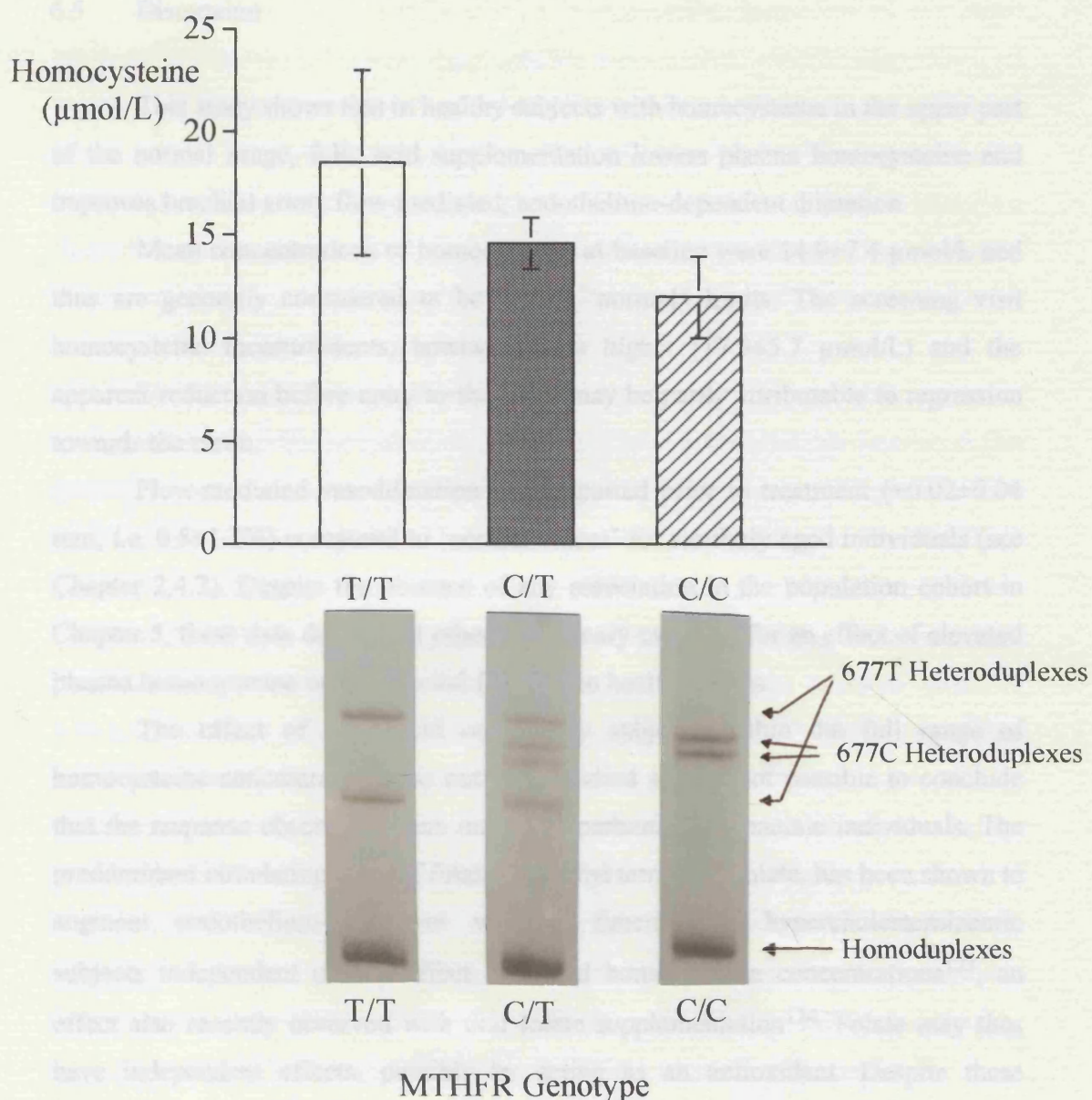
MTHFR = methylenetetrahydrofolate reductase genotype

#### 6.4.3 Methylenetetrahydrofolate reductase genotype

Six of the eighteen subjects were homozygous (T/T) for the thermolabile variant of methylenetetrahydrofolate reductase (i.e. 33% compared with approximately 12% in a local unselected population).

Homozygotes (n=6) had the highest mean plasma homocysteine at baseline, including the subject with the highest homocysteine level (Hcy =39.0  $\mu\text{mol/L}$ ) compared with heterozygotes (n=5) and wild type (n=7) subjects, respectively (T/T:  $18.5\pm11.0$   $\mu\text{mol/L}$  vs. C/T:  $14.6\pm2.8$   $\mu\text{mol/L}$  vs. C/C:  $12.0\pm5.2$   $\mu\text{mol/L}$ ) (Fig 6.5).

Flow-mediated vasodilatation was similar for the 3 genotypes at baseline (Table 6.2). Following folate treatment, homozygotes showed a trend towards the largest change in mean homocysteine concentration from  $17.2\pm11.3$  to  $8.0\pm1.2$   $\mu\text{mol/L}$  ( $\Delta$   $-9.2\pm10.3$   $\mu\text{mol/L}$ ) compared with  $13.8\pm2.8$  to  $8.8\pm2.5$   $\mu\text{mol/L}$  ( $\Delta$   $-5.0\pm1.5$ ) for heterozygotes and  $12.1\pm4.1$  to  $9.2\pm3.4$   $\mu\text{mol/L}$  ( $\Delta$   $-2.9\pm2.8$ ) for wild type subjects. There was a similar trend in flow-mediated responses in that homozygotes showed a larger increase in FMD cf. baseline after folate treatment (Table 6.2) ( $+0.11\pm0.06$  cf.  $+0.03\pm0.04$ , i.e.  $\Delta$   $+0.09\pm0.08$ ), in comparison to heterozygotes ( $\Delta$   $+0.06\pm0.08$ ) and wild type ( $\Delta$   $+0.04\pm0.06$ ) subjects but not to the point of statistical significance as numbers are small.



**Fig 6.5: Top Panel: Plasma total homocysteine according to methylenetetrahydrofolate reductase (MTHFR) genotype**

Genotypes for C677T mutation are T/T = Homozygous variant (n=6);

C/T = Heterozygous variant (n=5); C/C = Wild type (n=7)

Data are mean (SEM).

**Bottom Panel: Electrophoretic patterns of samples analysed by heteroduplex analysis** (see Ref 28).

## 6.5 Discussion

This study shows that in healthy subjects with homocysteine in the upper part of the normal range, folic acid supplementation lowers plasma homocysteine and improves brachial artery flow-mediated, endothelium-dependent dilatation.

Mean concentrations of homocysteine at baseline were  $14.9 \pm 7.4$   $\mu\text{mol/L}$  and thus are generally considered to be within “normal” limits. The screening visit homocysteine measurements, however, were higher ( $17.9 \pm 5.7$   $\mu\text{mol/L}$ ) and the apparent reduction before entry to the study may be partly attributable to regression towards the mean.

Flow-mediated vasodilatation was impaired prior to treatment ( $+0.02 \pm 0.04$  mm, i.e.  $0.5 \pm 1.2\%$ ) compared to ‘normal values’ for similarly aged individuals (see Chapter 2.4.2). Despite the absence of any association in the population cohort in Chapter 5, these data do support other preliminary evidence for an effect of elevated plasma homocysteine on endothelial function in healthy adults.

The effect of folic acid on healthy subjects within the full range of homocysteine concentrations has not been studied so it is not possible to conclude that the response observed occurs only in hyperhomocysteinaemic individuals. The predominant circulating form of folate, 5-methyl tetrahydrofolate, has been shown to augment endothelium-dependent vascular function in hypercholesterolaemic subjects independent of any effect on blood homocysteine concentrations<sup>123</sup>, an effect also recently observed with oral folate supplementation<sup>124</sup>. Folate may thus have independent effects, possibly by acting as an antioxidant. Despite these drawbacks, however, the data is consistent with experimental evidence that homocysteine has a direct effect on endothelium<sup>63,116</sup>.

The relatively high dose of folic acid (5mg daily which is the dosage readily available for prescription in the United Kingdom) was chosen to produce maximum changes in homocysteine concentration and resulted in a reduction of plasma homocysteine of 28% compared with placebo, similar to that reported in a meta-analysis of randomised trials of folic acid supplementation<sup>121</sup>.

A criticism of this study is that the six-week interval between treatments was too short to allow return to baseline of serum folate and plasma homocysteine. Therefore in the group of subjects where the placebo treatment period followed the folate period, these individuals were studied in a setting of continuing slow decline

in plasma folate and increase in plasma homocysteine. Inspection of the flow-mediated responses (Fig. 6.4) in those subjects who received placebo after folic acid shows that these subjects continued to have flow-mediated dilatation above baseline values (visit 4 vs. visit 1). This has the effect of lessening the difference between post-folate and post-placebo flow-mediated dilatation, thereby tending to obscure a “positive” result. Hence the demonstration of an effect despite the carryover limitations does not detract from the conclusion that folic acid treatment enhances endothelium-dependent flow-mediated vasodilatation.

Six of the eighteen subjects were homozygous for the thermolabile variant of methylenetetrahydrofolate reductase, MTHFR. There is considerable interest in this genetic variant as it occurs commonly in the general population resulting in impaired remethylation of homocysteine leading to elevated homocysteine levels, usually in conjunction with suboptimal vitamin intake<sup>25,27</sup>. In our local healthy population MTHFR C677T homozygosity occurs with a frequency of 12.5% (Clark-personal communication). In this study homozygotes had the highest baseline levels of homocysteine and showed a trend towards larger decreases following folate treatment, although numbers are small. Absolute and relative reductions in plasma homocysteine produced by folic acid supplements are greater at higher pre-treatment homocysteine concentrations, as observed in this study where the largest change in plasma homocysteine (from 39.0 to 9.8  $\mu\text{mol/L}$ ) was seen in a male MTHFR homozygote. These individuals may potentially benefit most from any associated improvement in vascular function, although whether the enzyme variant is associated with an increased risk of vascular disease remains controversial.<sup>29</sup>

With regard to the general population, increased folate intake may be of potential benefit for cardiovascular disease but a number of questions remain to be answered and further studies are required. Folic acid at a dose of 0.5-5 mg daily may have similar effects on lowering blood homocysteine concentrations and therefore a lower dose of folic acid could be sufficient to reduce cardiovascular risk and be practical for the fortification of everyday foodstuffs (e.g. bread, cereals) as has been recently implemented in the United States. The folate requirement for subjects with different MTHFR genotypes may be different<sup>125</sup>. Folic acid supplementation should not be given in isolation to individuals with hyperhomocysteinaemia due to vitamin B<sub>12</sub> deficiency because of the risk of subacute combined degeneration of the spinal cord.

This study shows that oral folic acid supplementation lowers plasma homocysteine and improves endothelium-dependent vasodilatation in mild hyperhomocysteinaemia. Further studies are required to investigate the effect of folate across the range of homocysteine concentrations, using lower doses of folate and to determine clinical endpoints. However, clinical trials of vitamin supplementation need to be large, performed in a high risk patient population, include an adequate dose and of optimal duration of treatment. The issues in the design of such trials have been reviewed<sup>122</sup> and one such UK study (SEARCH), is currently in progress. Homocysteine lowering by vitamin supplementation is being investigated alongside a comparison of low and high dose Simvastatin therapy. If the present data is confirmed by larger randomised trials, this potentially offers an important preventative and therapeutic role for folic acid in cardiovascular disease.

## CHAPTER 7

### Investigation Of The Mechanisms By Which Homocysteine Modulates Endothelium-Dependent Relaxation In Vitro

#### 7.1 Introduction

Homocysteine is formed by demethylation of methionine and is converted via a series of enzymatic reactions to cysteine in the transsulphuration pathway. S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH) are intermediate metabolites formed from methionine by the enzymes methionine adenosyltransferase and S-adenosylhomocysteine hydrolase respectively (see Chapter 1.2).

Experimentally, homocysteine injures endothelium but the effect may be non-specific and has been shown to occur with other thiols, such as cysteine<sup>56</sup>. There is some evidence to suggest that the intermediate metabolite, SAM may also be important as a protective factor in the development of coronary artery disease<sup>126</sup>. The formation of SAH leads to the generation of methyl groups, which may have the potential to methylate L-arginine, the substrate for the production of nitric oxide by nitric oxide synthase (NOS), forming methylarginines. Indeed, one such product, asymmetric dimethyl arginine has been reported to be a competitive inhibitor of NOS in humans<sup>127</sup> and elevated levels shown to be associated with endothelial dysfunction in patients with hypercholesterolaemia.<sup>128</sup> Mechanisms other than direct toxicity of homocysteine could therefore be involved in mediating endothelial dysfunction.

In the experiments described in this chapter, a conventional isolated rabbit aortic ring preparation is used to investigate the effects of the major constituents of the transsulphuration pathway (i.e. methionine, S-adenosylmethionine, S-adenosyl homocysteine, homocysteine and cysteine) on endothelium-dependent vascular smooth muscle relaxation.

It has been postulated that homocysteine-induced endothelial dysfunction may be mediated by an increase in oxidant stress<sup>66</sup>, possibly by the production of hydrogen peroxide or superoxide anions (see Chapter 1.7). There is also some preliminary evidence that changes in *intracellular* redox status may also be important in altering endothelial cell function<sup>70,129</sup>. Selected experiments were therefore repeated in the presence of superoxide dismutase, catalase, vitamin C, and



the intracellular superoxide scavenger, 4,5-dihydroxy-1,3-benzene disulfonic acid (Tiron).

## 7.2 Methods

### 7.2.1 Aortic ring preparations

The thoracic aortas of male New Zealand White rabbits (2-2.5kg) were removed into fresh Krebs buffer of the following composition (mmol): NaCl 138; KCl 5.3; KH<sub>2</sub>PO<sub>4</sub> 1.2; MgSO<sub>4</sub> 1.2; Glucose 15; NaHCO<sub>3</sub> 24; CaCl 1.5 and indomethacin 0.01, and gassed with 95% O<sub>2</sub>/5% CO<sub>2</sub> at 37°C. For isometric tension recording, 2- to 3-mm wide endothelium-intact (+E) or -denuded (-E) rings were mounted in 8mL tissue baths containing fresh Krebs buffer (see Fig 7.1). Resting tension was set at 2g and frequently readjusted for stress relaxation during a 60 min equilibration period. Force development was recorded using a MacLab™ set-up.

#### *Endothelium-intact tissues*

All tissues (except those when using the calcium ionophore, A23187) were preconstricted with a sub maximal concentration of Phenylephrine (PE 1 µmol/L). They were then exposed to increasing concentrations of acetylcholine (ACH 1nmol/L to 10 µmol/L) (Fig.7.2). After washing, tissues were re-equilibrated for 15 min and then incubated for 3 hours (a time based on preliminary experiments) with one of the following:-

- i. Homocysteine (1 mmol/L) - as prepared by method of van der Molen (see below) or vehicle
- ii. Methionine (1 mmol/L)
- iii S-adenosylmethionine (1mmol/L) or vehicle (0.4 mol/L Perchloric acid, PCA)
- iv. S-adenosylhomocysteine (1mmol/L) or vehicle (0.4 mol/L PCA)
- v. Cysteine (1 mmol/L)

After these various incubations, all aortic rings were reconststricted with PE (1µM) and the concentration responses to ACH (for all of the above) were repeated.

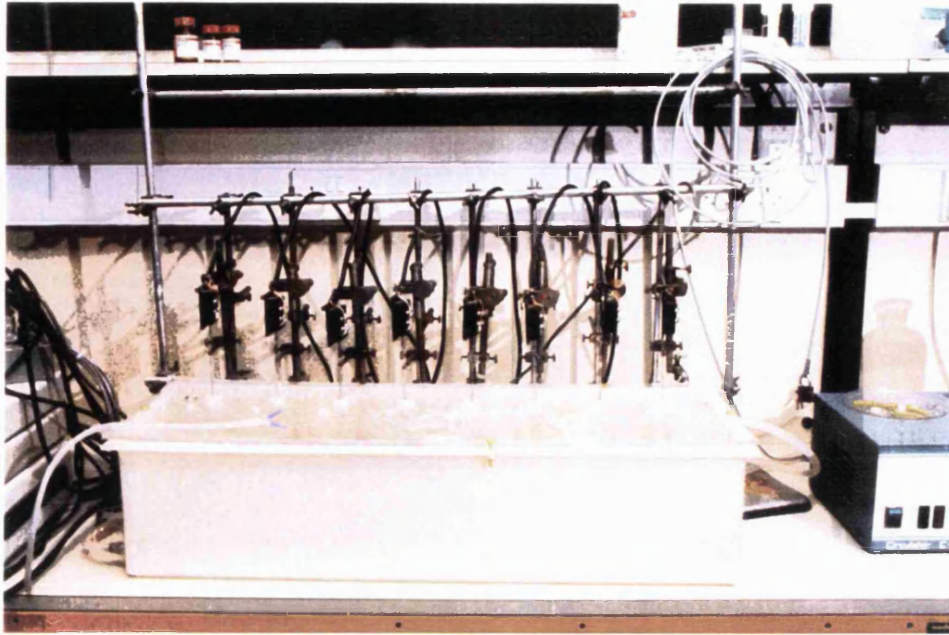
#### *Endothelium-denuded tissues*

Tissues were exposed to PE as described above, followed by sodium nitroprusside (SNP 1 nmol/L to 10 µmol/L). After they were washed, the tissues were

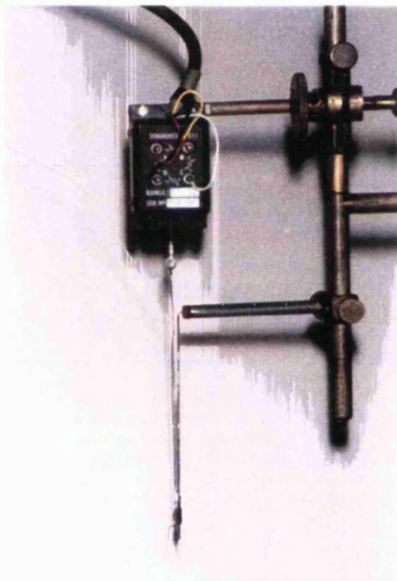
**Fig 7.1: Aortic ring preparation**

- A – main tissue bath with 8 individual incubation ports containing Krebs buffer in which aortic rings were suspended
- B – tension gauge allowing fine adjustment connected to MacLab™ setup
- C – the aortic ring is suspended between a lower and upper mount, the latter is attached to one of 8 individual tension gauges

A

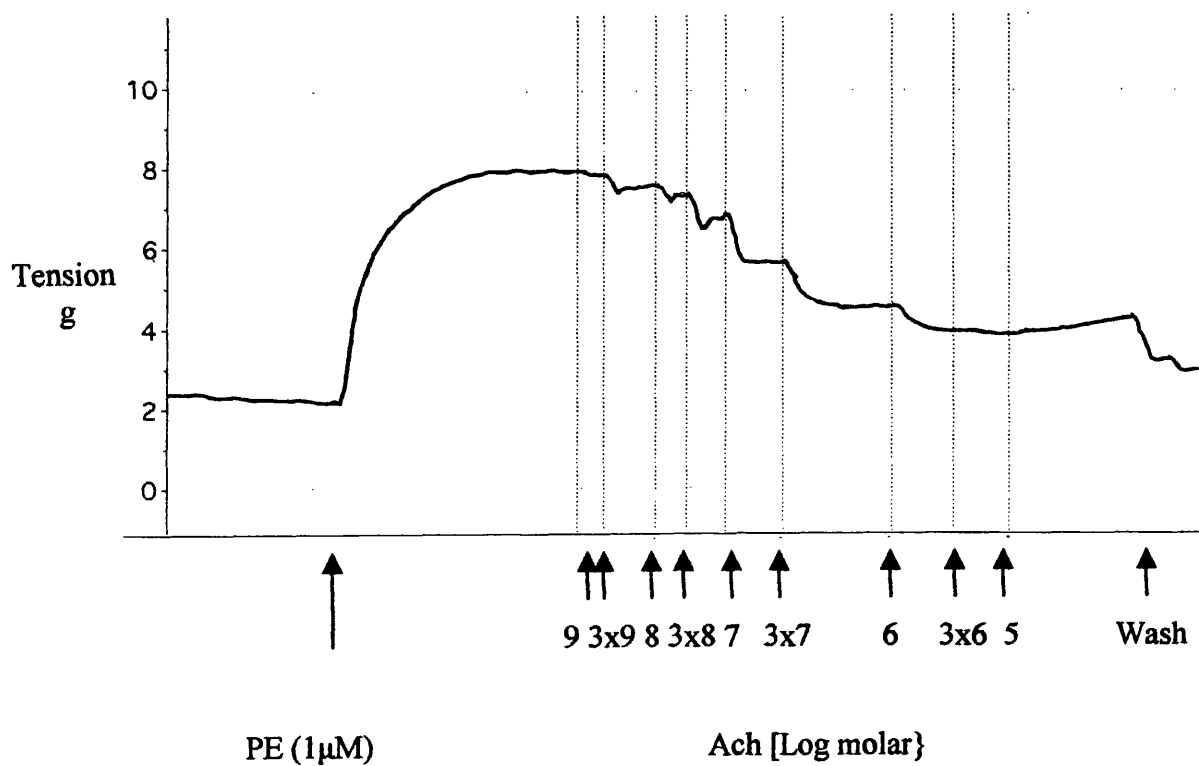


B



C





**Fig7.2:** Acetylcholine induced relaxation of PE (1  $\mu$ M)- precontracted aortic ring. Control response (no intervention)

incubated for 3 hrs with either vehicle, homocysteine alone (1 mmol/L) or cysteine (1 mmol/L), reconstituted with PE (1 $\mu$ M) and concentration responses to SNP repeated.

### 7.2.2 Sample preparation

All stock solutions were prepared to a concentration of 100mmol/L. An 80 $\mu$ L aliquot was then added to each 8mL bath containing each aortic ring to give a final concentration of 1mmol/L. L-methionine and L-cysteine were dissolved directly in Krebs buffer. S-adenosylmethionine and S-adenosylhomocysteine were dissolved in 0.4mol/L Perchloric acid (PCA). Free, reduced L-homocysteine was prepared by the method of van der Molen et al<sup>130</sup>. In brief, L-homocysteine thiolactone was incubated for 5 mins in 5 mol/L NaOH at 40°C, before neutralising with 0.1 mol/L phosphate buffer (pH 7.8) and 2.5 mol/L HCl and diluted to the appropriate concentration with Krebs buffer. This reaction has previously been demonstrated<sup>130</sup> to facilitate the complete conversion of L-homocysteine thiolactone to free reduced L-homocysteine. The vehicle was prepared in the same way, omitting L-homocysteine thiolactone.

L-methionine, S-adenosylmethionine, S-adenosylhomocysteine, L-homocysteine thiolactone, cysteine, Phenylephrine, acetylcholine and A23187 were obtained from Sigma Aldrich (Dorset, UK).

### 7.2.3 Experiments with antioxidants

A series of further experiments were performed to investigate the effect of homocysteine on endothelium-dependent relaxation in the presence of either superoxide dismutase (SOD 60U/mL), catalase (CAT 120U/mL), vitamin C (10 $\mu$ mol/L), or the intracellular superoxide anion scavenger Tiron (10 mmol/L).

As cysteine also had a significant (but less marked) effect on endothelium-dependent relaxation, experiments were also repeated in the presence of superoxide dismutase (SOD 60U/mL) and catalase (CAT 120U/mL). As the effect of cysteine was reversed with SOD (see below), Tiron experiments were omitted.

Each intervention was added to the tissue baths immediately prior to reduced homocysteine or cysteine.

Superoxide dismutase, catalase and Tiron were also obtained from Sigma Aldrich.

#### **7.2.4 Experiments with the calcium ionophore, A23187**

Rings were first incubated for 3 hours with either vehicle, homocysteine, or homocysteine + Tiron as described above. They were then constricted with PE (1  $\mu$ mol/L) and concentration responses to A23187 (1 nmol/L to 10  $\mu$ mol/L, +E) were performed.

#### **7.2.5 Statistical analysis**

Relaxation responses are expressed as a percentage of the phenylephrine-induced constriction. Concentration response curves were fitted to sigmoid curves using KaleidaGraph™ for the Apple Macintosh. This allowed for the calculation of the maximum relaxation response (Rmax) values for each concentration-response curve. Student's paired or unpaired t-tests were used to compare values where appropriate. All data are expressed as mean  $\pm$  SEM and  $n \geq 4$ .

### 7.3 Results

Precontraction with a sub maximal concentration of PE (1  $\mu\text{mol/L}$ ) produced a mean constriction of  $5.3 \pm 0.2\text{g}$  ( $n=80$ ).

#### 7.3.1 Homocysteine experiments (Table 7.1)

Incubation with vehicle caused a small but non-significant ( $P=0.42$ ) decrease in the maximum relaxation-response ( $R_{\text{max}}$ ) to the endothelium-dependent agonist ACH compared with the initial responses ( $77.2 \pm 2.6$  vs.  $74.7 \pm 12.1\%$ , i.e.  $\Delta -2.5 \pm 9.1\%$ , respectively). There was no decrease in  $R_{\text{max}}$  to the endothelium-independent agonist SNP following incubation with vehicle, but in fact a small significant increase ( $103.5 \pm 2.8$  vs.  $90.8 \pm 1.6\%$ , i.e.  $\Delta +12.7 \pm 3.0\%$ , respectively for SNP).

Incubation with homocysteine caused a highly significant ( $P<0.0001$ ) inhibition of the  $R_{\text{max}}$  to ACH compared to the initial ACH exposure ( $52.9 \pm 11.2$  vs.  $81.4 \pm 8.1\%$  respectively, i.e.  $\Delta -28.5 \pm 11.1\%$ ) (Fig. 7.3a). There was no significant decrease in the  $R_{\text{max}}$  to SNP observed following incubation with homocysteine, but again a small increase similar to vehicle ( $100.6 \pm 2.7$  vs.  $93.4 \pm 1.7\%$ , i.e.  $\Delta +7.2 \pm 1.0\%$ ,  $P=0.006$ ) (Fig. 7.3b).

In endothelium-intact rings, preincubation with homocysteine also significantly inhibited the relaxation-response to increasing concentrations of the calcium ionophore, A23187 (1  $\text{nmol/L}$  to 10  $\mu\text{mol/L}$ ),  $R_{\text{max}}$ :  $64.8 \pm 17.2\%$  vs. vehicle (control)  $R_{\text{max}}$ :  $80.2 \pm 5.3\%$ ,  $P=0.03$ ) (Table 7.1).

**Table 7.1: Homocysteine Experiments**

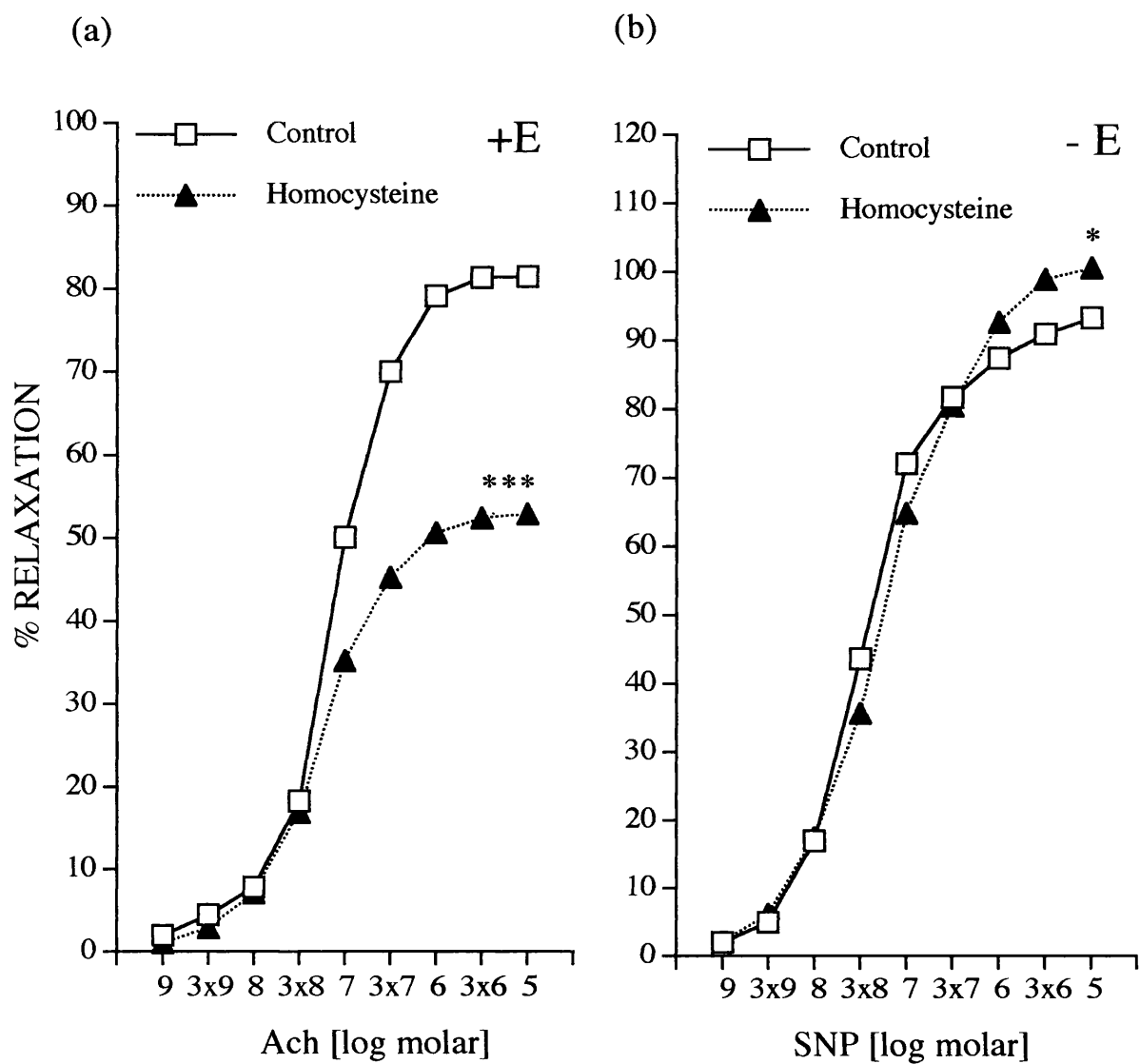
Effect Of Homocysteine On Vascular Smooth Muscle Cell Relaxation Induced By Acetylcholine, Sodium Nitroprusside (SNP) And The Calcium Ionophore, A23187.

	R max			P value
	Pre	Post	Δ%	
<b><u>Acetylcholine</u></b>				
Vehicle	77.2 (2.6)	74.7 (12.1)	-2.5 (9.1)	NS
Homocysteine	81.4 (8.1)	52.9 (11.2)	-28.5 (11.1)	<0.0001
<b><u>SNP</u></b>				
Vehicle	90.8 (1.6)	103.5 (2.8)	+12.7 (3.0)	0.004
Homocysteine	93.4 (1.7)	100.6 (2.7)	+7.2 (1.0)	0.006
<b><u>A23187</u></b>				
Vehicle	--	80.2 (5.3)	--	--
Homocysteine	--	64.8 (17.2)	-15.4*	0.03*

All data are mean (SD)

\*derived by comparison with vehicle [unpaired t test]





**Fig. 7.3: Relaxation responses to acetylcholine and sodium nitroprusside: control vs homocysteine**

Responses to acetylcholine [Ach] (left) and sodium nitroprusside [SNP] (right) for:

Ach (a) control (initial Ach response) and homocysteine, 1mM in endothelium intact rings (+E)

SNP (c) control (initial SNP response) and homocysteine, 1mM in endothelium denuded rings (-E)

\*p<0.01 \*\*\*p<0.0001 vs. control

### 7.3.2 Other transsulphuration pathway constituents (Table 7.2)

#### *Methionine*

There was a small significant inhibition of the maximum relaxation-response ( $R_{\max}$ ) to ACH with methionine incubation, compared to the initial ACH response ( $70.9 \pm 8.7$  vs.  $75.6 \pm 6.0\%$ , i.e.  $\Delta -4.7 \pm 3.1\%$ ,  $P=0.015$ ) (Fig. 7.4a).

#### *S-Adenosylmethionine (SAM)*

Incubation with SAM caused a small but significant decrease in the  $R_{\max}$  to ACH compared to the initial ACH responses ( $61.1 \pm 11.0$  vs.  $70.6 \pm 11.0\%$ , respectively, i.e.  $\Delta -9.5 \pm 5.3\%$ ,  $p=0.001$ ) (Fig. 7.4b), but markedly less than that observed following incubation with homocysteine [ $\Delta -9.5 \pm 5.3\%$  (SAM) vs.  $\Delta -28.5 \pm 11.1\%$  (Hcy)]. There was no effect of the vehicle used, 0.4M Perchloric acid (PCA), on  $R_{\max}$  to ACH ( $72.9 \pm 12.4$  vs.  $76.7 \pm 11.2\%$ , i.e.  $\Delta -3.8 \pm 6.4\%$ ,  $p=0.17$ ).

#### *S-Adenosyl homocysteine (SAH)*

Following incubation with SAH, there was no significant inhibition of the  $R_{\max}$  to ACH compared to the initial ACH response (Fig. 7.4c).

#### *Cysteine*

Incubation with cysteine caused a highly significant inhibition of the  $R_{\max}$  to ACH compared to the initial ACH exposure ( $63.5 \pm 7.6$  vs.  $77.4 \pm 3.8\%$ , respectively, i.e.  $\Delta -13.9 \pm 7.0\%$ ,  $P<0.0001$ ) (Fig 7.5a). There was no significant change in the  $R_{\max}$  to SNP observed following incubation with cysteine ( $98.9 \pm 2.1$  vs.  $100.7 \pm 2.2\%$ , respectively, i.e.  $\Delta -1.8 \pm 1.9\%$ ,  $P=NS$ ) (Fig. 7.5b).

**Table 7.2: Other Transsulphuration Pathway Constituents**

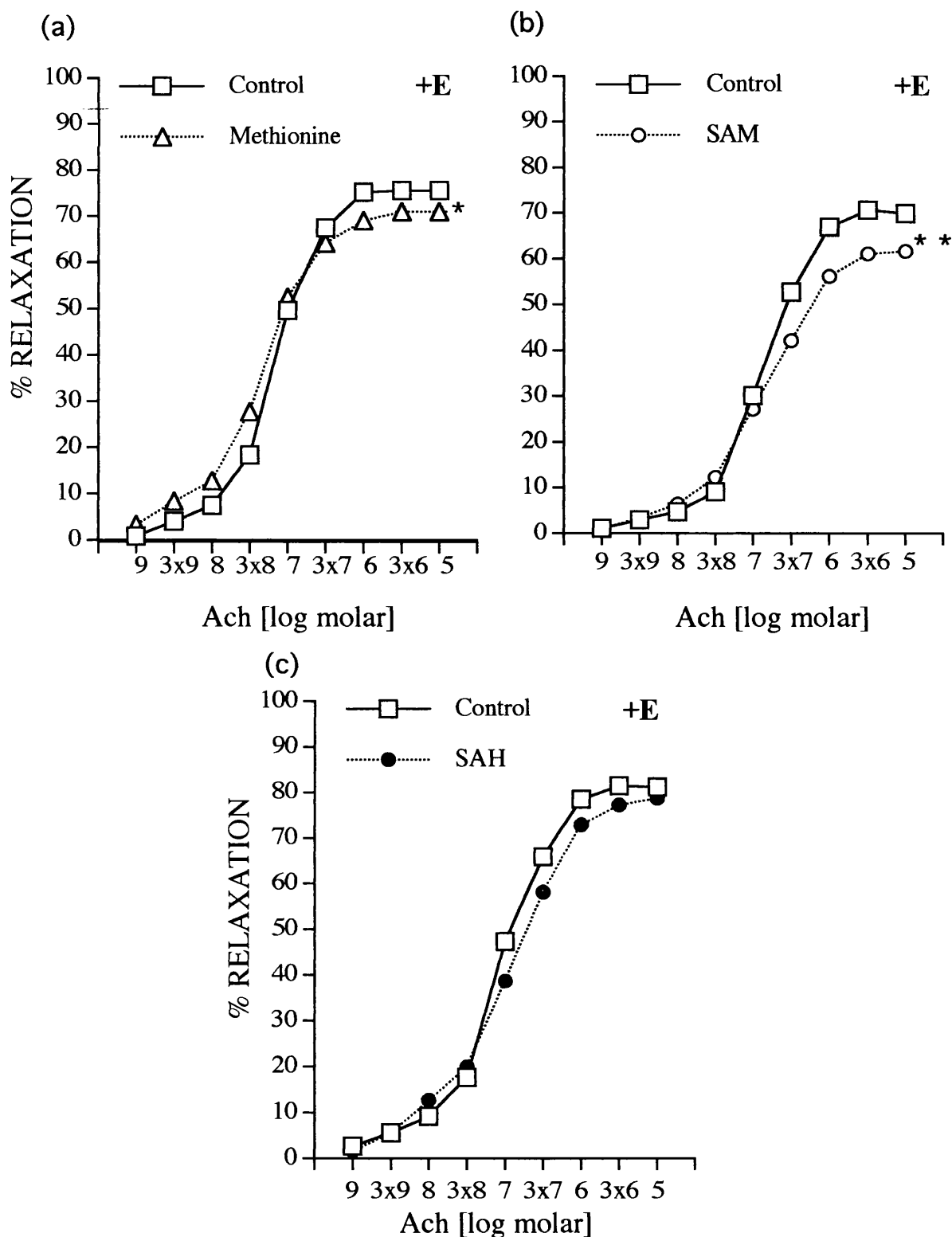
(a) Effect Of Methionine And The Homocysteine Intermediates S-Adenosyl-methionine (SAM) And S-Adenosylhomocysteine (SAH) On Vascular Smooth Muscle Cell Relaxation Induced By Acetylcholine.

	R max			P value
	Pre	Post	Δ%	
<b><u>Acetylcholine</u></b>				
Methionine	75.6 (6.0)	70.9 (8.7)	-4.7 (3.1)	0.015
S-Adenosyl Methionine	70.6 (11.0)	61.1 (11.0)	-9.5 (5.3)	0.001
S-Adenosyl Homocysteine	81.5 (0.9)	77.2 (5.8)	-4.3 (5.9)	NS
Vehicle: SAM/SAH	76.7 (11.2)	72.9 (12.4)	-3.8 (6.4)	NS

(b) Effect Of Cysteine On Vascular Smooth Muscle Cell Relaxation Induced By Acetylcholine And Sodium Nitroprusside (SNP).

	R max			P value
	Pre	Post	Δ%	
<b><u>Acetylcholine</u></b>				
Cysteine	77.4 (3.8)	63.5 (7.6)	-13.9 (7.0)	<0.0001
<b><u>SNP</u></b>				
Cysteine	100.7 (2.2)	98.9 (2.1)	-1.8 (1.9)	NS

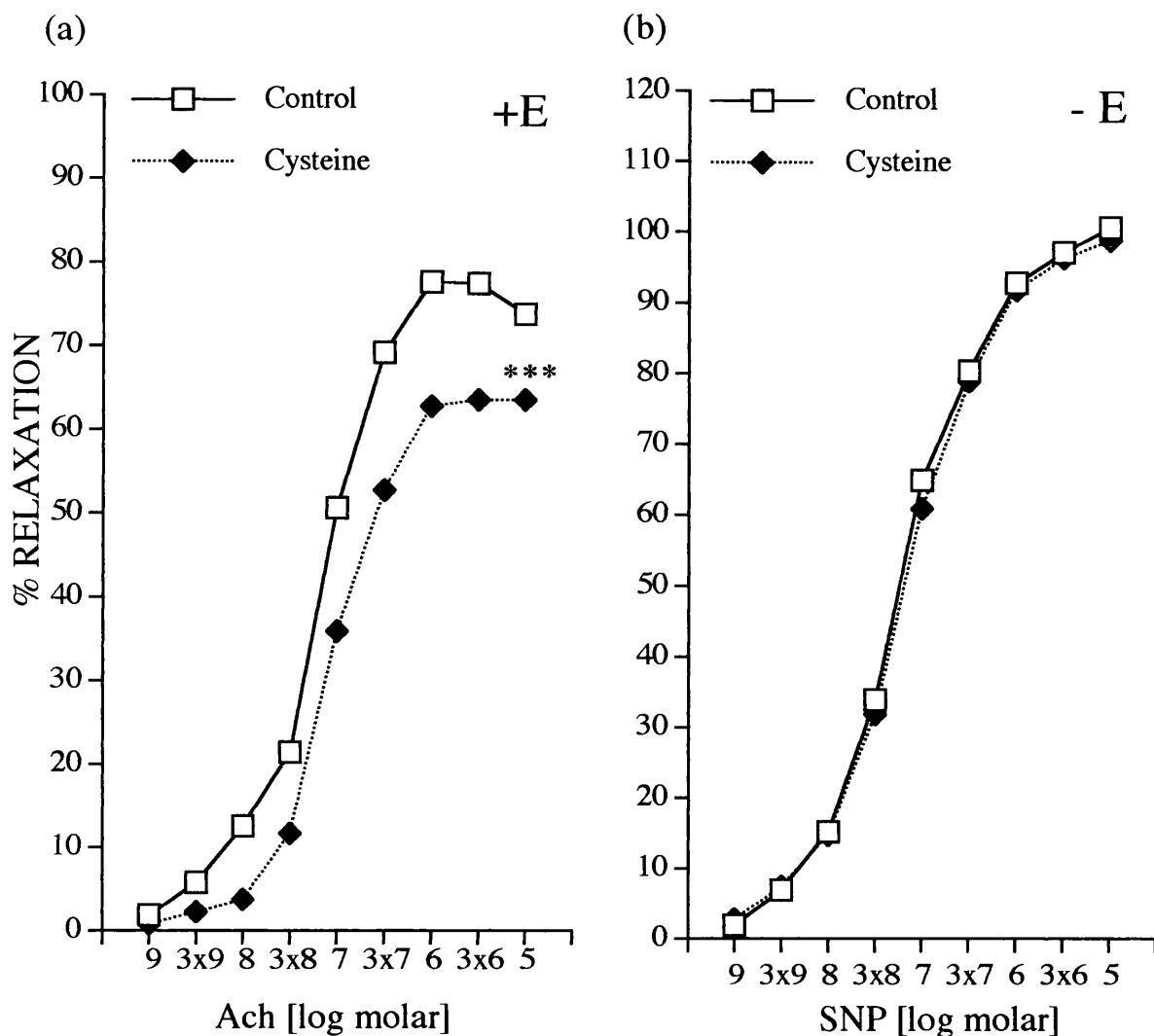
All data are mean (SD)



**Fig 7.4: Relaxation responses to acetylcholine: methionine, S-adenosyl methionine and S-adenosyl homocysteine**

- (a) in the absence of other interventions (control) (open squares) and following a 3hr incubation with methionine, 1mM (open triangles)
- (b) control and S-adenosyl methionine, 1mM (open circles)
- (c) control and S-adenosyl homocysteine, 1mM (closed circles)

\*P<0.05 \*\*\*P<0.01 vs control (+E = endothelium intact)



**Fig 7.5: Relaxation responses to acetylcholine and sodium nitroprusside: control vs cysteine**

Responses to acetylcholine [Ach] (left) and sodium nitroprusside [SNP] (right) for:

Ach (a) control (initial Ach response) and cysteine, 1mM in endothelium intact rings (+E)

SNP (c) control (initial SNP response) and cysteine, 1mM in endothelium denuded rings (-E)

\*\*\*p<0.0001 vs. control

### 7.3.3 Effect of prior incubation of homocysteine and cysteine with superoxide dismutase, catalase, vitamin C and Tiron (Table 7.3)

#### *Homocysteine and superoxide dismutase (SOD)*

The addition of SOD to the organ bath prior to incubation with homocysteine had no effect on the Rmax to ACH. There remained a highly significant decrease in Rmax versus the initial ACH response ( $21.2 \pm 5.4$  vs.  $62.1 \pm 7.8\%$ , respectively, i.e.  $\Delta -40.9 \pm 7.6\%$ ,  $P=0.002$ ) (Fig 7.6a).

#### *Homocysteine and superoxide dismutase with catalase*

Prior incubation with SOD + catalase did not prevent a significant reduction in Rmax to ACH following homocysteine [ $47.5 \pm 5.3$  (post) vs.  $56.6 \pm 2.8\%$  (pre),  $P=0.002$ ], however the effect of homocysteine was attenuated [i.e.  $\Delta -9.1 \pm 3.8\%$  (Hcy + SOD + catalase) vs.  $\Delta -28.5 \pm 11.1\%$  (Hcy)] (Fig 7.6b).

#### *Homocysteine and vitamin C*

Incubation with homocysteine in the presence of vitamin C also did not prevent a significant inhibition of the Rmax to ACH compared to the initial ACH exposure ( $51.2 \pm 12.9$  vs.  $65.9 \pm 15.2\%$ , respectively), but as was observed with catalase, the magnitude of the decrease in Rmax was attenuated [i.e.  $\Delta -14.7 \pm 5.4\%$  (Hcy+ Vit C) vs.  $\Delta -28.5 \pm 11.1\%$  (Hcy)] (Fig 7.6c).

#### *Homocysteine and Tiron (4,5-dihydroxy-1,3-benzene disulfonic acid)*

Incubation with Tiron did not completely inhibit the effect of homocysteine on ACH-induced relaxation. There was a small significant decrease in Rmax compared with the initial ACH response ( $71.8 \pm 6.6$  vs.  $79.9 \pm 6.8$ , respectively, i.e.  $\Delta -8.1 \pm 2.0\%$ ,  $P=0.02$ ) (Fig. 7.6d) but the change in Rmax was the smallest observed compared with SOD + catalase and vitamin C. Tiron completely inhibited the effect of homocysteine on A23187-induced relaxation compared with vehicle ( $75.2 \pm 15.8$  vs.  $80.2 \pm 5.3\%$ ,  $P=NS$ ).

**Table 7.3: Antioxidant Experiments**

(a) Effect of Prior Incubation Of Homocysteine With Superoxide Dismutase (SOD), Catalase and Other Antioxidants On Vascular Smooth Muscle Cell Relaxation.

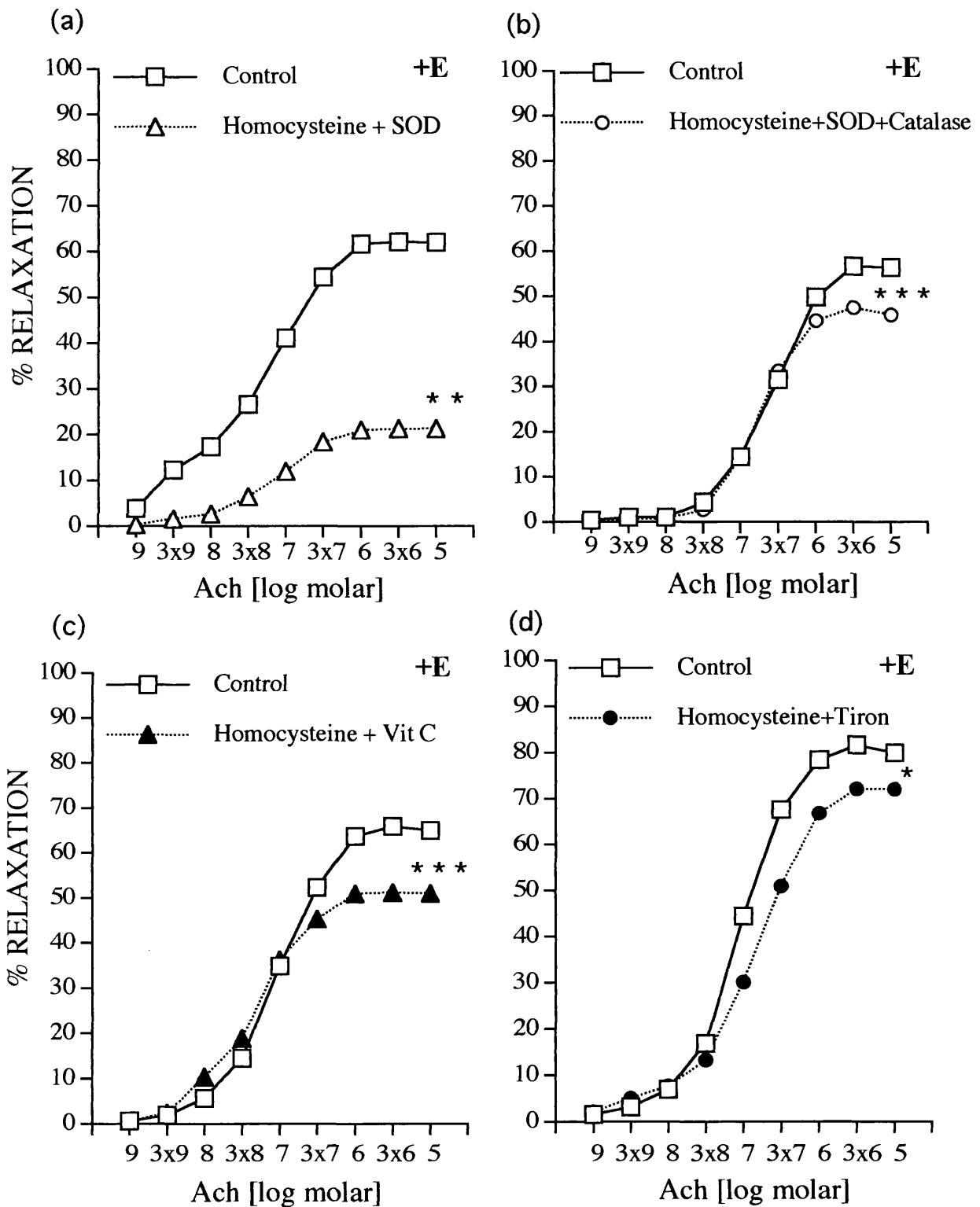
	R max			P value
	Pre	Post	Δ%	
<b><u>Acetylcholine</u></b>				
Homocysteine +SOD	62.1 (7.8)	21.2 (5.4)	-40.9 (7.6)	0.002
Homocysteine +SOD+Catalase	56.6 (2.8)	47.5 (5.3)	-9.1 (3.8)	0.0003
Homocysteine +Vitamin C	65.9 (15.2)	51.2 (12.9)	-14.7 (5.4)	<0.0001
Homocysteine +Tiron	79.9 (6.8)	71.8 (6.6)	-8.1 (2.0)	0.02
<b><u>A23187</u></b>				
Homocysteine +Tiron	—	75.2 (15.8)	-5.0*	NS*

All data are mean (SD)

\*derived by comparison with vehicle in Table 7.1 [unpaired t test]

(b) Effect of Prior Incubation Of Cysteine With Superoxide Dismutase (SOD) and Catalase On Vascular Smooth Muscle Cell Relaxation Induced By Acetylcholine.

	R max		$\Delta\%$	P value
	Pre	Post		
Cysteine +SOD	73.9 (11.3)	76.0 (15.8)	+2.1 (8.8)	NS
Cysteine +Catalase	72.0 (6.3)	78.7 (9.3)	+6.7 (8.9)	NS



**Fig 7.6: Homocysteine experiments: effects of superoxide dismutase, catalase vitamin C and Tiron**

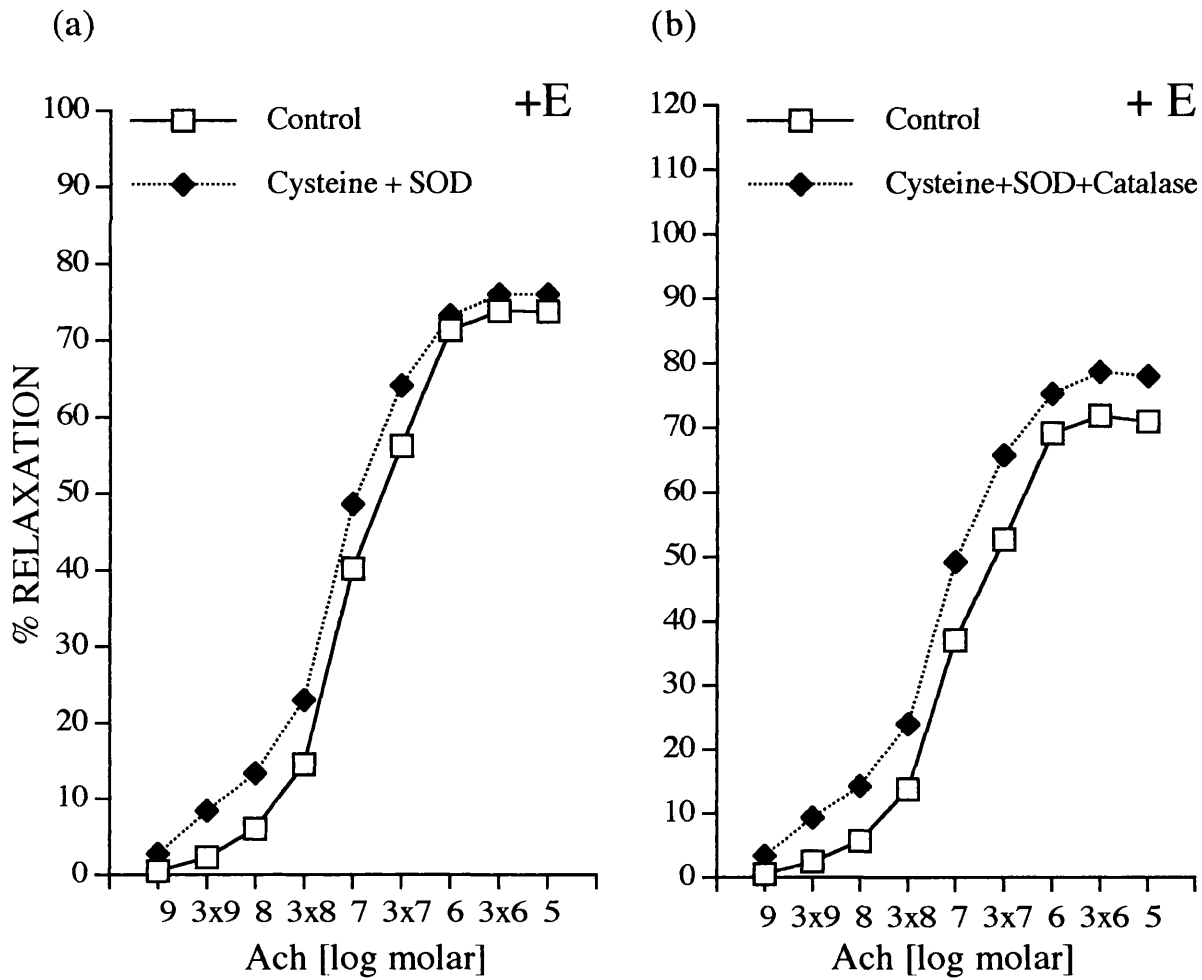
- (a) in the absence of other interventions (control) (open squares)  
and following a 3hr incubation with 1mM Hcy + SOD (open triangles)
- (b) control and 1 mM Hcy + SOD + Catalase (open circles)
- (c) control and 1 mM Hcy + Vitamin C (closed triangles)
- (d) control and 1 mM Hcy + Tiron (closed circles)

\* $p < 0.05$  \*\* $p < 0.01$  \*\*\* $p < 0.001$  vs. control (+E = endothelium intact)



### *Cysteine and superoxide dismutase*

The effect of cysteine on ACH-induced relaxation was inhibited by prior incubation of cysteine with SOD and catalase. No significant decrease in R<sub>max</sub> to ACH was observed with either intervention compared with the initial ACH responses (Fig. 7.7).



**Fig 7.7: Cysteine experiments: effect of superoxide dismutase and catalase**

(a) in the absence of other interventions (control) and following a 3 hr incubation with 1 mM cysteine + SOD

(b) control versus 1 mM cysteine + SOD + Catalase

## 7.4 Discussion

These data demonstrate that exposure to homocysteine (1mM) for 3 hours significantly inhibits endothelium-dependent relaxation to both the receptor agonist, acetylcholine (ACH) and the calcium ionophore A23187, in a receptor-independent manner. Homocysteine did not impair endothelium-independent relaxation to sodium nitroprusside.

Previous *in vitro* studies have shown that the effect of homocysteine is not specific<sup>56</sup> and that other thiols such as cysteine may also produce similar changes. This is confirmed in the current experiments. Cysteine, at the same concentration, also impaired endothelium-dependent relaxation, but to a lesser extent than homocysteine (i.e.  $\Delta$  -13.9 vs.  $\Delta$  -28.5%, respectively). A non-specific effect of the thiol group may be a result of the high concentrations of homocysteine and cysteine used. Indeed the concentration of homocysteine (1mM) is approximately 70 times that which is known to be associated with vascular disease in humans ( $>15\mu\text{M}$ ). Lower concentrations of Hcy ( $<1\text{mM}$ ) have been used in exploratory experiments (Dr D Lang–personal communication) but did not consistently impair vascular responses. However, despite the concentrations being non-physiological, this series of experiments does serve to demonstrate the differential effects on endothelium-dependent relaxation of the various intermediates in the transsulphuration pathway and provides a technique to explore possible mechanisms of action. Homocysteine and cysteine produced the most marked decrease in  $R_{\text{max}}$  to ACH with much smaller changes occurring after prior incubation with methionine and S-adenosyl-methionine (SAM). No effect was observed following S-adenosylhomocysteine (SAH).

S-adenosylmethionine is a methyl donor and its conversion to SAH may potentially lead to the methylation of a variety of substrates, notably arginine. L-arginine, the substrate for nitric oxide synthase (NOS) can be methylated to form the structural isomers asymmetric dimethyl arginine (ADMA) and symmetric dimethyl arginine (SDMA). ADMA, but not SDMA, has been shown to be a competitive inhibitor of NOS-III<sup>127</sup> and hence may impair production of nitric oxide (NO) and consequently NO-mediated vascular smooth muscle relaxation. Incubation of aortic rings with both methionine and SAM itself could possibly lead to a decrease in the  $R_{\text{max}}$  to ACH by the above mechanism. This is in keeping with the observed neutral

effect of SAH. An alternative explanation would be the formation of homocysteine in situ. If significant conversion of these intermediates to homocysteine were taking place, however, one would have expected some effect on the R<sub>max</sub> to ACH following incubation with SAH.

The mechanisms by which homocysteine inhibits endothelium-dependent relaxation are not well understood and are likely to be multifactorial. As described in Chapter 1.7, homocysteine may undergo auto-oxidation to both superoxide and hydrogen peroxide. There is also some preliminary evidence demonstrating an alteration in intracellular redox status<sup>70</sup>. Generation of increased concentrations of superoxide may lead to a decrease in NO bioavailability by the formation of peroxynitrite<sup>73</sup>. The generation of hydrogen peroxide may lead to consumption of intracellular reduced glutathione, free radical formation and impaired mitochondrial function.

In these experiments, the prior incubation of homocysteine with superoxide dismutase (SOD) alone did not reverse its effect on ACH-induced relaxation and in fact appeared to enhance its inhibitory action. The addition of catalase with SOD, however, did attenuate the inhibitory effect of homocysteine although a significant decrease in the R<sub>max</sub> remained. These data suggest that an elevation in both superoxide and hydrogen peroxide levels are, at least in part, responsible for the effect of homocysteine. Vitamin C also partially reversed the effect of homocysteine suggesting a significant role for reactive oxygen species.

The failure of SOD and catalase to completely reverse endothelial dysfunction implies that an alteration in intracellular redox status may be an additional important mechanism and the effect is not mediated purely by auto-oxidation of homocysteine as previously suggested. Addition of the intracellular superoxide scavenger, Tiron, prior to incubation of rings with homocysteine, also partially reversed the inhibitory effect of homocysteine on ACH-induced relaxation but a small significant decrease was still observed. The effect of homocysteine was completely reversed, however, in experiments with the calcium ionophore, A23187 compared with vehicle. Although the exact source of intracellular oxygen free radicals is not entirely clear, NADH and NADPH oxidase(s), eicosanoid metabolism, and respiratory chain enzymes are possible candidates. Superoxide may also be generated intracellularly, particularly when reduced flavins, for example xanthine oxidase, are reoxidised univalently by molecular oxygen, and from eNOS.

In contrast to homocysteine, the effect of cysteine on endothelium-dependent relaxation was completely reversed by prior incubation with SOD. This may suggest that homocysteine and cysteine (at the concentrations used here) affect endothelial cell function by different mechanisms. Auto-oxidation of the thiol group itself leading to extracellular superoxide formation may account for the inhibitory effect of cysteine. It is unlikely that cysteine *per se* is directly toxic to endothelium. Moreover, it is unlikely that there is any significant increase in the intracellular production of reactive oxygen species, as if this was the case a residual inhibitory effect after SOD should have been apparent.

In summary, the preliminary experiments in this chapter have confirmed that homocysteine *in vitro* directly impairs endothelium-dependent vascular smooth muscle cell relaxation by a receptor-independent mechanism and that precursors in the transsulphuration pathway when used at similar concentration do not produce an effect of similar magnitude. The mechanism by which homocysteine modulates endothelial function is likely to be multifactorial but may involve both the production of superoxide with hydrogen peroxide and also formation of intracellular reactive oxygen species. Further studies are however required to confirm these preliminary findings aiming to use more physiological concentrations of homocysteine, including a series of dose-response experiments.

## CHAPTER 8

### Summary, Conclusion And Future Perspectives

#### 8.1 Summary

A general introduction on homocysteine metabolism and its relationship to cardiovascular disease, with the likely pivotal role of endothelial dysfunction, was given in Chapter 1. Many epidemiological studies have been performed to assess the strength of the relationship between elevated homocysteine concentrations and vascular disease. Meta-analysed data reported by Boushey et al<sup>44</sup> indicated that a 5  $\mu\text{mol/L}$  increase in Hcy leads to a 60% higher risk of coronary artery disease, comparable to the effect of an increase of 0.5 mmol/L cholesterol. A more recent updated meta-analysis<sup>132</sup>, which included prospective studies, has reported more conservative estimates for an effect of elevated Hcy concentrations. The odds ratio of coronary heart disease for a 5 $\mu\text{mol/L}$  increase was 1.3 (95% CI 1.1-1.5) in prospective studies versus 1.9 (95% CI 1.6-2.3) in retrospective studies. Importantly, an elevated Hcy level has been shown to constitute a graded and independent risk factor for premature atherosclerosis, with strong interactive effects with smoking and hypertension<sup>50</sup>.

A number of plausible mechanisms have been proposed for the '*homocysteine hypothesis of vascular disease*' and there is increasing evidence suggesting the primary cellular mechanism for Hcy-mediated vascular injury appears to be toxicity to the endothelium leading to disruption of normal vasomotor function and the subsequent promotion of endothelium-mediated thrombosis.

The studies in this thesis investigated the effects of elevated Hcy levels as a result of abnormalities in different aspects of the homocysteine transsulphuration / remethylation pathways. Non-invasive measurement of endothelial function using the method of high-resolution ultrasonic wall-tracking was presented in Chapter 2. Brachial artery dilatation in response to hyperaemic blood flow was shown to be an endothelium-dependent mechanism by the inhibition of vasodilatation after the prior infusion of the NOS-III antagonist, N<sup>G</sup>-monomethyl L-arginine. Typical normal values for the local population were determined and *absolute change in arterial diameter* shown to be an appropriate index to represent flow- and GTN-mediated responses. Differences in normal values compared with published data were a consequence of 2 factors: (i)

improved resolution of arterial measurements and (ii) distal placement of the cuff used to induce hyperaemic blood flow thus avoiding brachial artery ischaemia.

To determine whether hyperhomocysteinaemia in humans is associated with impaired endothelium-dependent vasodilatation, studies were performed to investigate the effect of acute hyperhomocysteinaemia after an oral methionine load and mild hyperhomocysteinaemia in subjects with low vitamin B<sub>12</sub>/folate status. In Chapter 3, experimental hyperhomocysteinaemia following an oral methionine load (which increased Hcy levels 3-fold) was associated with impaired endothelium-dependent vasodilatation and a temporal relationship was demonstrated between the rise in plasma Hcy and a decrease in flow-mediated dilatation. The effect of methionine loading was attenuated by the prior oral administration of vitamin C, suggesting Hcy may have a role in oxidative damage to endothelium. A direct effect of methionine could not however be excluded. These data are supported by two recent publications where similar findings to those here (pre- and post-vitamin C therapy) have been reported<sup>133, 134</sup>.

Subjects with mildly elevated fasting Hcy levels due to low B<sub>12</sub>/folate status (leading to impaired remethylation of homocysteine) were studied in Chapter 4. Endothelium-dependent dilatation was impaired versus age and sex-group matched controls. Vitamin supplementation was not studied in this small cohort. However, in a homozygous homocystinuric patient with suboptimal vitamin levels, endothelium-dependent dilatation improved after 3 months vitamin supplementation, suggesting a role for vitamin supplementation in augmenting endothelial function in homocystinuric subjects at high risk of vascular complications.

In Chapter 5, a relatively large cross-sectional study was conducted to investigate the relationship between fasting Hcy and endothelial dysfunction across the range of Hcy concentrations in the general population. There was no inverse correlation demonstrated. As previously discussed, there are a number of possible explanations as to why an association between Hcy and endothelial dysfunction was not seen in this study. One possibility is the influence of other as yet unknown risk factors that may have independently led to abnormal flow-mediated dilatation in otherwise healthy subjects. Passive smoking and/or genetic factors may also have influenced the results.

An important omission, however, was not to include a methionine loading test in addition to fasting Hcy levels to identify subjects who may have impaired Hcy metabolism. Elevated plasma Hcy concentration in the fasting state is postulated to reflect abnormalities in Hcy remethylation, whereas an abnormal increase in Hcy after a

methionine load is considered to reflect aberrant Hcy transsulphuration<sup>10</sup>. On the basis of fasting Hcy concentrations alone, about 25-40% of subjects with methionine intolerance (and hence possible impaired endothelial function) may not have been detected<sup>16,50</sup>, possibly obscuring any positive association. Published data has now demonstrated an inverse relationship between hyperhomocysteinaemia and flow-related endothelial function both in elderly subjects<sup>116</sup> and in a Chinese cohort<sup>117</sup>. There is also recent data to show an interesting association between elevated Hcy levels and endothelial dysfunction in Asians<sup>135</sup>, a population known to have an increased risk of cardiovascular disease.

Serum folate concentrations were shown to be strongly correlated with plasma Hcy. Vitamin supplementation can lower plasma Hcy even in subjects with normal Hcy levels<sup>36</sup>. In Chapter 6, a double blind, placebo-controlled crossover trial demonstrated an improvement in endothelial function in association with a reduction in Hcy concentrations, providing preliminary evidence that folate might have beneficial cardiovascular effects in adults with mild hyperhomocysteinaemia. Large-scale trials of vitamin supplementation are awaited.

At the molecular level, the exact mechanisms whereby Hcy may promote atherosclerosis are only partially understood. Adverse effects of Hcy on endothelial function, thrombomodulin surface expression<sup>136</sup>, protein C activation<sup>137</sup>, tissue factor activity<sup>138</sup> and on susceptibility of low-density lipoproteins to oxidation<sup>139</sup> have all been described as possible mechanisms by which Hcy promotes atherosclerosis and thrombosis. In addition, studies have shown that Hcy promoted DNA synthesis in smooth muscle cells by induction of cyclin A gene expression<sup>59</sup>, and increased transcription of cyclin-dependent kinase, a regulatory protein in mitosis<sup>140</sup>. There is increasing evidence that oxidant stress and alterations in intracellular redox status may have a significant role in mediating the effects of Hcy on endothelial function<sup>66</sup>. In Chapter 7, the effect of Hcy on agonist-induced, endothelium-dependent relaxation of phenylephrine-precontracted rabbit aortic rings was investigated. Although the concentrations used were higher than those occurring *in vivo*, the series of experiments served to demonstrate the differential effects on endothelium-dependent relaxation of the various intermediates in the transsulphuration pathway and a preliminary technique to explore the effects of superoxide scavengers and intracellular antioxidants. Incubation of aortic rings with Hcy significantly impaired agonist-mediated endothelium-dependent relaxation. This effect was attenuated by prior incubation of Hcy with antioxidants and



the intracellular superoxide scavenger, Tiron, supporting the hypothesis that Hcy mediates endothelial injury by oxidant stress.

## 8.2 Conclusions

In relation to the hypotheses and aims stated in Chapter 1, the following conclusions can be made:-

(a) Endothelial function can be assessed non-invasively in humans by measuring changes in brachial artery end-diastolic diameter in response to hyperaemic blood flow using ultrasonic vessel wall tracking, a technique that improves the resolution of vascular measurements compared with previous methods. Changes in vessel size are best expressed as simple increments in vascular diameter and normal values have been determined.

(b) The experiments presented in this thesis provide additional evidence that hyperhomocysteinaemia (secondary to both acute experimental increases in plasma Hcy and mild elevations due to acquired and/or genetic causes) is associated with endothelial dysfunction. However, all the data is not consistent with this conclusion and does not prove causality.

(c) Lowering Hcy concentrations with folate supplements improves endothelium-dependent vasodilatation. As endothelial dysfunction is postulated to be a surrogate marker of future cardiovascular events, these findings may have important public health implications for those individuals at increased risk. Large scale randomised studies are required to examine the effect of vitamin supplementation on cardiovascular morbidity and mortality. If a causal role for Hcy in cardiovascular disease is confirmed, the simple and cheap intervention of oral vitamin supplementation may offer an intervention appropriate to both the population as a whole (food fortification) or to patients with cardiovascular disease (oral supplements).

(d) The mechanism whereby homocysteine modulates endothelium-dependent vasodilatation is likely to be multifactorial. A predominant role, however, for an increase in oxidant stress mediated by hyperhomocysteinaemia is supported by the observed protective effect of oral antioxidants in humans prior to methionine loading and experimentally has been demonstrated in preliminary studies using *in vitro* tissue bath preparations.

### 8.3 Future Perspectives

The oral methionine load was a useful method of producing an acute elevation in plasma Hcy concentration but one cannot completely exclude an additional effect of methionine on inducing endothelial dysfunction. Methionine may increase the concentration of methylated proteins and specifically could increase the concentration of asymmetric dimethyl arginine (ADMA), which has been shown to act as a competitive inhibitor of nitric oxide synthase<sup>127</sup>. Further studies are needed to measure concentrations of ADMA post methionine load to exclude this possibility. In addition, studies using oral homocysteine preparations in place of methionine could be performed thereby removing the effect of methionine. Studies aiming to produce physiological increments in plasma Hcy by oral intake of methionine-rich foods, thereby simulating post-prandial dietary changes are required.

Preliminary experiments using oral vitamin C attenuated the effect of the methionine load. Further studies are needed using larger numbers of subjects to elucidate the mechanisms whereby Hcy may promote oxidant stress. Additional experiments should include measurement of peroxynitrite concentrations or if possible experiments designed to measure free radical production directly, by means of sample collection in spin traps and electron spin resonance spectroscopy.

A larger intervention study to determine the beneficial effect of folate on endothelial function is needed, particularly examining its effect across the range of Hcy concentrations (including normal values), using a longer washout period to exclude any crossover effects (e.g. 120 days equivalent to red cell survival time, the predominant folate storage pool) and using smaller doses from 400 mcg upwards to define the optimum dose required. This may be achieved by dietary supplements in combination with vitamin preparations. There is evidence to suggest that folate may have an effect independent of Hcy concentrations and act as a potent antioxidant *per se*<sup>124,141</sup>.

The cross-sectional population study highlighted the possibility of independent genetic determinants of endothelial dysfunction whereby subjects with no known risk factors for vascular disease had markedly impaired flow-mediated responses. Family studies, where there is a strong history of premature vascular disease may help to further elucidate mechanisms, investigating possible genotypes that may be associated with the development of atherosclerosis.

As discussed in section 8.1, whether Hcy-lowering is clinically beneficial in terms of long term cardiovascular morbidity and mortality remains to be established in a large randomised clinical trial. A positive result will help to raise awareness of the importance of Hcy as a risk factor for vascular disease, something that at present is not well accepted in the clinical arena as a whole. A readily available, affordable, standardised assay will also be needed in all hospitals for accurate measurement and follow up of Hcy concentrations.

In regard to the experimental laboratory data and future research, most *in vitro* studies, including those presented here, have been performed with non-physiological concentrations of Hcy. Furthermore, in blood only about 1% of all Hcy moieties are present in its reduced state. *In vitro* experiments are needed to investigate whether the effect of Hcy is both dose- and time-dependent using lower, physiological doses of Hcy. The potential role of intracellular superoxide in Hcy-mediated endothelial dysfunction requires a method of quantitative measurement of superoxide anion production after Hcy incubation. *In vivo*, studies to investigate the exact pathological mechanism of hyperhomocysteinaemia will involve either normal animals fed high methionine diets or more specifically, transgenic animal models allowing the effect of hyperhomocysteinaemia on arterial wall and endothelial cell physiology to be determined.

## REFERENCES

1. du Vigneaud V, Ressler C, Rachele R. The biological synthesis of "labile methyl groups". *Science* 1950;**112**:267-271.
2. Carson NAJ, Neill DW. Metabolic abnormalities detected in a survey of mentally backward individuals in Northern Ireland. *Arch Dis Child* 1962;**37**:505-513.
3. Gibson JB, Carson NAJ, Neill DW. Pathological findings in homocystinuria. *J Clin Path* 1964;**17**:427-437.
4. Mudd SH, Levy HL, Skovby F. Disorders of transsulfuration. In: Scriver C, Beaudet A, Sly W, Valle D, eds. *The Metabolic and Molecular Bases of Inherited Disease*. 7th ed. New York: McGraw Hill, 1995: 1279-1323.
5. Mudd SH, Skovby F, Levy HL, Pettigrew KD, Wilcken B, Pyeritz RE, Andria G, Boers GHJ, Bromberg IL, Cerone R, Fowler B, Grobe H, Schmidt H, Schweitzer L. The natural history of homocystinuria due to cystathionine  $\beta$ -synthase deficiency. *Am J Hum Genet* 1985;**37**:1-31.
6. McCully KS, Wilson RB. Homocysteine theory of arteriosclerosis. *Atherosclerosis* 1975;**22**:215-227.
7. Wilcken DEL, Reddy SG, Gupta VJ. Homocysteinemia, ischemic heart disease, and the carrier state for homocystinuria. *Metabolism* 1983;**32**:363-370.
8. Livesey G. Methionine degradation: "anabolic and catabolic". *Trends Biochem Sci* 1984;**9**:27-29.
9. Finkelstein JD. Methionine metabolism in mammals. *J Nutr Biochem* 1990;**1**:228-237.
10. Finkelstein JD, Martin JJ. Methionine metabolism in mammals. Adaptation to methionine excess. *J Biol Chem* 1986;**261**:1582-1587.
11. Ueland PM, Refsum H, Stabler SP, Malinow MR, Andersson A, Allen RH. Total homocysteine in plasma or serum. Methods and clinical applications. *Clin Chem* 1993;**39**:1764-1779.
12. Brenton DP, Cusworth DC, Dent CE, Jones EE. Homocystinuria:clinical and dietary studies. *Q J Med* 1966;**35**:325-346.
13. Fowler B, Sardharwalla IB, Robins AJ. The detection of heterozygotes for homocystinuria by oral loading with L-methionine. *Biochem J* 1971;**122**:23P-24P.
14. Brattstrom L, Israelsson B, Norrving B, Bergqvist D, Thorne J, Hultberg B, Hamfelt A. Impaired homocysteine metabolism in early-onset cerebral and

peripheral occlusive arterial disease. Effects of pyridoxine and folic acid treatment. *Atherosclerosis* 1990;**81**:51-60.

15. Mansoor MA, Bergmark C, Svardal AM, Lonning PE, Ueland PM. Redox status and protein binding of plasma homocysteine and other aminothiols in patients with early-onset peripheral vascular disease. *Arterioscler Thromb Vasc Biol* 1995;**15**:232-240.

16. Bostom AG, Jacques PF, Nadeau MR, Williams RR, Ellison RC, Selhub J. Post-methionine load hyperhomocysteinemia in persons with normal fasting plasma homocysteine: initial results from the NHLBI Family Heart Study. *Atherosclerosis* 1995;**116**:147-151.

17. Selhub J, Jacques PF, Wilson PWF, Rush D, Rosenberg IH. Vitamin status and intake as primary determinants of homocysteinemia in an elderly population. *J Am Med Assoc* 1993;**270**:2693-2698.

18. Guttormson AB, Svarstad E, Ueland PM, Refsum H. Elimination of homocysteine from plasma in subjects with endstage renal failure. *Irish J Med Sci* 1995;**164**(Suppl.15):8.

19. Bostom A, Brosnan JT, Hall B, Nadeau MR, Selhub J. Net uptake of plasma homocysteine by the rat kidney in vivo. *Atherosclerosis* 1995;**116**:59-62.

20. Nygard O, Vollset SE, Refsum H, Stensvold I, Tverdal A, Nordrehaug JE, Ueland PM, Kvale G. Total plasma homocysteine and cardiovascular risk profile. *J Am Med Assoc* 1995;**274**:1526-33.

21. Nygard O, Refsum H, Ueland PM, Stensvold I, Nordrehaug JE, Kvale G, Vollset SE. Coffee consumption and plasma total homocysteine: The Hordaland Homocysteine Study. *Am J Clin Nutr* 1997;**65**:136-143.

22. Fenton WA, Rosenberg LE. Inherited disorders of cobalamin transport and metabolism. In: Scriver C, Beaudet A, Sly W, Valle D, eds. *The metabolic basis of inherited disease*. 6th ed. New York: McGraw-Hill, 1989.

23. Finkelstein JD, Mudd SH, Irreverre F, Laster L. Homocystinuria due to cystathionine synthetase deficiency: the mode of inheritance. *Science* 1964;**146**:785-786.

24. Fowler B, Kraus J, Packman S, Rosenberg LE. Homocystinuria: evidence for three distinct classes of cystathionine B-synthase mutants in cultured fibroblasts. *J Clin Invest* 1978;**61**:645-653.

25. Kang SS, Passen EL, Ruggie N, Wong PWK, Sora H. Thermolabile defect of methylenetetrahydrofolate reductase in coronary artery disease. *Circulation* 1993;**88**:1463-1469.
26. Kang SS, Zhou JZ, Wong PWK, Kowalisyn J, Strokosch G. Intermediate homocysteinemia: a thermolabile variant of methylenetetrahydrofolate reductase. *Am J Hum Genet* 1988;**43**:414-421.
27. Frosst P, Blom HJ, Milos R, Goyette P, Sheppard CA, Matthews RG, Boers GJH, den Heijer M, Kluijtmans LAJ, van den Heuvel LP, Rozen R. A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. *Nat Genet* 1995;**10**:111-113.
28. Clark ZE, Bowen DJ, Whatley SD, Bellamy MF, Collins PW, McDowell IFW. Genotyping method for methylenetetrahydrofolate reductase (C677T thermolabile variant) using heteroduplex technology. *Clin Chem* 1998;**44**:2360-2362.
29. Brattstrom L, Wilcken DEL, Ohrvik J, Brudin L. Common methylenetetrahydrofolate reductase gene mutation leads to hyperhomocysteinemia but not to vascular disease. *Circulation* 1998;**98**:2520-2526.
30. Ubbink JB, Vermaak WJH, van der Merwe A, Becker PJ. Vitamin B-12, vitamin B-6, and folate nutritional status in men with hyperhomocysteinemia. *Am J Clin Nutr* 1993;**57**:47-53.
31. Mudd SH, Poole JR. Labile methyl balances for normal humans on various dietary regimens. *Metabolism* 1975;**24**:721-735.
32. Kang SS, Wong PWK, Norusis M. Homocysteinemia due to folate deficiency. *Metabolism* 1987;**36**:458-462.
33. Chanarin I. The megaloblastic anaemias. 2nd ed. Oxford: Blackwell, 1979.
34. Refsum H, Helland S, Ueland PM. Fasting plasma homocysteine as a sensitive parameter of antifolate effect: a study of psoriasis patients receiving low-dose methotrexate treatment. *Clin Pharmacol Ther* 1989;**46**:510-520.
35. Miller LT, Dow MJ, Kokkeler SC. Methionine metabolism and vitamin B6 status in women using oral contraceptives. *Am J Clin Nutr* 1978;**31**:619-625.
36. Ubbink JB, Vermaak WJH, van der Merwe A, Becker PJ, Delport R, Potgieter HC. Vitamin requirements for the treatment of hyperhomocysteinemia in humans. *J Nutr* 1994;**124**:1927-1933.
37. Chu RC, Hall CA. The total serum homocysteine as an indicator of vitamin B12 and folate status. *Am J Clin Path* 1988;**60**:446-449.

38. Jacobsen DW, Gatautis VJ, Green R, Robinson K, Savon SR, Secic M, Ji J, Otto JM, Taylor LM Jr. Rapid HPLC determination of total homocysteine and other thiols in serum and plasma: sex differences and correlation with cobalamin and folate concentrations in healthy subjects. *Clin Chem* 1994;**40**:873-881.
39. Robinson K, Arheart K, Refsum H, Brattstrom L, Boers G, Ueland P, Rubba P, Palma-Reis R, Meleady R, Daly L, Witteman J, Graham I. Low circulating folate and vitamin B6 concentrations, risk factors for stroke, peripheral vascular disease and coronary artery disease. *Circulation* 1998;**97**:437-443.
40. Rimm EB, Willett WC, Hu FB, Sampson L, Colditz GA, Manson JE, Hennekens C, Stampfer MJ. Folate and vitamin B6 from diet and supplements in relation to risk of coronary heart disease among women. *J Am Med Assoc* 1998;**279**:359-364.
41. Selhub J, Miller JW. The pathogenesis of homocysteinaemia: interruption of the coordinate regulation by S-adenosylmethionine of the remethylation and transsulfuration of homocysteine. *Am. J. Clin. Nutr.* 1992;**55**:131-8.
42. Moustapha A, Naso A, Nahlawi M, Gupta A, Arheart KL, Jacobsen DW, Robinson K, Dennis VW. Prospective study of hyperhomocysteinemia as an adverse cardiovascular risk factor in end-stage renal failure. *Circulation* 1998;**97**:138-141.
43. Sevvitt LH, Hoffbrand AV. Serum folate and vitamin B12 levels in acute and chronic renal disease. Effect of peritoneal dialysis. *Br Med J* 1969;**ii**:18-21.
44. Boushey CJ, Beresford SAA, Omenn GS, Motulsky AG. A quantitative assessment of plasma homocysteine as a risk factor for vascular disease. *J Am Med Assoc* 1995;**274**:1049-1057.
45. Arnesen E, Refsum H, Bonna KH, Ueland PM, Forde OH, Nordrehaug J E. Serum total homocysteine and coronary heart disease. *Int J Epidemiol* 1995;**24**:704-709.
46. Stampfer MJ, Malinow MR, Willet WC, Newcomer LM, Upson B, Ullmann D, Tishler PV, Hennekens CH. A prospective study of plasma homocysteine and risk of myocardial infarction in US physicians. *J Am Med Assoc* 1992;**268**:877-881.
47. Wald NJ, Watt HC, Law MR, Weir DG, McPartlin J, Scott JM. Homocysteine and ischaemic heart disease. Results of a prospective study with implications regarding prevention. *Arch Intern Med* 1998;**158**:862-867.
48. Alfthan G, Pekkanen J, Juahianen M, Pitkaniemi J, Karvonen M, Tuomilehto J, Salonen JT, Ehnholm C. Relation of serum homocysteine and lipoprotein(a)

concentrations to atherosclerotic disease in a prospective Finnish population based study. *Atherosclerosis* 1994;**106**:9-19.

49. Evans RW, Shaten J, Hempel JD, Cutler JA, Kuller LH. Homocysteine and risk of cardiovascular disease in the Multiple Risk Factor Intervention Trial. *Arterioscler Thromb Vasc Biol* 1997;**17**:1947-1953.

50. Graham IM, Daly LE, Refsum HM, Robertson K, Brattstrom LE, Ueland PM, Palma-Reis RJ, Boers GHJ, Sheahan RG, Israelsson B, Uiterwaal CS, Meleady R, McMaster D, Verhoef P, Witteman J, Rubba P, Bellet H, Wautrecht JC, de Valk HW, Sales Luis AC. Plasma homocysteine as a risk factor for vascular disease. *J Am Med Assoc* 1997;**277**:1775-1781.

51. Busse R, Fleming I. Endothelial dysfunction in atherosclerosis (Review). *J Vasc Res* 1996;**33**:181-194.

52. Palmer R, Ashton D, Moncada S. Vascular endothelial cells synthesize nitric oxide from L-arginine. *Nature* 1988;**333**:664-666.

53. Flavahan NA. Atherosclerosis and lipoprotein-induced endothelial dysfunction. *Circulation* 1992;**85**(2):1927-1938.

54. Ross R. The pathogenesis of atherosclerosis - an update. *N Engl J Med* 1986;**314**:488-500.

55. Wall RT, Harlan JM, Harker LA, Striker GE. Homocysteine-induced endothelial cell injury in vitro: a model for the study of vascular injury. *Thromb Res* 1980;**18**:113-121.

56. Dudman NPB, Hicks C, Wang J, Wilcken DEL. Human arterial endothelial cell detachment in vitro: its promotion by homocysteine and cysteine. *Atherosclerosis* 1991;**91**:77-83.

57. Harker LA, Ross R, Slichter SJ, Scott CR. Homocysteine-induced arteriosclerosis. The role of endothelial cell injury and platelet response in its genesis. *J Clin Invest* 1976;**58**:731-741.

58. Ross R. The pathogenesis of atherosclerosis: a perspective for the 1990's. *Nature* 1993;**362**:801-809.

59. Tsai JC, Wang H, Perrella MA, Yoshizumi M, Sibinga NES, Tan LC, Haber E, Chang TH, Schlegel R, Lee ME. Induction of cyclin A gene expression by homocysteine in vascular smooth muscle cells. *J Clin Invest* 1996;**97**:146-153.

60. Rubanyi GM, Romero JC, Vanhoutte PM. Flow-induced release of endothelium-derived relaxing factor. *Am J Physiol* 1986;**250**:H1145-H1149.60.



61. Stamler JS, Loscalzo J. Endothelium-derived relaxing factor modulates the atherothrombogenic effects of homocysteine. *J Cardiovasc Pharm* 1992;**20**(Suppl 12):S202-S204.
62. Stamler JS, Osborne JA, Jaraki O, Rabbani LE, Mullins M, Singel D, Loscalzo J. Adverse vascular effects of homocysteine are modulated by endothelium-derived relaxing factor and related oxides of nitrogen. *J Clin Invest* 1993;**91**:308-318.
63. Lentz SR, Sobey CG, Piegors DJ, Bhopatkhar MY, Faraci FM, Malinow MR, Heistad DD. Vascular dysfunction in monkeys with diet-induced hyperhomocyst(e)inemia. *J Clin Invest* 1996;**98**:24-29.
64. Celermajer DS, Sorensen K, Ryalls M, Robinson J, Thomas O, Leonard JV, Deanfield JE. Impaired endothelial function occurs in the systemic arteries of children with homozygous homocystinuria but not their heterozygous parents. *J Am Coll Cardiol* 1993;**22**:854-858.
65. Joannides R, Haefeli WE, Linder L, Richard V, Bakkali EH, Thuillez C, Luscher TF. Nitric oxide is responsible for flow-dependent dilatation of human peripheral conduit arteries in vivo. *Circulation* 1995;**91**:1314-1319.
66. Loscalzo J. The oxidant stress of hyperhomocysteinaemia [editorial]. *J Clin Invest* 1996;**98**:5-7.
67. Olszewski AJ, McCully KS. Homocysteine metabolism and the oxidative modification of proteins and lipids. *Free Rad Biol and Med* 1993;**14**:683-693.
68. Starkebaum G, Harlan JM. Endothelial cell injury due to copper-catalyzed hydrogen peroxide generation from homocysteine. *J Clin Invest* 1986;**77**:1370-1376.
69. Blundell G, Jones BG, Rose FA, Tudball N. Homocysteine mediated endothelial cell toxicity and its amelioration. *Atherosclerosis* 1996;**122**:163-172.
70. Upchurch GR, Welch GN, Fabian AJ. Homocysteine decreases bioavailable nitric oxide by a mechanism involving glutathione peroxidase. *J Biol Chem* 1997;**272**:17012-17017.
71. Dudman NPB, Wilcken DEL, Stocker R. Circulating lipid hydroperoxide levels in human hyperhomocysteinaemia. *Arteriosclerosis and Thrombosis* 1993;**13**:512-516.
72. Blom HJ, Kleinveld HA, Boers GHJ, Demacker PNM, Hak-Lemmers HLM, Te Poele-Pothoff MTWB, Trijbels JMF. Lipid peroxidation and susceptibility of low-density lipoprotein to in vitro oxidation in hyperhomocysteinaemia. *Eur J Clin Invest* 1995;**25**:149-154.

73. Jia L, Furchgott RF. Inhibition by sulphhydryl compounds of vascular relaxation induced by nitric oxide and endothelium-derived relaxing factor. *J Pharmacol Exp Ther* 1993;**267**:371-378.
74. Beckman JS, Koppenol WH. Nitric oxide, superoxide and peroxynitrite: the good, the bad and the ugly. *Am J Physiol* 1996;**271**:C1424-C1437.
75. Miles AM, Bohle DS, Glassbrenner PA, Hansert B, Wink DA, Grisham MB. Modulation of superoxide-dependent oxidation and hydroxylation reactions by nitric oxide. *J Biol Chem* 1996;**271**:40-47.
76. Ku DD, Liu S, Dai J. Coronary vascular and antiplatelet effects of peroxynitrite in human tissues. *Endothelium* 1995;**3**:309-319.
77. Wu MN, Pritchard KA, Kaminski PM, Fayngersh RP, Hintze TH, Wolin MS. Involvement of nitric oxide and nitrosothiols in relaxation of pulmonary arteries to peroxynitrite. *Am J Physiol* 1994;**266**:H2108-H2113.
78. Pryor WA, Squadrito GL. The chemistry of peroxynitrite: a product from the reaction of nitric oxide and superoxide. *Am J Physiol* 1995;**268**:L699-L722.
79. Beckman JS, Beckman TW, Chen J, Marshall PA, Freeman BA. Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. *Proc Natl Acad Sci USA* 1990;**87**:1620-1624.
80. Ischiropoulos H, Al-Mehdi AB. Peroxynitrite-mediated oxidative protein modifications. *FEBS Lett* 1995;**364**:279-282.
81. Ma XL, Lopez BL, Liu GL, Christopher TA, Gao F, Guo YP, Feuerstein GZ, Ruffolo RR, Barone FC, Yue TL. Hypercholesterolemia impairs a detoxification mechanism against peroxynitrite and renders the vascular tissue more susceptible to oxidative injury. *Circ Res* 1997;**80**:894-901.
82. Rees MM, Rodgers GM. Homocysteinaemia: association of a metabolic disorder with vascular disease and thrombosis. *Thrombosis Res.* 1993;**71**:337-359.
83. Mandel H, Brenner B, Berant M, Rosenberg N, Lanir N, Jakobs C, Fowler B, Seligsohn U. Coexistence of hereditary homocystinuria and factor V leiden-effect on thrombosis. *N Engl J Med* 1996;**334**:763-768.
84. Harpel PC, Chang VT, Borth W. Homocysteine and other sulfhydryl compounds enhance the binding of lipoprotein(a) to fibrin: a potential biochemical link between thrombosis, atherogenesis and sulfhydryl compound metabolism. *Proc Natl Acad Sci* 1992;**89**:10193-10197.

85. Kielty CM, Wilson DG, Stuart G, Musumeci F, Jones CJ, Davies S, Shuttleworth A. Fibrillin expression and deposition by vascular endothelial cells: implications for the Marfan syndrome [abstract]. *Circulation* 1996;**94**(Suppl):I-350.
86. Wilson DG, Bellamy MF, Ramsey MW, Goodfellow J, Brownlee M, Davies S, Wilson JF, Lewis MJ, Stuart AG. Endothelial function in Marfan syndrome. Selective impairment of flow-mediated vasodilatation. *Circulation* 1999;**99**:909-915.
87. Lubec B, Fang-Kircher S, Lubec T, Blom HJ, Boers GHJ. Evidence for McKusick's hypothesis of deficient collagen cross-linking in patients with homocystinuria. *Biochim Biophys Acta* 1996;**1315**:159-162.
88. Furchgott RF, Zawadzki JV. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle. *Nature* 1980;**288**:373-376.
89. Palmer RM, Ferrige AG, Moncada S. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature* 1987;**327**:524-526.
90. Quyyumi AA, Dakak N, Mulcahy D, Andrews NP, Husain S, Panza JA, Cannon RO. Nitric oxide activity in the atherosclerotic human coronary circulation. *J Am Coll Cardiol* 1997;**29**:308-317.
91. van den Berg M, Boers GHJ, Franken DG, Blom HJ, van Kamp GJJ, Jakobs C, Rauwerda JA, Kluft C, Stehouwert CDA. Hyperhomocysteinaemia and endothelial dysfunction in young patients with peripheral arterial occlusive disease. *Eur J Clin Invest* 1995;**25**:176-181.
92. Pohl U, Holtz J, Busse R, Bassenge E. Crucial role of endothelium in the vasodilator response to increased flow in vivo. *Hypertension* 1986;**8**:37-47.
93. Anderson TJ, Uehata A, Gerhard MD, Meredith IT, Knab S, Delagrang D, Lieberman EH, Ganz P, Creager MA, Yeung AC, Selwyn AP. Close relation of endothelial function in the human coronary and peripheral circulations. *J Am Coll Cardiol* 1995;**26**:1235-1241.
94. Celermajer DS, Sorenson KE, Gooch VM. Non-invasive detection of endothelial dysfunction in children and adults at risk of atherosclerosis. *Lancet* 1992;**340**:1111-1115.
95. Sorensen KE, Celermajer DS, Spiegelhalter DJ, Georgakopoulos D, Robinson J, Thomas O, Deanfield JE. Non-invasive measurement of human endothelium

dependent arterial responses: accuracy and reproducibility. *Br Heart J* 1995;**74**:247-253.

96. Corretti MC, Plotnick GD, Vogel RA. Technical aspects of evaluating brachial artery vasodilatation using high-frequency ultrasound. *Am J Physiol* 1995;**268**:H1397-H1404.

97. Hoeks APG, Brands PJ, Smeets FAM, Reneman RS. Assessment of the distensibility of superficial arteries. *Ultrasound in Med & Biol* 1990;**16**:121-128.

98. Ramsey MW, Goodfellow J, Jones CHJ, Luddington LA, Lewis MJ, Henderson AH. Endothelial control of arterial distensibility is impaired in chronic heart failure. *Circulation* 1995;**92**:3212-3219.

99. Goodfellow J, Ramsey MW, Luddington LA, Jones CJH, Lewis MJ, Henderson AH. Flow-related endothelial function and serum cholesterol - non-invasive markers of atherogenesis "Abstract". *Circulation* 1994;**90**:I-573.

100. Leeson CPM, Thorne S, Donald AE, Mullen MJ, Clarkson P, Deanfield JE. Non-invasive measurement of endothelial function: effect on brachial artery dilatation of graded endothelial dependent and independent stimuli. *Heart* 1997;**78**:22-27.

101. Celermajer DS, Sorensen KE, Spiegelhalter DJ, Georgakopoulos D, Robinson J, Deanfield JE. Aging is associated with endothelial dysfunction in healthy men years before the age-related decline in women. *J Am Coll Cardiol* 1994;**24**:471-476.

102. Celermajer DS, Sorensen KE, Georgakopoulos D, Bull C, Thomas O, Robinson J, Deanfield JE. Cigarette smoking is associated with dose-related and potentially reversible impairment of endothelium-dependent dilation in healthy young adults. *Circulation* 1993;**88**:2149-2155.

103. Tsai MY, Garg U, Key NS, Hanson NQ, Suh A, Schwichtenberg K. Molecular and biochemical approaches in the identification of heterozygotes for homocystinuria. *Atherosclerosis* 1996;**122**:69-77.

104. Levine GN, Frei B, Koulouris SN, Gerhard MD, Keaney JF, Vita JA. Ascorbic acid reverses endothelial vasomotor dysfunction in patients with coronary artery disease. *Circulation* 1996;**93**:1107-1113.

105. Kuo AK, Still R, Cale S, McDowell IFW. Standardisation (external and internal) of HPLC assay for plasma homocysteine. *Clin Chem* 1997;**43**:1653-1655.

106. Khan J, Brennand DM, Bruckdorfer KR, Jacobs M. Nitrotyrosine-like immunoreactivity in human plasma of cigarette smokers and non-smokers. *Br J Pharmacol* 1995;**116**:328P.
107. Hills M, Armitage P. The two-period cross-over clinical trial. *Br J Clin Pharmacol* 1979;**8**:7-20.
108. Beckman JS, Ischiropoulos H, LZhu, van der Woerd M, Smith C, Chen J, Harrison J, Martin JC, Tsai M. Kinetic of superoxide dismutase and iron-catalysed nitration of phenolics by peroxynitrite. *Arch Biochem Biophys* 1992;**2**:438-445.
109. Griendling KK, Alexander RW. Oxidative stress and cardiovascular disease [Editorial]. *Circulation* 1997;**96**:3264-3265.
110. Frei B, England L, Ames BN. Ascorbate is an outstanding antioxidant in human blood plasma. *Proc Natl Acad Sci USA* 1989;**86**:6377-6381.
111. Retsky KL, Freeman MW, Frei B. Ascorbic acid oxidation products protect human low-density lipoprotein against atherogenic modification. *J Biol Chem* 1993;**268**:1304-1309.
112. Meister A. Glutathione-ascorbic acid antioxidant system in animals. *J Biol Chem* 1994;**269**:9397-9400.
113. Winkler BS, Orselli SM, Rex TS. The redox couple between glutathione and ascorbic acid: a chemical and physiologic perspective. *Free Radic Biol Med* 1994;**17**:333-339.
114. Ghigo D, Alessio P, Foco A, Bussolino F, Costamagna C, Heller R, Garbarino G, Pescarmona GP, Bosia A. Nitric oxide synthesis is impaired in glutathione-depleted human umbilical vein endothelial cells. *Am J Physiol* 1993;**265**:C728-C732.
115. den Heijer M, Koster T, Blom HJ, Bos GMJ, Briet E, Reitsma PH, van den Broucke JP, Rosendaal FR. Hyperhomocysteinaemia as a risk factor for deep-vein thrombosis. *N Engl J Med* 1996;**334**:759-762.
116. Tawakol A, Omland T, Gerhard M, Wu JT, Creager MA. Hyperhomocysteinemia is associated with impaired endothelium-dependent vasodilation in humans. *Circulation* 1997;**95**:1119-1121.
117. Woo KS, Chook P, Lolin YI, Cheung ASP, Chan LT, Sun YY, Sanderson JE, Metreweli C, Celermajer DS. Hyperhomocysteinemia is a risk factor for arterial endothelial dysfunction in humans. *Circulation* 1997;**96**:2542-2544.

118. Clarkson P, Celermajer DS, Powe AJ, Donald AE, Henry RMA, Deanfield JE. Endothelium-dependent dilatation is impaired in young healthy subjects with a family history of premature coronary disease. *Circulation* 1997;**96**:3378-3383.
119. Zeiher AM, Schachinger V, Minners J, Halle M, Roschmann E, Niessner M, Volk BA. Angiotensin-converting enzyme gene deletion polymorphism is associated with impaired coronary endothelial vasodilator function. *Circulation* 1995;**92**:1-96.
120. Celermajer DS, Adams MR, Clarkson P. Passive smoking and impaired endothelium-dependent arterial dilatation in healthy young adults. *N Engl J Med* 1996;**334**:150-154.
121. Homocysteine Lowering Trialists Collaboration. Lowering blood homocysteine with folic acid based supplements: meta-analysis of randomised trials. *BMJ* 1998;**316**:894-898.
122. Clarke R and Collins R. Can dietary supplements with folic acid or vitamin B<sub>6</sub> reduce cardiovascular risk? Design of clinical trials to test the homocysteine hypothesis of vascular disease. *J Cardiovascular Risk* 1998;**5**:249-255
123. Verhaar MC, Wever RMF, Kastelein JJP, van Dam T, Koomans HA, Rabelink TJ. 5-Methyltetrahydrofolate, the active form of folic acid, restores endothelial function in familial hypercholesterolemia. *Circulation* 1998;**97**:237-241.
124. Verhaar MV, Wever RMF, Kastelein JJP, van Loon D, Milstien S, Koomans HA, Rabelink TJ. Effects of folic acid supplementation on endothelial function in familial hypercholesterolaemia. *Circulation* 1999;**100**:335-338.
125. Malinow MR, Nieto FJ, Kruger WD, Duell PB, Hess DL, Gluckman RA, Block PC, Holzgang CR, Anderson PH, Seltzer D, Upson B, Lin QR. The effects of folic acid supplementation on plasma total homocysteine are modulated by multivitamin use and methylenetetrahydrofolate reductase genotypes. *Arterioscler Thromb Vasc Biol* 1997;**17**:1157-1162.
126. Loehrer FMT, Angst CP, Haefeli WE, Jordan PP, Ritz R, Fowler B. Low whole-blood S-adenosylmethionine and correlation between 5-methyltetrahydrofolate and homocysteine in coronary artery disease. *Arterioscler Thromb Vasc Biol* 1996;**16**:727-733.
127. Vallance P, Leone A, Calver A, Collier J, Moncada S. Endogenous dimethylarginine as an inhibitor of nitric oxide synthesis. *J Cardiovasc Pharmacol* 1992;**20**(Suppl 12):S60-S62.

128. Boger RH, Bode-Boger SM, Szuba A, Tsao PS, Chan JR, Tangphao O, Blaschke TF, Cooke JP. Asymmetric Dimethylarginine (ADMA): a novel risk factor for endothelial dysfunction. *Circulation* 1998;**98**:1842-1847.
129. Wever RMF, Luscher TF, Cosentino F, Rabelink TJ. Atherosclerosis and the two faces of endothelial nitric oxide synthase. *Circulation* 1998;**97**:108-112.
130. van der Molen EF, van den Heuvel LPWJ, Te Poele Pothoff MTWB, Monnens LAH, Eskes TKAB, Blom HJ. The effect of folic acid on the homocysteine metabolism in human umbilical vein endothelial cells (HUVECS). *Eur J Clin Invest* 1996;**26**:304-309.
131. Cejka J. Enzyme immunoassay for factor VIII-related antigen. *Clin Chem* 1982;**28**:1356-1358.
132. Danesh J and Lewington S. Plasma homocysteine and coronary heart disease: systematic review of published epidemiological studies. *J Cardiovascular Risk* 1998;**5**:229-232.
133. Chambers JC, McGregor A, Jean-Marie J, Obeid OA, Kooner JS. Demonstration of rapid onset vascular endothelial dysfunction after hyperhomocysteinemia: an effect reversible with vitamin C therapy. *Circulation* 1999;**99**:1156-1160.
134. Kanani PM, Sinkey CA, Browning RL, Allaman M, Knapp HR, Haynes WG. Role of oxidant stress in endothelial dysfunction produced by experimental hyperhomocyst(e)inemia in humans. *Circulation* 1999;**100**:1161-1168.
135. Chambers JC, McGregor A, Jean-Marie J, Kooner JS. Abnormalities of vascular endothelial function may contribute to increased coronary heart disease risk in UK Indian Asians. *Heart* 1999;**81**:501-504.
136. Lentz SR and Sadler JE. Inhibition of thrombomodulin surface expression and protein C activation by the thrombogenic agent homocysteine. *J Clin Invest* 1991;**88**:1906-1914.
137. Rodgers GM and Conn MT. Homocysteine, an atherogenic stimulus, reduces protein C activation by arterial and venous endothelial cells. *Blood* 1990;**75**:895-901.
138. Fryer RH, Wilson BD, Gubler DB, Fitzgerald LA, Rodgers GM. Homocysteine, a risk factor for premature vascular disease and thrombosis, induces tissue factor activity in endothelial cells. *Arterioscler Thromb* 1993;**13**:1327-1333.
139. McKully KS. Homocysteine and vascular disease. *Nature Med* 1996;**2**:386-389.

140. Lubec B, Labudova O, Hoeger H, Muehl A, Fangkircher S, Marx M, Mosgoeller W, Gialamas J. Homocysteine increases cyclin-dependent kinase in aortic rat tissue. *Circulation* 1996;**94**:2620-2625.
141. Verhaar MC, Rabelink TJ. Future for folates in cardiovascular disease. *Eur J Clin Invest* 1999;**29**:657-658.



## **PUBLICATIONS**

### **Peer-Reviewed Papers**

1. Bellamy MF, McDowell IFW. Putative mechanisms for vascular damage by homocysteine. *J Inher Metab Dis* 1997;**20**:307-315.
2. Bellamy MF, McDowell IFW, Ramsey MW, Brownlee M, Newcombe R, Lewis MJ. Hyperhomocysteinaemia after an oral methionine load acutely impairs endothelial function in healthy adults. *Circulation* 1998;**98**:1848-1852.
3. Bellamy MF, IFW McDowell, Ramsey MW, Brownlee M, Newcombe RG, Lewis MJ. Oral folate enhances endothelial function in hyperhomocysteinaemic subjects. *Eur J Clin Invest* 1999;**29** (8):659-662.
4. Clark ZE, Bowen DJ, Whatley SD, Bellamy MF, Collins PW, McDowell IFW. Genotyping method for methylenetetrahydrofolate reductase (C677T thermolabile variant) using heteroduplex technology. *Clin Chem* 1998;**44**:2360-2362.
5. Lang D, Kredan MB, Moat SJ, Hussain SA, Powell CA, Bellamy MF, Powers HJ, Lewis MJ. Homocysteine-induced inhibition of endothelium-dependent relaxation in rabbit aorta - role for superoxide anions. *Arterioscler Thromb Vasc Biol* 2000;**20**:422-427

### **Abstracts**

1. Bellamy MF, Ramsey MW, Goodfellow J, Brownlee M, Gorman ST, McDowell IF and Lewis MJ. Hyperhomocysteinaemia and endothelial dysfunction with low vitamin B12/folate status. *Circulation* 1996; **94**(Suppl):I-462.
2. Bellamy MF, Ramsey MW, Brownlee M, Gorman ST, Kuo K, Goodfellow J, McDowell I, Lewis MJ. Abnormal flow-mediated brachial artery responses in subjects with hyperhomocysteinaemia and low vitamin B12 / folate status. *J. Inher. Metab. Dis.* 1996;**19**(Suppl. 1):p23.

3. Bellamy MF, Gorman ST, Brownlee M, Ramsey MW, Goodfellow J, Lewis MJ, Henderson AH. Non-invasive assessment of endothelial function: normal values and the effect of age. *Eur Heart J.* 1996;17(Suppl):p190.

4. Bellamy MF, Ramsey MW, Brownlee M, McDowell IFW, Lewis MJ. Acute hyperhomocysteinaemia following an oral methionine load impairs flow-related endothelial function in man. *Eur Heart J.* 1997; 18 (Suppl):p4.

5. Bellamy MF, Ramsey MW, Brownlee M, RG Newcombe, McDowell IFW, Lewis MJ. Hyperhomocysteinaemia following an oral methionine load acutely impairs endothelial function in healthy adults. *Circulation* 1997;96 (Suppl):I-355.

6. Bellamy MF, McDowell IFW, Ramsey MWR, Brownlee M, Lewis MJ. Folic acid supplements lower plasma homocysteine and improve flow-mediated endothelial function in healthy adults. *Netherlands J Med.* 1998;52(Suppl): S14.

7. McDowell IFW, Clark ZE, Bowen DJ, Bellamy MF, Burr ML. Heteroduplex analysis for C677T genotyping of methylenetetrahydrofolate reductase: a case-control study in men with angina. *Netherlands J Med.* 1998;52(Suppl): S26.

