Homocysteine and Vascular Disease

Dr Steven Carl MARTIN

BSc, MBChB, DrMedSc, MRCPath, EurClinChem.

Department of Clinical Biochemistry and Immunology, Heartlands Hospital, Birmingham, UK

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Abstract

Cardiovascular disease is multifactorial. The main risk factors for developing cardiovascular disease (age, sex, smoking, diabetes, hyperlipidaemia and hypertension) do not explain its development in everyone. New risk factors are continually being sought in order to better understand and treat the disease process. In recent years homocysteine has been proposed as a risk factor for the development of premature cardiovascular disease as a consequence of the accelerated arterial and venous thrombotic disease seen in homocystinuria as a result of a single gene defect. This theory has been difficult to test because patients with premature cardiovascular disease are thankfully rare and because of the difficulties in measuring homocysteine itself.

We propose that, if homocysteine is a causative risk factor for atherothrombosis, it will be involved in the development of cardiovascular disease regardless of age and have therefore studied affected patients from routine hospital clinics. Homocysteine analysis has become easier over the past decade with the development of HPLC methods utilising fluorescent detection, but these methods involve toxic chemicals and suffer from high background fluoresence. I have developed an HPLC method more suited to a routine hospital laboratory utilising coulometric detection for measuring plasma total homocysteine and used it to investigate the relationship between homocysteine levels and both micro- and macro-vascular atherothrombotic disease.

Retinal vessel occlusion was chosen as the paradigm for microvascular disease and the homocysteine levels were found to be higher in 70 patients with retinal artery or vein occlusion than controls, (18.4 v 9.5 μmol/l; $p = 0.0002$, for arterial occlusions) and (13.8 v 9.5 μmol/l; $p < 0.0001$, for venous occlusions). Multivariate analysis confirmed the link between homocysteine and retinal vein occlusion with a mean 32% increase (95% confidence intervals, 14% – 52%; $p = 0.0002$) in homocysteine levels in the subjects compared to controls. This is the first time that homocysteine has been linked to microvascular disease and it is possible that B vitamins may be of use in the prevention of further occlusions.

Homocysteine levels were also significantly elevated in unselected patients with peripheral vascular disease (PVD) attending vascular clinics in the Midlands. (17.4 v 12.3 μmol/l, $p < 0.01$). Logistic regression analysis confirmed the link between PVD and homocysteine with an odds ratio favouring PVD of 4.1 (95% CI, 1.5 – 11.1) per μmol/l homocysteine increase over the mean. These results confirm that homocysteine appears to be strongly linked to peripheral vascular disease regardless of age.

Two hundred and eighty patients undergoing coronary angiography were recruited from a Chest Pain clinic. Homocysteine levels were significantly elevated in patients with ischaemic heart disease compared to the controls (11.5 v 10.1 μmol/l, $p = 0.008$). Homocysteine levels were linked to myocardial infarction in females (11.9 v 9.4 μmol/l, $p = 0.016$), but not males (11.1 v 10.9 μmol/l, $p = 0.72$); nor were they linked to angina in either sex. Logistic regression analysis confirmed that a 1 μmol/l increase in homocysteine resulted in an odds ratio for ischaemic heart disease of 1.5

(95% CI, 1.03 – 55.2, p = 0.019) for both sexes and 1.3 (95% CI, 1.04 – 35.4; p = 0.033) for females. Only total cholesterol was significantly linked to ischaemic heart disease in males. These findings suggest that homocysteine may be a more important risk factor for ischaemic heart disease in females than males. Most previous studies have concentrated on men and the outcomes of prospective studies have been largely negative. These findings suggest that future prospective studies of homocysteine and IHD should include more women.

The mechanism by which homocysteine causes athero-thrombosis is unclear. One theory is that the thiol group is involved in free radical formation. The literature, however, is divided between those who find homocysteine to be a pro-oxidant and those who find homocysteine to be an antioxidant. I found that physiological concentrations of homocysteine added to freshly prepared lipoproteins markedly prolonged the lag phase for conjugated diene formation (and therefore acted as an antioxidant). Repetition of the experiment after ageing the lipoproteins overnight at +4°C showed that homocysteine acted as a pro-oxidant. We believe that oxidation of the thiol group keeps the antioxidant vitamin E reduced while the lipoproteins are fresh, but once the vitamin E is exhausted, homocysteine oxidation generates oxygen free radicals. This would also help to explain why the literature on the subject is equivocal.

Hyperhomocysteinaemia is readily treated by low doses of folate and vitamin B_{12}. I investigated the effects of folate supplementaion on lipoprotein oxidation in a group of volunteers from the top (Q4) and bottom (Q1) quartiles of the homocysteine reference interval. The subjects in Q4, were given 400

μg of folate daily for 4 weeks, whilst the others were not treated. Homocysteine levels fell by 23% in the treatment group as expected, and the lag phase for conjugated diene formation increased, significantly, by 33%, while the control group's homocysteine levels and lag phases did not change over the course of the investigation. This pilot study builds on the knowledge that folate reduces homocysteine levels to imply that this also has an effect on the oxidisation of low-density lipoproteins. A larger study is planned to explore the possibility of inexpensive B vitamin treatment for atherothrombotic disease.

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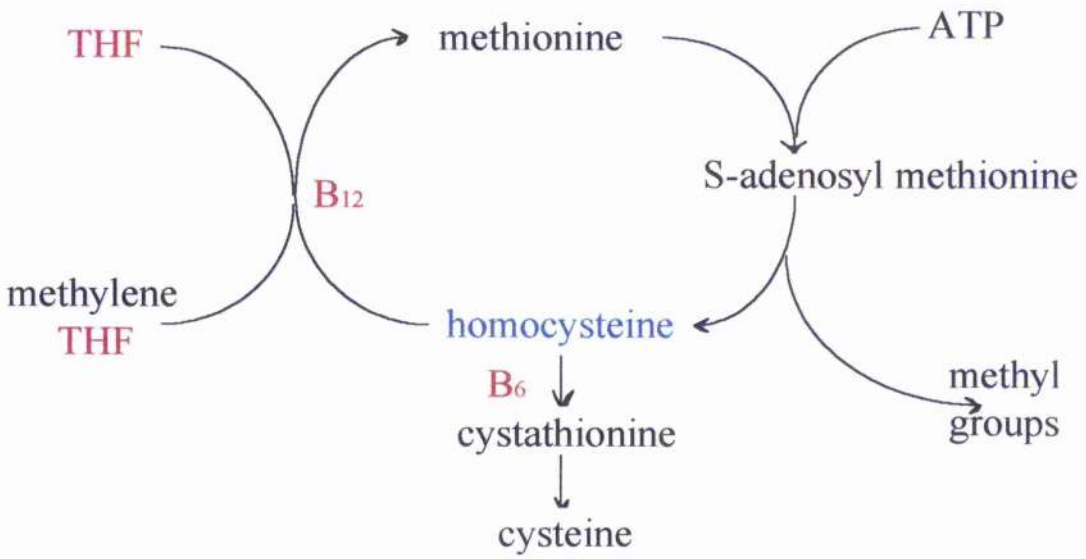
Introduction

Atherosclerosis, and its complication thrombosis (athero-thrombosis), is the largest cause of morbidity and mortality in the UK. It is a multifactorial disease with hundreds of identified risk factors. Despite this, only about half of athero-thrombotic disease can be explained by the well-established risk factors of smoking, age, gender, hyperlipidaemia, hypertension and diabetes mellitus. Hyperhomocysteinaemia has been proposed as an additional independent risk factor for athero-thrombotic disease[1], however, this has not yet been fully established. If a causative relationship between hyperhomocysteinaemia and vascular disease was proved, treatment with B group vitamins might provide a safe and inexpensive treatment.

Homocysteine (HCySH) is a thiol-containing amino acid and an intermediary metabolite in methionine metabolism (Figure 1). It arises from the demethylation of S-adenosyl methionine and either undergoes remethylation to methionine, a process dependent on folic acid (as methylene tetrahydrofolate) and vitamin B_{12}, or in the presence of excess methionine, enters the trans-sulphuration pathway. Here it is converted to cysteine, and may be further metabolised to CO_2, NH_4, H_2O and SO_4. The rate-limiting enzyme in this pathway, cystathionine β-synthase, is dependent on pyridoxine, vitamin B_6, as cofactor. Unlike methionine and cysteine, homocysteine is not a constituent of proteins and only a small amount enters the circulation, where the highly reactive thiol group forms disulphide bonds with other thiol-containing molecules, primarily albumin (~70%). Less than 1% of the total plasma homocysteine circulates in its free form[2].

Figure 1

The Methionine Cycle



Homocysteine is the de-methylation product of methionine. Methionine is activated by reaction with adenosine triphosphate to produce the high-energy intermediate, S-adenosyl methionine, which can donate methyl groups to nucleic acids and proteins. Homocysteine can either be metabolised to cysteine (Trans-sulphuration pathway) or remethylated to methionine (Remethylation pathway). Both pathways involve vitamins (shown in red) B_6 = pyridoxine; B_{12} = cobalamin; THF = tetrahydrofolate.

Homocysteine is eventually metabolised by the kidney[3]. Normal adult levels for plasma total HCySH are between 5 & 15 μM[2], increasing with age. Interest in HCySH as a risk factor for atherothrombotic disease has arisen because children born with homocystinuria, an inherited metabolic disease resulting in plasma HCySH levels being elevated above 100 μM, die in early adulthood from widespread arterial and venous thromboses[4]. It was proposed that homocysteine was the direct cause of this outcome[5], as there were no other biochemical abnormalities to account for the athero-thrombosis. Skeletal abnormalities, mental retardation and lens dislocation are also associated with classical homocystinuria. The defective enzyme is cystathionine β-synthase, which is the rate-limiting enzyme in the trans-sulphuration pathway. Treatment with the enzyme's cofactor, pyridoxine, ameliorates the condition in many patients, and is the primary treatment. Defects in the remethylation pathway have also occasionally been described, which result in similar syndromes to classical homocystinuria.

The situation is different in adults, where folate and/or vitamin B_{12} deficiencies are thought to underlie more moderate degrees of hyperhomocysteinaemia (15 – 100 μM). Studies show that supplements of 400 μg of folic acid daily can reduce fasting total HCySH levels by up to 30%, while vitamin B_{12} produces a smaller fall (< 10%), but pyridoxine has little effect[2]. This suggests that borderline folate deficiency may be common, and that the remethylation pathway is likely to be more important than cystathionine β-synthase defects in the pathogenesis of adult hyperhomocystinaemia[2].

There have been numerous cross-sectional studies linking hyperhomocysteinaemia with stroke[1,6], peripheral vascular disease (PVD)[1,7,8], deep venous thrombosis[9,10] and ischaemic heart disease (IHD)[1,11,12]. The results of prospective studies have been less conclusive, with most studies failing to detect any relationship between HCySH and athero-thrombosis[13-15].

Cross-sectional studies[1,6-8,11] have concentrated on the rare cases of premature vascular disease (onset before the age of 60) as heterozygotes for homocystinuria were believed to be at increased risk of athero-thrombotic disease since it was assumed that their plasma HCySH levels would be moderately elevated. This is a dubious proposition for two reasons. First, classical homocystinuria is due to a defect in the trans-sulphuration pathway, yet adult hyperhomocystinaemia seems to be associated with defects in the remethylation pathway. Moderate hyperhomocysteinaemia is associated with borderline B vitamin deficiency, which becomes more prevalent with age[16]. Second, there is no evidence that relatives of children with homocystinuria are at increased risk of developing athero-thrombotic disease[17].

Another problem with many published studies is that they report plasma homocysteine concentrations following methionine loading, either alone or in combination with fasting homocysteine levels[1,6,8,9]. Methionine loading tests are unstandardised, unphysiological and are also impractical for most large-scale studies. Classification of hyperhomocystinaemia from methionine loading usually differs from classification based on fasting homocysteine levels. This discrepancy in the definition of

hyperhomocystinaemia has hindered the investigation of the underlying pathophysiology.

There can be little doubt that HCySH does cause the athero-thrombotic disease in classical homocystinuria. If hyperhomocysteinaemia is a risk factor for athero-thrombosis in the general population, then it will be apparent in the general clinic population and it will almost certainly be due to a defect in the remethylation pathway, causing elevated fasting plasma homocysteine concentrations.

The measurement of HCySH has been technically difficult and although the link with athero-thrombotic disease was proposed by McCully over thirty years ago[5], it is only in the last decade that HPLC methods for the assay of HCySH have made clinical studies practicable. Previous techniques, such as amino acid analysis, have lacked the sensitivity to measure homocysteine concentrations in the normal population and researchers had to resort to methionine loading to stress the methionine cycle and ensure plasma homocysteine levels fell within the analytical range of their methodology. Recent studies have measured total plasma homocysteine after a 12 hour overnight fast, which allows standardisation on a reproducible, physiological measurement.

Most commonly available HPLC detection methods utilise fluorescent labelling of the thiol group of HCySH, but the drawbacks are high background fluorescence and the use of hazardous chemicals (fluorophores, sodium tetrahydroborate, mercaptoethanol). This has restricted the availability of the assay. I perceived a need for a new method utilising the HPLC equipment

and expertise readily available in NHS Clinical Biochemistry laboratories, which could be easily introduced into routine service.

Various theories have been proposed to account for the atherogenicity of homocysteine. Endothelial toxicity[18], procoagulant effect on coagulation factors[19], or free radical generation[20,21]. Most of the studies have been conducted at non-physiological homocysteine concentrations and the results are therefore unreliable. A particular interest of our Clinical Biochemistry department has been oxidative stress and free radical formation, I therefore chose to study HCySH and lipoprotein oxidation as a mechanism to explain the link between HCySH and atherosclerosis.

I set out to develop a novel HPLC assay for HCySH and use it to investigate the relationship between hyperhomocysteinaemia and athero-thrombotic disease in older, more typical, hospital clinic populations. I also investigated whether HCySH was linked to microvascular disease, as all previous studies have concentrated on macrovascular disease, and experimented with the effects of HCySH on lipoprotein oxidation to gain an understanding of the potential effects of hyperhomocystinaemia.

Materials and Methods

Subjects

All of the patient studies in this thesis had been approved by the relevant hospital ethics committee and all patients gave prior informed consent. Details of any power calculations and the specific inclusion or exclusion criteria are given in connection with each study.

Analyses

Homocysteine was analysed by reversed-phase, ion-pair HPLC with electrochemical detection as described in detail below. Other analytes were measured by routine laboratory methods using commercially available reagents, as described in the relevant section. Quality control was ensured by internal and, when available, external QC schemes.

Lipoprotein ultracentrifugation was performed on EDTA plasma. A fraction containing LDL was isolated by density gradient ultracentrifugation using a Centrikon T1065 centrifuge (Kontron Instruments, Watford, UK) with a swing out rotor (TST 55.5) and 5 ml polyallomer tubes. Plasma (3 ml) was mixed with 1 ml 1.182 kg/l density solution (2.4 mol/l NaBr in 1.006 kg/l density solution) and overlaid with 0.5 ml 1.006 kg/l density solution (0.193 mol/l NaCl, 1 mmol/l NaOH and 0.25 mmol/l EDTA) in the centrifuge tubes. These were centrifuged at 40,000 rpm for 18 hours at 15°C. The orange band containing LDL was aspirated in a volume of less than 0.5 ml. Samples were run in duplicate and the LDL fractions combined. The lipoproteins were further purified from EDTA, water soluble antioxidants e.g. urate and other low molecular weight contaminants by gel filtration chromatography using

PD-10 columns (Pharmacia Ltd, Milton Keynes, UK). Fractions of 0.5 ml were collected following elution with phosphate buffered saline (PBS), pH 7.4. LDL containing fractions (identified by orange / brown coloration) were pooled.

LDL Oxidation

The copper (II) [Cu (II)] catalysed oxidation of LDL was monitored by measuring the change in absorbance at 234 nm in a Philips Unicam automatic eight position changer spectrophotometer. Absorbance readings were taken every 3 minutes over a three hour period. The output from the spectrophotometer was entered into an Excel spreadsheet. LDL (final concentration 50 mg/l) was added to a quartz cuvette and incubated in PBS at 37°C in the presence of 10 μmol/l copper sulphate. A negative control without added copper ions or thiol was included in every experiment.

Statistics

Homocysteine and lipoprotein (a) levels were log-transformed before statistical analysis to normalise the data. Univariate analyses used the Microsoft Excel and Astute software packages. Multivariate analysis was by SPSS for Windows. Lag times for lipoprotein oxidation were compared by Mann Whitney U test.

RESULTS

Method development

HPLC assay

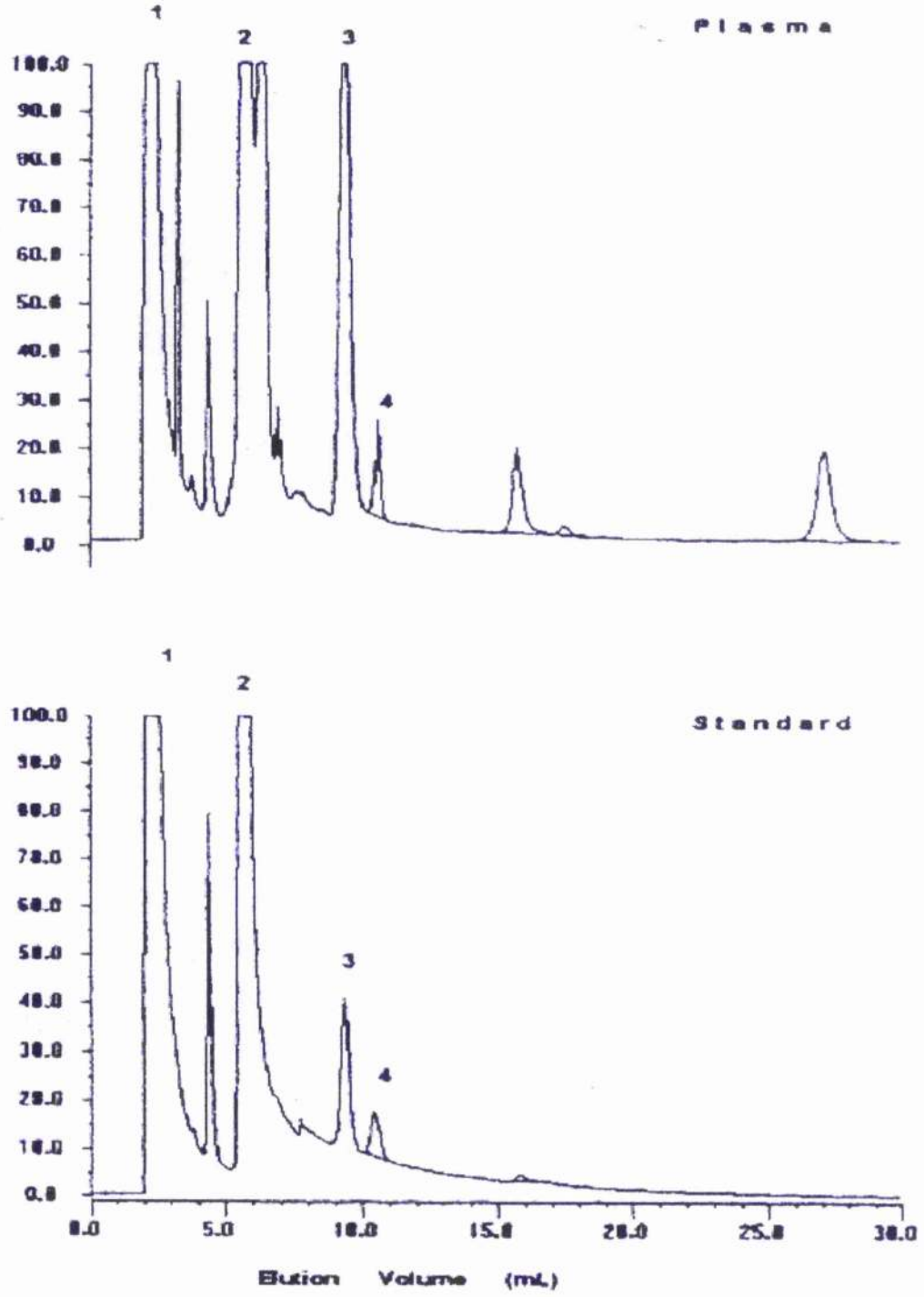
Dithiothreitol was chosen as the reducing agent (to liberate HCySH from albumin and the other disulphides) since it is non-hazardous. The final concentration chosen (10 mmol/l) was half that used by Andersson[22], because higher concentrations lead to gel formation. The procedure was to mix 100 µl of 50 mmol/l DTT with 400 µl of EDTA plasma and incubate at 37°C in a water bath for 15 minutes. One hundred microlitres of 150 g/l sulphosalicylic acid was then added to precipitate the protein, before centrifugation in a benchtop microfuge. Twenty microlitres of supernatant were injected onto a 250 x 4.6 mm octadecyl silica column (Spherisorb ODS-2, particle size 5 µm (Jones Chromatography Ltd., Hengoed, UK), with a 10 x 4.6 mm guard column packed with the same material. The mobile phase (flow rate 1 ml/min) consisted of 10 mM sodium dihydrogen phosphate adjusted to pH 2.75 with orthophosphoric acid, 15% (v/v) methanol and 12 mmol/l octane sulphonic acid (Sigma Chemical Co., Poole, UK). The mobile phase was not recycled to prevent the accumulation of DTT, which otherwise caused a rapid deterioration in the chromatographic separation.

The detector was an ESA Coulochem 5100A (ESA Analytical Ltd., Cambridge, UK) and was used with an ESA 5010 coulometric cell. Electrode 1 (E1) was set at a screening potential of +0.4 V and electrode 2 (E2) set at + 1.0 V, based on Harvey[23]. An ESA 5020 guard cell (+ 1.1 V) was installed before the autosampler (model ISS 200, Perkin-Elmer, Norwalk, CT, USA). Gilson Unipoint software (Anachem Ltd., Luton, UK) was used to integrate

the signal (peak area) measured at E2. HCySH standards (2.5 – 40 μmol/l) were prepared from L-homocystine (Sigma Chemical Co., Poole, UK). Internal quality control was achieved using HCySH-spiked, time-expired, fresh, frozen plasma (West Midlands Blood Transfusion Service) at 7 and 20 μmol/l.

Each component of the mobile phase was independently assessed to achieve optimal separation of HCySH from the other components. Sulphosalicylic acid was not retained on the column. DTT gave rise to an early reduced, and a later oxidised peak. Phosphate concentrations greater than 10 mmol/l decreased the retention times of all peaks without changing the order in which they eluted. Increasing the concentration of the ion-pairing reagent, octanesulphonic acid (OSA), did have an effect on retention times, more so on HCySH than on the DTT peaks. At 6 mmol/l OSA, the eluting order changed as HCySH was retained on the column longer than the oxidised DTT peak. Further increases in the OSA concentration up to 12 mmol/l improved the separation between HCySH and oxidised DTT. The optimal mobile phase pH was found to be 2.75, and allowed HCySH to be detected down to 2.5 μmol/l. Increasing the methanol concentration reduced the retention times of all the peaks without changing their eluting order. 15% (v/v) methanol was chosen for optimal separation within a reasonable run-time. Typical chromatograms are shown in Figure 2. The purity of the HCySH peak was established

Figure 2



Chromatograms of plasma and a 20 μmol/l aqueous standard. Key: 1, sulphosalicylic acid; 2, reduced dithiothreitol; 3, oxidised dithiothreitol; 4, homocysteine

electrochemically by plotting the peak areas of the presumed HCySH peak (plasma samples) against those of a pure standard at various E2 applied voltages. The correlation coefficient (r^2) of the resulting regression line was 0.995. This shows that the presumed HCySH peak had similar electrochemical characteristics to the pure standard and provides some evidence of specificity. Recovery experiments from spiked plasma showed HCySH recoveries of 97 –100%.

Imprecision data gave intra-assay CV's of 3.9% at 20 μmol/l (n = 20) and 2.3% at 50 μmol/l (n = 20). Inter-assay CV's were 10.7% at 7 μmol/l and 11.0 % at 20 μmol/l (both n = 19)[24].

The assay was subsequently improved by the incorporation of an internal standard[25]. Penicillamine was chosen because it does not occur naturally in the body and has a similar structure to homocysteine. Refinements were also made to the mobile phase with the substitution of diammonium hydrogen phosphate for sodium phosphate. It was found that the ammonium ions helped to block residual uncapped silanol groups on the column packing, resulting in improved peak symmetry. As before, starting with 400 μl of plasma, 100 μl of 50 mmol/l DTT was added to reduce the disulphide bonds and liberate the homocysteine, but it also contained 200 μmol/l penicillamine, as the internal standard. The mobile phase consisted of 10 mmol/l diammonium phosphate, adjusted to pH 2.58 with orthophosphoric acid, 13.75% (v/v) methanol and 12 mmol/l OSA. The method was otherwise unchanged as were the detector settings Each component of the mobile phase was once again individually adjusted to ensure optimal separation of HCySH from the other components, (Table 1). Under these conditions the

limit of detection for HCySH was reduced to less than 0.6 μmol/l. Imprecision data gave improved intra-assay CV's of 2.2% at 7 μmol/l and 2.4% at 20 μmol/l (both n = 20). Inter-assay CV's were 8.6% at 8.5 μmol/l and 6.8% at 25 μmol/l (both n = 8). Typical chromatograms are shown in Figure 3.

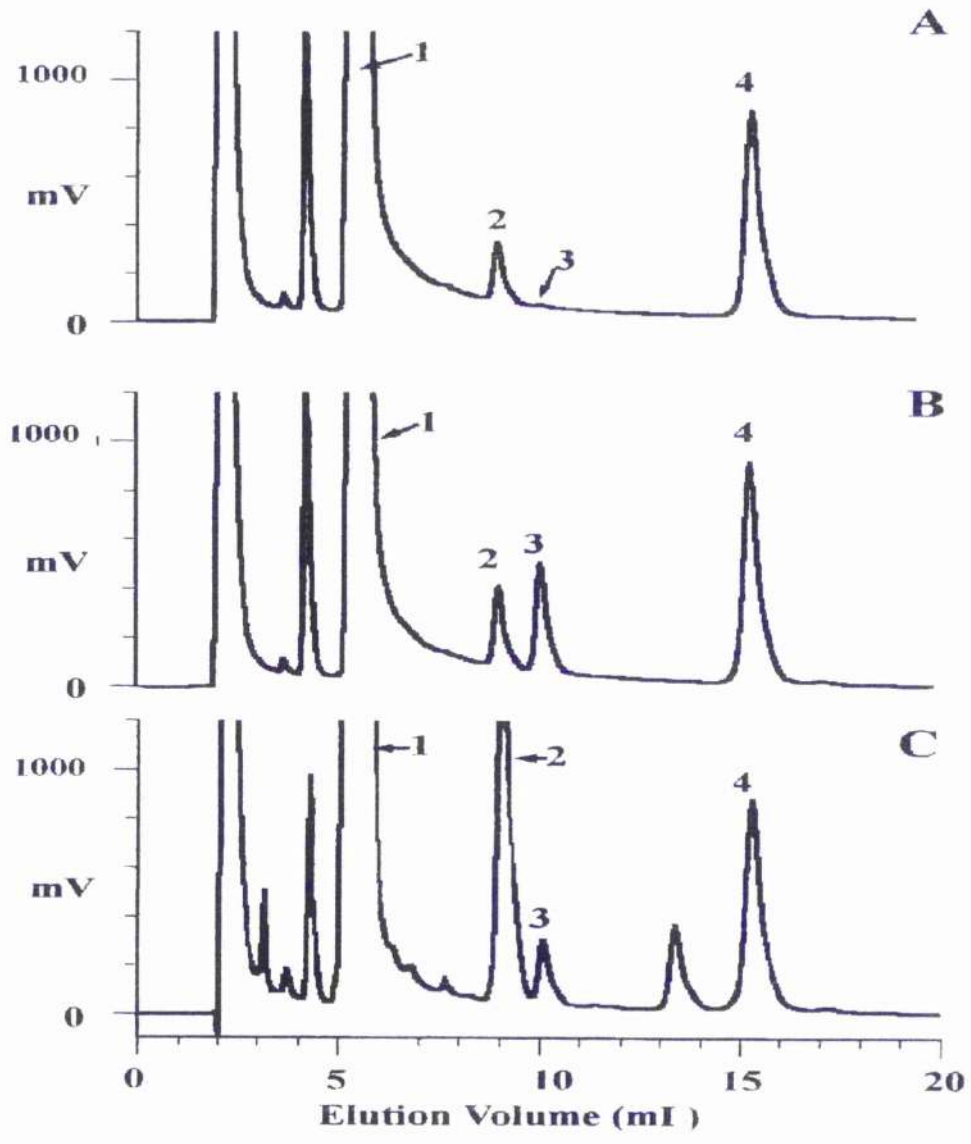
Table 1

The effect of varying pH on peak retention times (min).

pH	DTT Reduced	DTT oxidised	HCySH	Penicillamine
3.25	5.7	9.5	---[a]	6.8
3.00	5.6	9.3	6.2	8.5
2.85	5.7	9.4	7.4	10.5
2.75	5.7	9.6	8.6	12.6
2.65	5.7	9.5	---[b]	14.5
2.58	7.3	12.6	16.3	29.0
2.50	6.6	11.2	15.6	26.9

[a] the HCySH peak co-eluted with reduced DTT
[b] the HCySH peak co-eluted with oxidised DTT

Figure 3



Typical chromatograms achieved with a mobile phase consisting of 10 mmol/l diammonium phosphate (pH 2.58) containing 137.5 ml/l methanol and 12 mmol/l octane sulphonic acid. The flow rate was 1 ml/min. **A** 1.25 μmol/l calibrator; **B** 40 μmol/l calibrator; **C** plasma containing 20 μmol/l HCySH. Peak 1, reduced DTT; peak 2, oxidised DTT; peak 3, HCySH; peak 4, penicillamine (internal standard).

Patient Studies

1 *Reference Interval*

The modified method with internal standard was adopted for the clinical studies and volunteers were recruited from the laboratory staff and from patients attending Heartlands hospital without a history of athero-thrombotic disease to construct a HCySH reference interval. The mean HCySH levels for the 38 men and 47 women did not differ significantly (10.3 ± 1.5 v 9.0 ± 1.4 µmol/l, p = 0.10) and are similar to published ranges from around the world. However the HCySH levels did

Table 2

Age-related reference interval for plasma total homocysteine

Age (yr)	mean [sd] (µmol/l)	n
20-29	7.87 [1.35]	11
30-39	8.14 [1.26]	10
40-49	8.11 [1.35]	11
50-59	9.93 [1.50]	23
60-69	10.56 [1.46]	22
70-79	11.85 [1.55]	7

One-way analysis of variance shows that the difference between the group means is significant (p = 0.046). The single subject aged > 80 years was excluded.

increase significantly with age, Table 2 and therefore an age-related reference interval was constructed. The rate of increase with age was similar to that published for a German cohort[26].

2 *Homocysteine and microvascular disease*

The reference interval was also used in a study to investigate whether homocysteine levels were elevated in microvascular disease, with arterial or venous retinal vascular occlusive disease (RVOD) being chosen as the paradigm. These conditions, although rare, present as sudden-onset blindness (with or without pain) and consequently make an ideal study group. RVOD has previously been associated with diabetes, hypertension and hyperlipidaemia[27] and there have also been case reports that homocysteine may be associated with RVOD[28,29]. Seventy patients presenting with RVOD were recruited from the General and Medical Ophthalmology clinics at the Heartlands and Priory Hospitals in Birmingham. All subjects underwent complete ophthalmological and medical examinations. The diagnosis was confirmed by ophthalmoscopy after pupillary dilation and 36 patients were diagnosed as having central retinal vein occlusions (CRVO), 24 had a diagnosis of branch retinal vein occlusion (BRVO) and the remaining 10 had retinal artery occlusions (RAO).

All of the groups had equal male/female ratios and there were no significant differences between the RVOD subgroups as regards mean age, serum total cholesterol, triglycerides, glucose, cigarettes smoked or ethanol intake. The control group were, however, significantly younger than the RVO group [51.5 (15.4) yr v 65.6 (12.5) yr, $p<0.0001$] or the RAO group [51.5

(15.4) yr v 69.8 (6.7) yr, p<0.0001]. Univariate analysis revealed that homocysteine levels were significantly raised in both arterial and venous occlusions compared to controls (Table 3). This relationship remained after grouping the patients and controls into decades of age.

Table 3

Mean values and standard deviations for age, plasma total homocysteine and serum creatinine in patients with retinal vascular disease and controls.

	Age (years)	HCySH(μmol/l)	creatinine(μmol/l)
controls	51.5 +/- 15.4	9.5 +/- 1.5	105.8 +/- 18.9
RAO	69.8 +/- 6.74*	18.4 +/- 1.5**	115.1 +/- 23.7
RVO	65.6 +/- 12.5*	13.8 +/- 1.5*	97.7 +/- 31.1

* p<0.0001 v control
** p=0.0002 v control

Multiple linear regression analysis revealed that the presence of retinal vessel occlusion (p= 0.0002), serum creatinine (p= 0.001) and age (p= 0.003) were all significantly linked to HCySH levels, while gender was not, Table 4.

Subgroup analysis of the RVOD group revealed that those patients with CRVO had significantly higher HCySH levels than BRVO (not shown) as had patients with RAO. There were no differences between the groups as

regards age, total cholesterol, triglycerides, cigarette smoking or ethanol consumption. However, both the mean systolic and diastolic blood pressures

Table 4

Multiple linear regression analysis with log homocysteine as the dependent variable

variable	B	95% confidence intervals for B	p value
RVOD	0.119878	0.057825 - 0.181930	0.0002
Creatinine	0.003243	0.001348 - 0.005137	0.001
Age	0.004605	0.001595 - 0.007614	0.003
Gender	0.003449	-0.062867 - 0.069766	0.918

B represents the slope of the regression line.
The multiple R value was 0.607.

were significantly elevated in the BRVO group compared to either the CRVO or RAO groups as has previously been reported[27].

3 *Homocysteine and peripheral vascular disease (PVD).*

An association between PVD and hyperhomocysteinaemia has been established in middle-aged men[30]. Such patients are not generally representative of those with vascular disease, who are typically elderly and

hence may be B vitamin deficient[16,25,30]. There have been no studies performed on homocysteine in such vascular patients.

There was a reported association between hyperhomocysteinaemia and neointimal hyperplasia (NIH) in 19 patients with infrainguinal lower limb vascular bypass grafts[31]. NIH is a form of vein graft stenosis, which may develop postoperatively in about 30% of grafts and is due to excessive vascular smooth muscle cell proliferation occurring for unknown reasons. The endothelium remains intact, but the vessel lumen is narrowed. This study investigated HCySH levels in unselected vascular clinic patients with PVD or NIH.

PVD was confirmed by clinical examination and an ankle-brachial blood pressure index (ABPI) < 0.8. The disease spectrum ranged from mild claudication to end-stage critical ischaemia. The presence of NIH in infrainguinal vein grafts that had developed critical vein graft stenosis was defined by a peak systolic velocity ratio greater than 2.5 after one post-operative year. All vein grafts had been part of a surveillance programme using colour-coded duplex imaging[32].

Clinical and demographic data were obtained. Investigations included chest X-ray and electrocardiograph. Fasting biochemistry and haematological indices were measured by standard laboratory methodology.

One hundred subjects were recruited: 48 with PVD (male:female 32:16), 22 with NIH (14:8) and 30 controls (15:15).

The PVD group had varying degrees of ischaemia with resting ABPI ranging from 0.21 to 0.7. Twelve patients had critical ischaemia and four had had amputations. Three patients also had symptomatic PVD manifest by

non-disabling claudication and two had asymptomatic disease detected by ABPI < 0.8. All patients in the NIH group had infrainguinal vein graft bypasses with stenoses producing a peak systolic velocity ratio > 2.5 within one postoperative year and all subsequently had angioplasty.

There were no significant differences between the groups in haemoglobin, white cell count, platelets, haematocrit, fibrinogen, vitamin B_{12} levels, electrolytes, albumin, bilirubin, transaminases, alkaline phosphatase, fasting glucose or triglycerides.

There were significant differences between the groups in age, body mass index, systolic blood pressure, smoking habit, serum creatinine, total cholesterol, HCySH and folate levels, (Table 5). The NIH group was significantly older than the controls. Both patient groups had a higher percentage of smokers than the control group, even though the percentage of smokers in the control group was itself unusually high at 55%. The PVD group had significantly higher systolic blood pressures than the controls

Renal function, as indicated by serum creatinine levels, was worse in the patient groups compared to controls, and serum total cholesterol levels were significantly higher in the NIH group.

HCySH levels in the control group were comparable to the age-matched laboratory reference interval (data not shown). The mean HCySH level in the PVD group was significantly higher than the level in the control group, while the HCySH levels for the NIH group did not differ significantly from the controls (Table 6). Serum folate levels were significantly lower in the PVD and higher in the NIH groups than in the controls.

Logistic regression analysis confirmed these findings, with peripheral

vascular disease being related to HCySH levels (table 6) such that the odds

Table 5

Measured parameters, mean (sd), which differ from controls

	Controls	PVD	NIH
Age (years)	65.2 (11.1)	64.6 (10.3)	72.6 (9.5)*
BMI	27.1 (7.0)	25.1 (5.1)	27.5 (6.7)
Smokers (%)	55	88***	85*
SBP(mmHg)	141.9 (22.8)	152.0 (17.3)*	143.0 (19.8)
Cre (μmol/l)	87 (18)	112 (57)*	133 (48)****
TChol (mmol/l)	4.91 (1.15)	5.52 (1.19)	6.10 (1.04)*
HCySH (μmol/l)	12.3 (1.4)	17.4 (1.7)**	10.6 (1.5)
Folate (ng/l)	5.14 (1.90)	4.26 (1.55)*	7.55 (3.81)*

*p<0.05 **p<0.01 ***p<0.001 ****p<0.0001

ratio for PVD increased fourfold for each 1 μmol/l increase in HCySH (95% CI 1.49 – 11.07, p = 0.0061). Fasting glucose levels were also related to PVD, with an odds ratio of 1.51 per mmol/l (95% CI 1.06 – 2.15, p = 0.0233). Neointimal hyperplasia was found to be related to male sex, haemoglobin concentration and serum folate levels, but only weakly and negatively to HCySH.

4 *Homocysteine and IHD*

IHD is known to be multifactorial in origin and HCySH has been purported to be an independent risk factor for IHD which is quantitatively as important as smoking[1]. A variety of other additional risk factors for IHD have been

Table 6

A *Logistic regression analysis with peripheral vascular disease as the dependent variable*

Variable	B	SE B	OR (95% CI)	p
log HCySH	14.03	5.11	4.07 (1.49-11.07)*	0.0061
Glucose	0.41	0.18	1.51 (1.06-2.15)	0.0233
Haemoglobin	-0.18	0.10	0.83 (0.69-1.01)	0.0578

B *Logistic regression analysis with neointimal hyperplasia as the dependent variable*

Variable	B	SE B	OR (95% CI)	p
Male sex	2.16	0.93	8.70 (1.40-54.2)	0.0204
Haemoglobin	0.76	0.35	2.15 (1.09-4.23)	0.0272
Serum folate	0.86	0.41	2.37 (1.05-5.32)	0.0367
log HCySH	-7.91	4.24	0.45 (0.20-1.04)*	0.0624

* odds ratio per μmol/l HCySH

B denotes the estimated coefficient used in the analysis

proposed including lipoprotein (a)[33] and angiotensin converting enzyme (ACE) genotype[34]. None of these have yet been proven to be causative and most studies have examined each factor in isolation and compared disease-free control groups to groups with IHD. This study involved 280 patients who underwent coronary angiography at Birmingham Heartlands Hospital Chest Pain Clinic. The largest group (110) were those undergoing further investigation after myocardial infarction. Eighty patients proved to have no angiographic evidence of atheroma and were designated as controls because of their good prognosis[35]. Power calculations showed that for triglycerides and lipoprotein (a), the assays with the poorest precision, 50 subjects were required for 95% significance at 90% power to demonstrate a difference from the controls. All patients had fasting blood samples taken for the measurement of serum creatinine, total cholesterol, triglyceride, glucose, lipoprotein (a), ACE activity, ACE genotype and total plasma homocysteine. Diabetes mellitus was defined using the new World Health Organisation / American Diabetic Association criteria as a fasting glucose ≥ 7.0 mmol/l. Hypertension was defined as a blood pressure greater than 160/90 mmHg. Lipoprotein (a) and ACE activity were measured on a Monarch analyser (IL, Warrington, UK) using Randox (Ardmore, UK) and Sigma (Poole, UK) reagent kits, respectively. ACE genotyping was kindly perfomed by Dr. F. MacDonald of the Women's Hospital, Birmingham according to the method of Cambien[34]. Table 7 shows the demographic and biochemical data for the study population. The control and IHD groups had similar ages, percentages of smokers and hypertensives. The proportion of those with diabetes in the IHD

Table 7

Variables measured in the study population

	IHD –ve (controls)	IHD +ve (angio +ve)	p value	Odds ratio (95% CI)
Age, years (sd)	58.5 (11.0)	58.4 (9.7)	0.93	
Males/females	37/43	152/48	0.002	
BP > 160/90 (%)	40.8	37.7	0.74	
Smokers (%)	15.8	19	0.73	
Diabetes (%)	8.8	16.6	0.2	
Glu, mmol/l	5.4 (1.4)	5.8 (1.9)	0.09	
Creat, μmol/l	103 (20)	114 (38)	0.004	1.99 (1.24–2.74)
M chol, mmol/l)	5.6 (1.2)	6.0 (1.0)	0.034	2.01 (1.17–2.85)
F chol, mmol/l)	6.4 (1.1)	7.3 (1.4)	0.001	3.79 (2.51–5.07)
Trigs, mmol/l)	2.1 (1.1)	2.6 (1.6)	0.002	1.71 (0–3.46)
HCySH, μmol/l)	10.1 (1.5)	11.5 (1.4)	0.008	1.50 (1.06–1.93)
Lp (a), mg/dl)	14.7 (3.5)	17.0 (3.0)	0.39	1.08 (0.57–1.58)
ACE act, U/l	38.9 (16.6)	38.3 (19.6)	0.8	
ACE, II (%)	24.0	25.0	0.99	
ACE, DD (%)	20.0	27.4	0.28	

Data is presented as mean (sd)
Angio +ve = coronary artery stenosis. BP = blood pressure. DM = diabetes mellitus. Glu = fasting glucose. Creat = serum creatinine. M chol (F chol) = male (female) total cholesterol levels. Trigs = fasting triglyceride levels. HCySH = plasma total homocysteine. Lp (a) = lipoprotein (a). ACE act = angiotensin converting enzyme activity. ACE, II (DD) = ACE genotypes.

Table 8

The association between some proven and putative risk factors and acute myocardial infarction (AMI).

A Males

	Controls (n = 37)	AMI (n = 88)	p value
Cholesterol, mmol/l	5.6 (1.2)	6.0 (0.9)	0.044
Triglycerides, mmol/l	2.3 (1.4)	2.7 (1.4)	0.17
HCySH, μmol/l	10.9 (1.6)	11.1 (1.3)	0.72

B Females

	Controls (n = 43)	AMI (n = 22)	p value
Cholesterol, mmol/l	6.4 (1.1)	7.0 (1.7)	0.10
Triglycerides, mmol/l	1.9 (0.7)	3.0 (2.5)	0.07
HCySH, μmol/l	9.4 (1.4)	11.9 (1.4)	0.016

Data is presented as mean (sd)

group, although almost double that in the control group, was not statistically significantly different and the mean serum glucose values were almost identical in the two groups. As expected, there was a male preponderance in the IHD group with significantly fewer females having proven IHD. Serum total cholesterol, creatinine, triglyceride and plasma HCySH levels were

Table 9

The association between some proven and putative risk factors and stable angina

A Males

	Controls (n = 37)	Angina (n = 57)	p value
Cholesterol, mmol/l	5.6 (1.2)	6.1 (1.0)	0.017
Triglycerides, mmol/l	2.3 (1.4)	2.2 (1.2)	0.66
HCySH, μmol/l	10.9 (1.6)	12.1 (1.5)	0.26

B Females

	Controls (n = 43)	Angina (n = 24)	p value
Cholesterol, mmol/l	6.4 (1.1)	7.5 (1.1)	<0.001
Triglycerides, mmol/l	1.9 (0.7)	3.2 (1.6)	0.001
HCySH, μmol/l	9.4 (1.4)	11.2 (1.4)	0.07

Data is presented as mean (sd)

significantly elevated in the IHD group, but lipoprotein (a), ACE levels and ACE genotype were not significantly different between the two groups. Apart from serum cholesterol, there were no sex differences in the levels of the measured variables.

Further analysis focussed on HCySH and the established risk factors cholesterol and triglycerides. Table 8 details the associations between these risk factors and acute myocardial infarction in males and females. Total cholesterol, as expected, was significantly related to acute myocardial

infarction in men, but not women. Only HCySH was linked to myocardial infarction in women. In contrast, table 9 shows that cholesterol was associated with stable angina in both sexes, serum triglycerides were linked to angina in females only and HCySH was not associated with angina in either sex.

Table 10

The association between some proven and putative risk factors and coronary artery occlusions

A Males

	Controls (n = 37)	Occ -ve (n = 77)	Occ +ve (n = 68)	p value
Cholesterol, mmol/l	5.6 (1.2)	6.1 (0.9)*	6.0 (1.0)	0.026
Triglycerides, mmol/l	2.3 (1.4)	2.4 (1.3)	2.5 (1.4)	---
HCySH, µmol/l	10.9 (1.6)	11.4 (1.4)	11.8 (1.4)	---

B Females

	Controls (n = 43)	Occ -ve (n = 27)	Occ +ve (n = 19)	p value
Cholesterol, mmol/l	6.4 (1.1)	7.3 (1.1)*	6.8 (1.3)	0.001
Triglycerides, mmol/l	1.9 (0.7)	3.5 (2.3)*	2.2 (0.8)	0.002
HCySH, µmol/l	9.4 (1.4)	10.6 (1.4)	12.2 (1.5)*	0.019

Data is presented as mean (sd)
Occ +ve (-ve) = coronary artery occlusion present (or absent).
* denotes significantly different from controls.

When the angiographic findings were included in the analysis, cholesterol levels were linked to coronary artery occlusions (table 10) in both sexes, but triglycerides and HCySH were related to occlusions in female patients only.

Table 11

The association between risk factors and the number of coronary arteries involved

A Males

	Controls (n = 37)	1 vessel (n = 41)	2 or more (n = 108)	p value
Cholesterol, mmol/l	5.6 (1.2)	6.0 (0.9)	6.0 (1.0)*	0.033
Triglycerides, mmol/l	2.3 (1.4)	2.4 (1.3)	2.4 (1.3)	---
HCySH, µmol/l	10.9 (1.6)	11.1 (1.4)	11.6 (1.4)	---

B Females

	Controls (n = 43)	1 vessel (n = 21)	2 or more (n = 27)	p value
Cholesterol, mmol/l	6.4 (1.1)	6.9 (1.0)	6.8 (1.3)*	0.002
Triglycerides, mmol/l	1.9 (0.7)	3.0 (2.2)	3.2 (2.0)*	0.003
HCySH, µmol/l	9.4 (1.4)	10.6 (1.4)	12.3 (1.4)*	0.003

Data is presented as mean (sd)
* denotes significantly different from controls.

A similar picture was seen for the number of atheromatous coronary arteries, table 11, where cholesterol was significantly elevated in patients of either sex with 2 or more atheromatous coronary arteries. Plasma HCySH again was elevated only in females and only significantly raised in those with 2 or more diseased vessels. Serum triglycerides were also higher, but only in females, and were significantly elevated regardless of the number of vessels involved.

Table 12

The association between risk factors and the number of coronary arteries involved

	B	SE	OR	95% CI	sig
A					
Sex (F)	-5.068	0.179	0.006	0.004-0.009	<0.001
Cholesterol	0.523	0.157	1.69	1.24-2.30	<0.001
Log HCySH	2.526	1.078	1.52	1.03-55.2	0.019
B					
Cholesterol	0.440	0.210	1.55	1.03-2.34	0.036
C					
Cholesterol	0.762	0.254	2.14	1.30-3.53	0.003
Age	-0.071	0.030	0.93	0.88-0.99	0.018
Log HCySH	4.178	1.959	1.29	1.04-35.4	0.033

OR = odds ratio. 95% CI = 95% confidence intervals. Sig = significance. Log HCySH = logarithm of plasma total homocysteine concentration. B denotes the estimated coefficient used in the analysis

Multivariate analysis (logistic regression) of all measured variables was performed with IHD as the dependent variable and the results are shown in table 12. Table 12A demonstrates that IHD is related to male sex, cholesterol and homocysteine levels. A 1 μmol/l increase in HCySH concentration results in a 1.52-fold (95% CI 1.03 to 55.2, p= 0.019) increase in the odds ratio for IHD. Table 12B confirms that only cholesterol levels were significantly linked to IHD in males, whereas HCySH contributes to IHD in females (table 12C), where a 1 μmol/l increase in HCySH increases the odds ratio for IHD by a factor of 1.29 (CI 1.04 –35.4, p= 0.033).

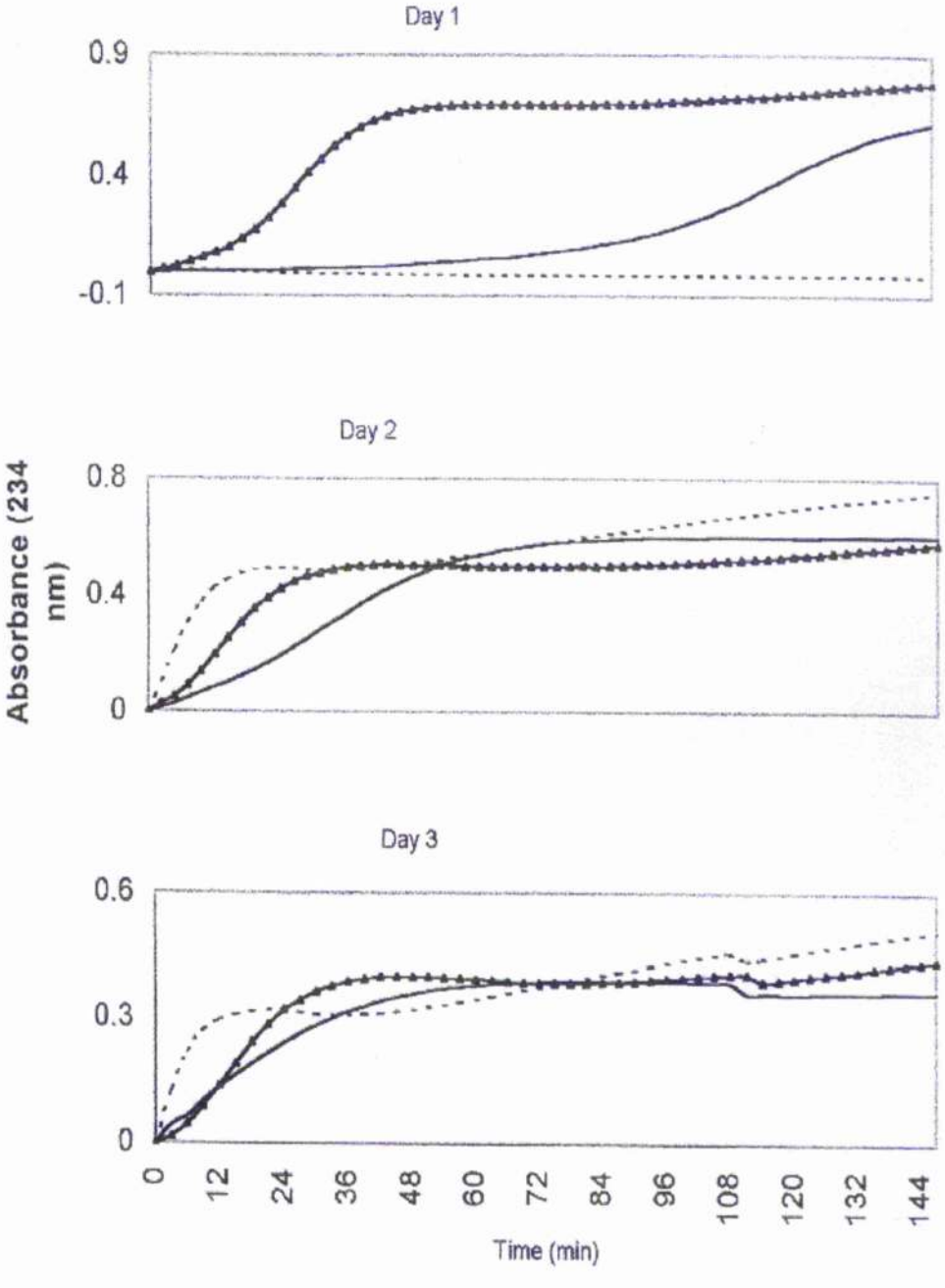
5 *Homocysteine and lipoprotein oxidation*

Native low density lipoprotein (LDL) is relatively non-atherogenic but oxidative modification renders it atherogenic[38]. Auto-oxidation of thiols generates oxygen free radicals that may result in oxidative modification of LDL. Cellular LDL oxidation *in vitro* has been found to require thiols [37,38]. It is thought that thiols may promote LDL oxidation by generating reactive oxygen species. Studies have shown that superoxide ($O_2^{\cdot -}$)[39], hydrogen peroxide (H_2O_2)[20,21,40] and hydroxyl radical ($OH^{\cdot}$)[41,42] can be produced by the oxidation of thiols and that reduced HCySH is rapidly oxidised in plasma to give homocystine and the mixed disulphide (cysteine-homocysteine)[43]. Heinecke *et al.*[44] observed that superoxide dismutase (SOD) prevented a copper (II) and cysteine dependent increase in macrophage degradation of ^{125}I labelled LDL, implicating $O_2^{\cdot -}$ in lipid peroxidation. However, not all workers have found that thiols promote the transition metal ion (TMI)

catalysed oxidative modification of LDL[45,46]. LDL is usually considered to be the main atherogenic lipoprotein found in plasma. Agarose gel electrophoresis of the lipoprotein fraction isolated by the ultra-centrifugation technique employed in the present study showed that, although it contained predominantly LDL, there was also some contamination with VLDL. It has recently been reported that VLDL is also susceptible to copper (II) catalysed oxidation, and as with LDL, the reaction produces conjugated dienes with lag phases approximately double those observed with LDL[47]. It has been proposed by one group that VLDL might also be involved in the development of atherosclerotic plaques, possibly depositing larger quantities of lipid into the developing foam cells than would LDL[48]. The presence of VLDL in the preparation therefore, would not appear to prejudice the significance of the findings.

We observed that with freshly isolated LDL, both 20 μmol/l HCySH and the same concentration of cysteine (Cys) acted as anti-oxidants by significantly increasing the lag times of LDL oxidation (calculated as in McDowell *et al*[49]) compared to incubations without added thiol (>180 min versus 17.5 min (8-60), [median (range)], n=10; $p<0.0001$ for HCySH and 88.5 min (66-123) versus 9 min (4-19), n=6; $p<0.001$ for Cys). With LDL aged by storage at 4°C, 20 μmol/l HCySH acted as a pro-oxidant, shortening lag times (5 min (4 -17) v 7 min (6-23), median (range), n=10; $p<0.05$), whereas 20 μmol/l Cys had no significant effect (6 min (5-17) v 6 min (4-7), n=6; NS). Examples of the effect of HCySH and Cys on the kinetics for LDL oxidation are given in Fig. 4.

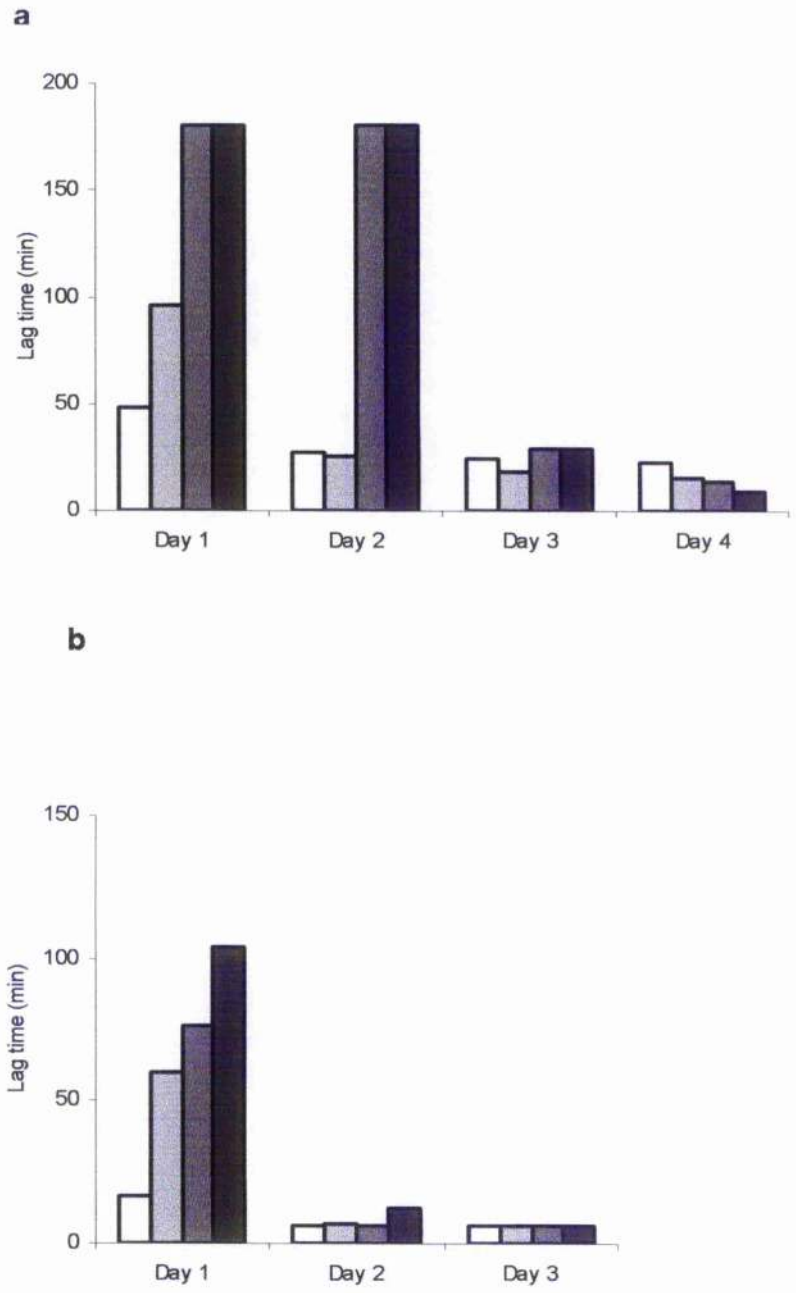
Figure 4



Copper (II) catalysed LDL oxidation (conjugated diene formation) on fresh and aged LDL. 20 μmol/l homocysteine, dotted lines; 20 μmol/l cysteine, solid lines; control, solid lines with filled triangles.

The effects of HCySH and Cys on LDL oxidation were found to be concentration dependent. The concentration range for HCySH and Cys used in this study was chosen to include the plasma reference range for HCySH and to extend up to that found in moderate hyperhomocysteinaemia. On day 1 the lag time of LDL oxidation was the shortest in the incubation without HCySH (Fig. 5a). Inclusion of 2.5 μmol/l HCySH increased the lag time, whilst 10 and 20 μmol/l HCySH prevented oxidation over the three hour study period. By day 2, the incubations with the lowest concentration of HCySH had a similar lag time to the incubation without HCySH. On day 3 oxidation occurred for the first time in incubations containing 10 μmol/l and 20 μmol/l HCySH, but with a longer lag time than in the absence of HCySH. By day 4 all incubations containing HCySH had a shorter lag time than in the absence of HCySH, with the shortest lag time being seen with the highest concentration of HCySH. In the case of Cys the lag time of LDL oxidation was shortest in the incubation without Cys on day 1 (Fig. 5b). Inclusion of 2.5 μmol/l, 10 μmol/l and 20 μmol/l Cys increased the lag time in a concentration-dependent fashion, however, unlike HCySH, oxidation still occurred at all concentrations within the three hour period of the study. On day 2 the lag time for 2.5 μmol/l and 10 μmol/l Cys was similar to that in the absence of Cys whilst the lag time in the presence of 20 μmol/l was slightly longer. On day 3 the lag time in the presence of all concentrations of Cys was the same. Cys was not studied on day 4 as previous studies had shown that it failed to become a pro-oxidant.

Figure 5



Variation in the lag phases for copper(II) catalysed oxidation of LDL at various thiol concentrations, with fresh or aged LDL. Key: white bars, no thiol; light gray bars, 2.5 μmol/l thiol; dark grey bars, 10 μmol/l thiol and black bars, 20 μmol/l thiol. Graph **a** shows the effects of homocysteine, while **b** represents the effects of cysteine.

The vitamin E concentration in three LDL preparations was measured according to the method of Thurnham *et al*[50] by Mr. D. Mayoss-Hurd, City Hospital, Birmingham to see if it decreased following storage at 4°C. The vitamin E concentration in freshly isolated LDL was 16.0 nmol/mg protein (8.7 – 22.7), median (range). On day 3 this had declined to 11.2 nmol/mg protein (3.2 – 14.7), median (range).

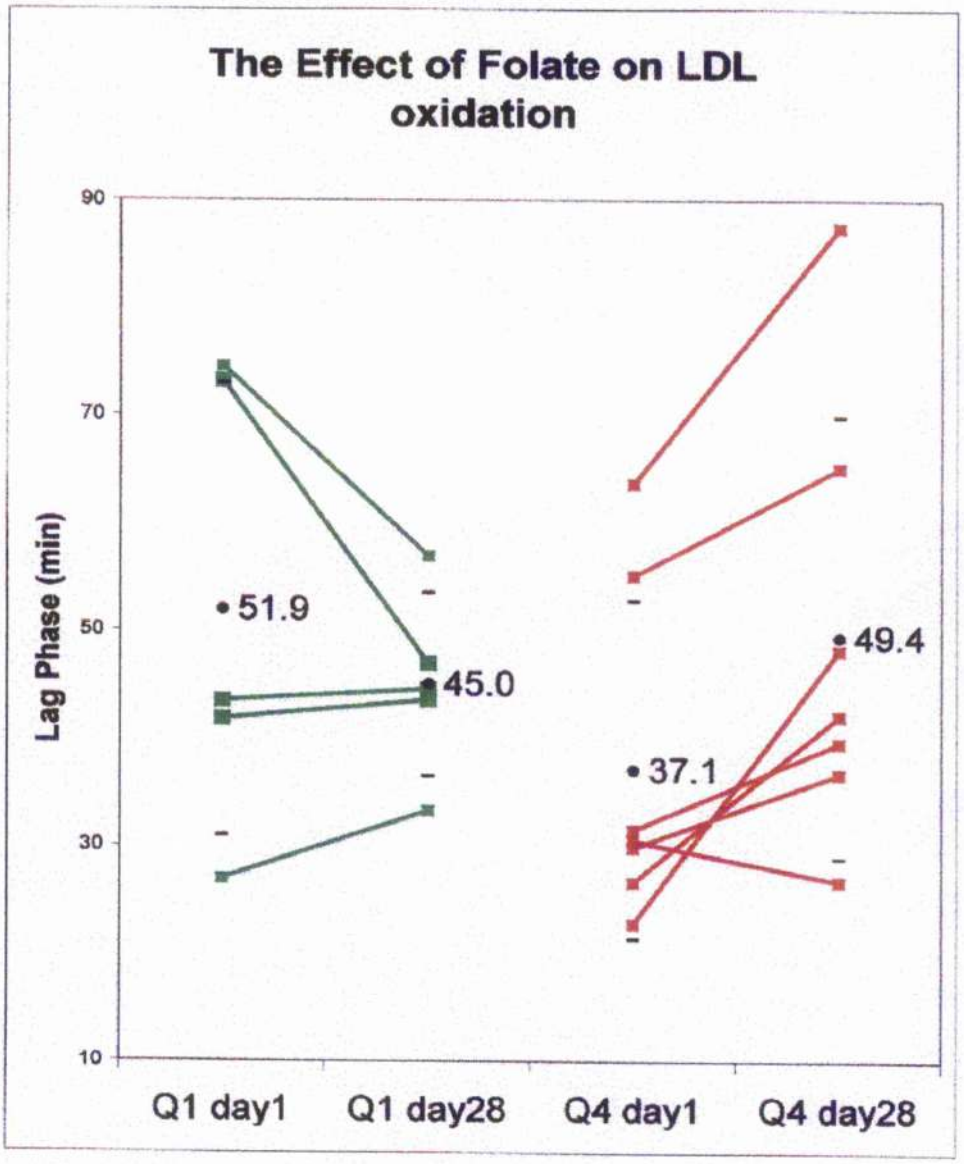
To investigate whether auto-oxidation of thiols was a possible mechanism for the copper (II) catalysed oxidation of LDL reduced and total HCySH were assayed in the incubation mix. Reduced HCySH was almost immediately oxidised, irrespective of whether LDL was present or not. In the presence of copper (II) ions only 25% of the HCySH remained in the reduced form at zero minutes and by 120 minutes, reduced HCySH could no longer be detected. All of the reduced HCySH in these samples was oxidised to homocystine as there was no significant decline in the amount of total HCySH (after reduction with DTT) measured in parallel. The addition of reduced HCySH to PBS again resulted in its oxidation, but at a slower rate than in the presence of copper (II) ions, with 68% of the reduced form present at zero minutes and 39% at 120 minutes.

6 *Effect of folic acid on lipoprotein oxidation*

Supplemental folic acid has been shown to significantly reduce plasma HCySH levels. As little as 400 μg per day is effective in reducing HCySH levels[51] and higher doses do not appear to result in any greater reductions[2]. I investigated the effect of 4 weeks treatment with 400 μg of folic acid on the

lipoprotein lag phases of volunteers. The subjects were healthy laboratory staff who had previously had their HCySH measured for the reference interval study. Seven subjects from the highest quartile (Q4) of HCySH levels and 6 from the lowest quartile (Q1, controls) agreed to participate. The lag phase for the copper (II) catalysed oxidation of freshly prepared LDL was measured by the methods described above. The Q4 subjects had the shortest lag phases for LDL oxidation, mean 37.1 min (sd 15.7 min) versus a mean of 51.9 (21.0) min for the controls. The subjects were given 400 μg folic acid per day (the controls received no intervention). After 4 weeks the lag phase for LDL oxidation was re-measured in both groups. Data from 1 control was lost due to a centrifuge failure. While there had been no significant change in the length of the lag-phase in the control group, 45.0 (8.5) min; $p = 0.34$, there was a significant increase in the length of the lag phase, Figure 6, for the folate-treated group, 49.4 (20.5) min; $p = 0.018$ v day 1. The average increase was 33.2%, compared to –13.3% for the controls, making the length of the lag-phase comparable in the 2 groups. Over the same period there had been a mean reduction in the HCySH levels of the folate treatment group of 23.0%, in agreement with the literature.

Figure 6



Copper (II) catalysed LDL oxidation at day 1 and day 28 of the experiment. The control subjects in quartile 1(Q1) are shown in green, while the subjects from quartile 4 (Q4), who took folic acid are depicted in red. The mean lag phase and 1sd are shown in black.

DISCUSSION

A sensitive HPLC method for measuring plasma total homocysteine has been developed. HPLC was chosen because the other methods for measuring HCySH are either expensive (GC-MS) and not readily available in routine clinical laboratories, or too insensitive (aminoacid analysers). Fluorometric HPLC methods for measuring HCySH often have very high background fluorescence and utilise toxic fluorophores and other hazardous chemicals such as sodium borohydride and β-mercaptoethanol. This limits their usefulness to a hospital laboratory. HPLC using electrochemical detection appeared to offer a better solution. Gold/mercury electrodes are easily 'poisoned' and require regular regeneration to restore activity, whereas coulometric detection has the advantage of familiarity, as it is already widely used by hospital laboratories measuring urine catecholamines, therefore there is an available pool of expertise and equipment to draw on. Dithiothreitol (DTT) was chosen as the reducing agent as it is safer than sodium borohydride and does not require to be handled in a fume-cabinet, unlike β-mercaptoethanol. The concentration of DTT used was halved from 20 mmol/l in the original paper[22] to avoid gel formation and to reduce the background signal. Even lower concentrations were tried (down to 5 mmol/l) but concern about potential incomplete total HCySH release resulted in 10 mmol/l being used in all the experiments described in this thesis. Even at this DTT concentration, the background was so low that it was possible to accurately determine HCySH concentrations as low as 0.625 μmol/l in aqueous solutions.

Using this HPLC method, I investigated whether there was a relationship between HCySH levels (and other proposed risk factors) and IHD in high-risk subjects and the strengths of any relationships. We studied ACE levels and genotype, lipoprotein (a) and plasma total HCySH along with established risk factors to see if the newer analytes were linked to proven coronary artery disease in a population of selected high-risk individuals. All of the patients were undergoing investigation at a Chest Pain Clinic and the controls consisted of a sub-group of those patients with angiographically normal coronary arteries, a group known to have a good prognosis (cardiological syndrome X)[35]. A criticism of previous studies investigating the relationship between HCySH and IHD has been the selection of a disease-free control group, which can accentuate any differences in HCySH (or other analyte) levels and introduce bias. The control group in this study was, therefore, very closely matched to the subjects.

Univariate analysis revealed that the control and IHD groups were well matched for age, hypertension, smoking and glycaemic control. There was the anticipated male predominance in the IHD group where diabetes mellitus was also more common, although not significantly so. Serum cholesterol and triglycerides were both significantly elevated in the IHD group as expected, but there were no significant differences in ACE activity, ACE genotype or lipoprotein (a) levels. Plasma total HCySH levels were significantly raised in the IHD group suggesting that this putative risk factor may be linked to IHD, whereas the evidence does not support a link between lipoprotein (a), ACE activity or genotype and IHD. These results contradict some previous

reports[33,34] but others have questioned the strength of the proposed link between Lp(a)[52] and ACE genotype[53] and IHD.

Further univariate sub-group analysis concentrated on HCySH, cholesterol, triglycerides and gender, because of the significant sex difference seen for total cholesterol levels. These analyses revealed that cholesterol levels in men and HCySH levels in women were significantly elevated in acute myocardial infarction. Cholesterol levels in both sexes were raised in stable angina as were triglycerides in females but there was no significant link between angina and HCySH in either sex. A comparison of the angiographic findings with the results revealed that cholesterol was higher in patients of either sex with a coronary artery occlusion, but that HCySH and triglycerides were only elevated in female patients with occlusions. When the levels of the various analytes are compared to the number of atheromatous coronary arteries, cholesterol is significantly raised in patients of both sexes with more than 1 vessel involved. Triglycerides are additionally elevated in women as is HCySH. Taken together these findings suggest a link between HCySH and acute thrombotic events (myocardial infarction or occlusion) in females with 2 or more atheromatous coronary arteries. There is a consistent lack of association between HCySH levels and IHD in males in contrast to some previous studies[13,54].

Multivariate analysis confirmed the link between HCySH and IHD with a 1.5-fold increase in the odds ratio for IHD for each μmol/l increase in HCySH. This was similar to the 1.7-fold increase seen per mmol/l serum total cholesterol. The link between IHD and HCySH appeared to exist particularly

in females, where the odds ratio increased 1.3 times for each μmol/l increase in HCySH, but not in males.

Whether the link between HCySH levels and IHD is causative is still a matter of debate. Prospective studies of HCySH and acute myocardial infarction have given conflicting results[13,14,15,54], but these studies have almost exclusively involved males and our results suggest that HCySH is more closely linked to myocardial infarction in females. Indeed, in our study, HCySH was the only analyte significantly linked to myocardial infarction in women.

Multivariate analysis confirmed the link between HCySH and IHD. HCySH is known to increase both with age[55,56] and serum creatinine levels[3,55]. The link between HCySH and the number of vessels affected is in agreement with Nygård *et al*[54].

In conclusion, HCySH, but not lipoprotein (a), ACE activity or ACE genotype is associated with IHD in a high-risk group who underwent coronary angiography. Further, HCySH appears to be particularly linked to thrombotic disease and IHD in females. Future prospective studies should include more females to confirm the hypothesis that HCySH causes IHD.

Previous studies have shown that total plasma homocysteine levels are elevated in selected younger patients (aged less than 50 years) with peripheral vascular disease[8] or neointimal hyperplasia[7]. We studied a more typical vascular clinic population to investigate whether hyper-homocysteinaemia was related to vascular disease in an unselected (and therefore older) vascular clinic population.

The sex ratios of the groups were not identical, but as post-menopausal women have been found to have similar homocysteine levels to men of the same age[57], this was not considered important. The study groups had a significantly higher proportion of smokers than the control group. Both groups also had significantly higher serum creatinine values than controls and the NIH group was significantly older. Surprisingly, perhaps, only the NIH group had significantly higher total cholesterol levels than the controls, there being only a non-significant trend ($p = 0.08$) towards higher cholesterol levels in the PVD group. The PVD group had significantly higher HCySH levels than the controls and NIH group. The difference between the means was surprisingly large at 5.1 μmol/l (141% higher than the control mean). Other studies linking HCySH to atherosclerotic disease have usually only shown a 1 – 3 μmol/l difference from controls. The negative association between hyperhomocysteinaemia and NIH contrasts with previous reports and is even more surprising considering the age of the NIH patients. HCySH levels are known to increase by approximately 1 μmol/l per decade[55] and therefore the mean HCySH level in the NIH group would be expected to be at least 0.5 μmol/l higher than the controls instead of 1.6 μmol/l lower. Folate deficiency has been implicated in the pathogenesis of hyperhomocysteinaemia, so the differences in HCySH between the PVD and NIH groups may be a reflection of the serum folate levels in each group. This also implies that HCySH is not involved in the pathophysiology underlying the development of NIH, despite a previously reported link[31].

When all the parameters were included in the multivariate analyses, HCySH was shown to be closely linked to PVD with an odds ratio of 4 for

each 1 μmol/l increase. The fasting glucose level was also positively linked to PVD with an odds ratio of 1.5 per mmol/l. Diabetes mellitus is known to be an independent risk factor for peripheral vascular disease and these results suggest that HCySH may also be an important independent risk factor for the development of PVD.

Male sex was the strongest predictor of NIH with an odds ratio of almost 9, while haemoglobin and folate levels each gave odds ratios of around 2. HCySH was negatively associated with NIH, in keeping with the univariate analysis, but narrowly failed to reach significance ($p = 0.0624$).

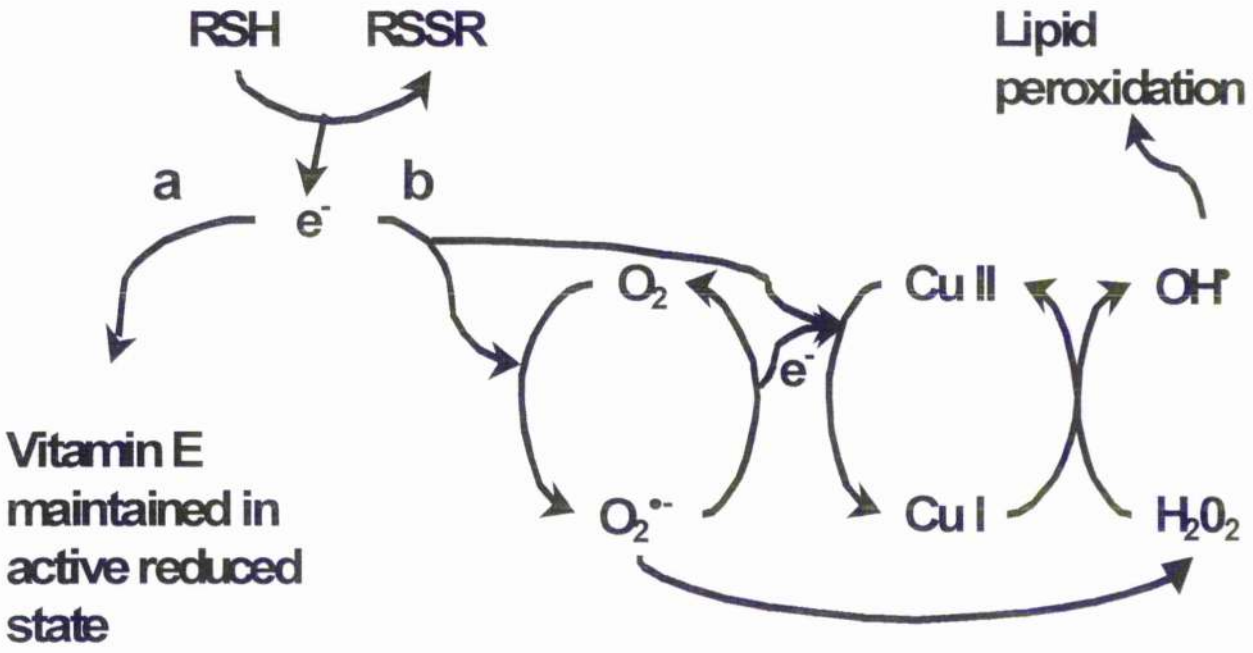
In summary, HCySH levels are significantly elevated in atherosclerotic vascular disease in a typical, elderly patient population, the association is particularly strong for PVD and may be causative. There was no association between HCySH and NIH, in contrast to earlier reports.

It is known that the risk factors for retinal vascular disease (RVOD) are similar to the risk factors for macrovascular disease. Therefore it was possible that HCySH might be linked to the development of microvascular disease as well as macrovascular disease. We demonstrated that fasting plasma total HCySH levels are significantly elevated RVOD. The homocysteine levels seen in our controls are similar to those reported for elderly German subjects[10] again suggesting that there is a general effect of age on homocysteine metabolism. One suggestion, based on tissue culture experiments with cystathionine β-synthase, is that there may be an decline in enzyme activity with age[12].

The subgroup analysis showed that the HCySH levels were highest in RAO, and lowest in BRVO. As previously reported, the BRVO group were significantly hypertensive compared to the other groups and this has been proposed as the main cause for branch retinal vein occlusions[6]. In this study, we found that the HCySH level in BRVO was still significantly higher than in the controls (12.2 v 9.5 umol/l; p=0.001) despite being lower than the levels found in CRVO (15.0 umol/l) or RAO (18.4 umol/l). This is the first study to demonstrate a link HCySH and microvascular disease and suggests that HCySH should be considered as an independent risk factor for retinal vascular disease and an additional risk factor to hypertension in BRVO.

Although there is good epidemiological evidence that links the plasma concentration of HCySH to the development of both athero-thrombotic diseases, the underlying pathological mechanisms remain uncertain. I investigated whether the atherogenic potential of LDL was influenced by HCySH and found that HCySH may have both anti-oxidant and pro-oxidant effects on LDL oxidation, and that this may contribute to the pathological effects seen in hyperhomocysteinaemia. Using freshly isolated LDL replete with vitamin E, HCySH and Cys both acted as anti-oxidants. However, after ageing LDL by storage at 4°C, HCySH became a pro-oxidant whereas Cys had no significant effect. Figure 7 proposes an explanation for the effect of thiols on the Cu (II) catalysed oxidation of LDL. The anti-oxidant effect may result from auto-oxidation of thiols, reducing vitamin E thereby maintaining its anti-oxidant properties. It has been shown that blocking thiol groups on apolipoprotein B with MAL6 enhances the rate of vitamin E depletion[58]. The

Figure 7



Legend Oxidation of the free thiol group in the presence of vitamin E (a) results in reduction of vitamin E. In the absence of vitamin E the electrons are donated to molecular oxygen or copper II ions to generate hydroxyl radicals.

pro-oxidant effect may only be observed when vitamin E has been partially or wholly depleted, when electrons may be transferred to molecular oxygen, generating radicals which participate in lipid peroxidation by Fenton-like reactions[59] or to copper (II) ions thereby reductively activating them. Thiyl radicals (formed from the reaction of thiols with transition metal ions) are also able to directly oxidise PUFA[60], it is possible that in the absence of vitamin E these may persist for longer. A requirement for reduced thiols has been shown by Heinecke *et al.*[44], who observed that copper (II) catalysed lipid peroxidation was increased by the inclusion of Cys and HCySH but not by the presence of homocystine or cystine with their oxidised thiol groups.

Cysteine was a less potent anti-oxidant than HCySH and unlike HCySH failed to become a pro-oxidant. Differences in the effect of HCySH and Cys on LDL oxidation have been observed previously[44,46,61], and it is possible that Cys is less susceptible to auto-oxidation than HCySH. Thus Cys would be a less potent anti-oxidant and pro-oxidant than HCySH. Andersson *et al*[43] concluded that the half-life for reduced HCySH in plasma was shorter than that of reduced Cys, but this has not been confirmed[62]. Chain length may affect the redox activity of thiols. Increasing the chain length between the thiol group and charged groups has been found to enhance the ability of thiyl radicals to oxidise polyunsaturated fatty acids and thus the HCySH radical would be a better oxidant agent than the Cys radical[60].

The relevance of the pro- and anti-oxidant effects of HCySH on atherosclerosis *in vivo* is unknown. These observations imply a more complicated relationship than has been proposed to date and a potential

interaction with vitamin E. Perhaps there is an interplay between hyperhomocysteinaemia and small, dense LDL, which is known to be atherogenic and is depleted in vitamin E, that increases the risk of athero-thrombosis. Whereas the same HCySH level in a subject with normal LDL and lipoprotein vitamin E levels would not increase the subject's cardiovascular risk.

I tested the hypothesis that LDL oxidation would be responsive to alterations in HCySH levels by giving folate supplements to a group in the highest quartile for HCySH. After 4 weeks of 400 μg folate daily, the mean HCySH level had fallen by 23% and the lag phase for LDL oxidation was 33.2% higher than on day 1, $p = 0.018$. This resulted in the length of the lag phase becoming comparable to the values in the control group.

As yet there are no published prospective trials of the treatment of hyperhomocysteinaemia on coronary heart disease events, and the evidence from those trials of vitamin E supplementation has been disappointing. It is of interest that a recent trial of vitamin E therapy on CHD events in those with endstage renal failure[63], a group of patients known to have particularly high homocysteine levels, showed large reductions in the risk of CHD, whilst other trials have shown little[64] or no benefit[65]. Homocysteine was not measured in any of these studies, and we suggest that future studies should allow for a potential interaction between HCySH, vitamin E and LDL oxidation.

In conclusion, this thesis documents a novel HPLC method using coulometric detection to measure plasma total homocysteine in unselected

patients attending hospital outpatient clinics with various atherothrombotic conditions more suited to hospital laboratories than other available HPLC methods.

Using this method, HCySH levels were for the first time shown to be elevated in microvascular disease[55] (subsequently confirmed by Cahill et al[66]) in addition to macrovascular disease. HCySH levels were also found to be linked to IHD in women, but not in men, which might help to explain why the largely male prospective studies have generally failed to link HCySH levels to the development of IHD. The association between hyperhomocysteinaemia and PVD was confirmed, but the puzzling link to neointimal hyperplasia was disproved. I have also attempted to investigate why HCySH might be atherogenic through its ability to promote free radical production in vitamin E depleted lipoproteins resulting in oxidised LDL formation. Finally, the results of a pilot study, indicated that low dose folate consumption not only reduced HCySH levels, but also resulted in the production of LDL that was more resistant to oxidation.

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