Homocysteinaemia (heterozygous state) in the Chinese Population

THESIS

SUBMITTED BY

CHENG Sau-kwan

in partial fulfilment of the degree of Master of Science in Clinical Biochemistry in The Chinese University of Hong Kong

February 1994

DEPARTMENT OF CHEMICAL PATHOLOGY

THE CHINESE UNIVERSITY OF HONG KONG
Thesis
WG
500
C43
1994
CONTENTS

LIST OF TABLES

LIST OF FIGURES

ACKNOWLEDGEMENTS

ABSTRACT .......................................................... 1

CHAPTER ONE ....................................................... 3

1.1 Introduction .................................................... 3

1.1.1 Sources of homocysteine and origins of deficiency or excess in the human body 3

1.1.2 Homocysteine metabolism ................................... 4

1.2 Causes of and clinical syndromes in homocysteinaemia .................. 12

1.2.1 Deficiency of cystathionine β-synthase .................. 12

1.2.1.1a Homozygous homocysteinaemia ..................... 13

1.2.1.1b Heterozygous hyperhomocysteinaemia ....... 17

1.2.2 Deficiency of 5, 10 methylenetetrahydrofolate reductase .......... 20

1.2.3 Defects of cobalamin synthesis .......................... 21

1.3 Standardised oral methionine load test ........................ 23

1.4 Treatment and prospects for homocysteinaemia ..................... 25

1.4.1 Homozygous homocysteinaemia ............................ 25

1.4.2 Heterozygous homocysteinaemia ............................ 27

1.5 Pathogenesis of vascular disease in homocystinuria .................. 28

1.6 Aim of the study ............................................... 30
<table>
<thead>
<tr>
<th>Chapter Two</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1 Patient's criteria</td>
<td>31</td>
</tr>
<tr>
<td>2.2 Control's criteria</td>
<td>31</td>
</tr>
<tr>
<td>2.3 Exclusion criteria for patients and controls</td>
<td>32</td>
</tr>
<tr>
<td>2.4 The methionine loading test and additional investigations carried out</td>
<td>33</td>
</tr>
<tr>
<td>2.5 Statistics used for data analyses</td>
<td>35</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Chapter Three</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1 Sample collection</td>
<td>38</td>
</tr>
<tr>
<td>3.2 Analytical methods for homocysteine determination</td>
<td>39</td>
</tr>
<tr>
<td>3.2.1 Cyanide nitroprusside test</td>
<td>39</td>
</tr>
<tr>
<td>3.2.2 Radioenzymic Assays</td>
<td>40</td>
</tr>
<tr>
<td>3.2.3 Gas chromatography - Mass spectrometry</td>
<td>41</td>
</tr>
<tr>
<td>3.2.4 HPLC with Electrochemical detection</td>
<td>42</td>
</tr>
<tr>
<td>3.2.5 HPLC and postcolumn derivatization</td>
<td>43</td>
</tr>
<tr>
<td>3.2.6 Precolumn derivatization, HPLC and fluorescence detection</td>
<td>44</td>
</tr>
<tr>
<td>3.3 The method used in this study</td>
<td>47</td>
</tr>
<tr>
<td>3.3.1 Materials</td>
<td>48</td>
</tr>
<tr>
<td>3.3.2 Reagents</td>
<td>49</td>
</tr>
<tr>
<td>3.3.3 Instrumentation</td>
<td>49</td>
</tr>
<tr>
<td>3.3.4 Sample preparation</td>
<td>50</td>
</tr>
<tr>
<td>3.3.4.1 Reduction</td>
<td>50</td>
</tr>
<tr>
<td>3.3.4.2 Derivatization</td>
<td>50</td>
</tr>
<tr>
<td>3.3.5 Chromatographic conditions</td>
<td>51</td>
</tr>
<tr>
<td>Section</td>
<td>Title</td>
</tr>
<tr>
<td>---------</td>
<td>-----------------------------------------------------------------------</td>
</tr>
<tr>
<td>3.3.6</td>
<td>Standard preparation</td>
</tr>
<tr>
<td>3.4</td>
<td>Method Optimization</td>
</tr>
<tr>
<td>3.4.1</td>
<td>Choice of reducing agent</td>
</tr>
<tr>
<td>3.4.1.1</td>
<td>Dithiotreitol (DTT)</td>
</tr>
<tr>
<td>3.4.1.2</td>
<td>Sodium borohydride</td>
</tr>
<tr>
<td>3.4.2</td>
<td>Choice of precipitating reagent</td>
</tr>
<tr>
<td>3.4.3</td>
<td>Optimization of chromatographic conditions</td>
</tr>
<tr>
<td>3.4.3.1</td>
<td>Flow rate, temperature and organic composition of mobile phase</td>
</tr>
<tr>
<td>3.4.3.2</td>
<td>pH of the mobile phase</td>
</tr>
<tr>
<td>3.4.4</td>
<td>Confirmation of homocysteine peak</td>
</tr>
<tr>
<td>3.5</td>
<td>Analysis of results</td>
</tr>
<tr>
<td>3.6</td>
<td>Method validation</td>
</tr>
<tr>
<td>3.6.1</td>
<td>Linearity</td>
</tr>
<tr>
<td>3.6.2</td>
<td>Precision</td>
</tr>
<tr>
<td>3.6.3</td>
<td>Recovery</td>
</tr>
<tr>
<td>CHAPTER FOUR</td>
<td>RESULTS</td>
</tr>
<tr>
<td>4.1</td>
<td>The pre- and post-methionine loading plasma homocysteine concentrations in patients and controls</td>
</tr>
<tr>
<td>4.2</td>
<td>The frequency distributions of hyperhomocysteinaemia in patients and controls</td>
</tr>
<tr>
<td>4.2.1</td>
<td>The distributions of homocysteinaemia in patients and controls</td>
</tr>
<tr>
<td>4.2.2</td>
<td>The frequency distributions of fasting hyperhomocysteinaemia in patients and controls</td>
</tr>
<tr>
<td>4.2.3</td>
<td>The frequency distributions of post-methionine hyperhomocysteinaemia in patients and controls</td>
</tr>
</tbody>
</table>
4.2.4 The frequency distributions of the abnormal methionine tolerance in patients and controls.............................. 75

4.3 The frequency distributions of hypertension and hyperlipidaemia in controls and, including smoking, in patients without and with hyperhomocysteinaemia........... 77

4.3.1 The frequency distribution of hypertension and hyperlipidaemia in patients and controls............................. 77

4.3.2 The frequency distributions of hyperlipidaemia, hypertension, smoking and gender in patients with vascular disease with and without hyperhomocysteinaemia............ 79

4.4 The comparison of the age, haematological and biochemical indices and the blood pressure between the patients and controls................................. 81

4.4.1 The comparison of the patients' age at presentation and plasma lipids following recovery from the acute episode with those in controls at the time of methionine loading.. 81

4.4.2 The comparison of the age at presentation and the plasma lipids in patients with and without hyperhomocysteinaemia............ 83

4.4.3 The comparison of the $B_{12}$, serum folate and RBC folate in patients and controls at the time of presentation.............. 84

4.4.4 The comparison of the $B_{12}$, serum folate and RBC folate levels in patients with and without hyperhomocysteinaemia and in controls.................... 85

CHAPTER FIVE DISCUSSION........................................ 87

REFERENCES......................................................... 98
<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1 Preparation of working standard</td>
<td>61</td>
</tr>
<tr>
<td>3.2 Total plasma homocysteine imprecision studies</td>
<td>64</td>
</tr>
<tr>
<td>3.3 Total plasma homocysteine recovery studies</td>
<td>65</td>
</tr>
<tr>
<td>4.1 The pre- and post-methionine loading plasma homocysteine concentrations and the rise in the homocysteine concentrations after methionine in patients and controls</td>
<td>67</td>
</tr>
<tr>
<td>4.2 The frequency of fasting hyperhomocysteinaemia in patients and controls</td>
<td>72</td>
</tr>
<tr>
<td>4.3 The frequency of post-methionine loading hyperhomocysteinaemia in patients and controls</td>
<td>74</td>
</tr>
<tr>
<td>4.4 Frequency of abnormal methionine tolerance in patients and controls</td>
<td>76</td>
</tr>
<tr>
<td>4.5 The frequency distribution of hypertension and hyperlipidaemia and the gender distribution in patients and controls</td>
<td>78</td>
</tr>
<tr>
<td>4.6 The frequency distribution of hyperlipidaemia, hypertension, smoking and gender in patients with vascular disease</td>
<td>80</td>
</tr>
<tr>
<td>4.7 Age and lipid concentrations in patients and controls</td>
<td>82</td>
</tr>
<tr>
<td>4.8 The age and plasma lipids concentrations in patients at presentation</td>
<td>83</td>
</tr>
<tr>
<td>4.9 The $B_{12}$, serum folate and red blood cell folate between patients and controls</td>
<td>84</td>
</tr>
<tr>
<td>4.10 The frequency distribution of low and normal RBC folate concentrations in controls and patients with 'normal' and raised homocysteine levels</td>
<td>85</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1 A simplified diagram of the metabolic pathway of methionine</td>
<td>5</td>
</tr>
<tr>
<td>1.2 The synthesis of S-adenosylmethionine from methionine</td>
<td>6</td>
</tr>
<tr>
<td>1.3 The conversion of S-adenosylmethionine to homocysteine</td>
<td>7</td>
</tr>
<tr>
<td>1.4 Formation of sulphate from homocysteine</td>
<td>9</td>
</tr>
<tr>
<td>1.5 Remethylation of homocysteine to L-methionine through the betaine pathway</td>
<td>10</td>
</tr>
<tr>
<td>1.6 Remethylation of homocysteine through 5-methyltetrahydrofolate pathway</td>
<td>11</td>
</tr>
<tr>
<td>1.7 Reactions catalysed by cobalamin coenzymes</td>
<td>22</td>
</tr>
<tr>
<td>3.1 A typical plasma homocysteine standard curve</td>
<td>62</td>
</tr>
<tr>
<td>4.1 The distribution of the fasting plasma homocysteine concentrations in patients and controls</td>
<td>69</td>
</tr>
<tr>
<td>4.2 The distribution of the post-methionine loading plasma homocysteine concentrations in patients and controls</td>
<td>70</td>
</tr>
<tr>
<td>4.3 The distribution of the methionine tolerance in patients and controls</td>
<td>71</td>
</tr>
</tbody>
</table>
ACKNOWLEDGEMENTS

During the work on this project, I have accumulated many debts. First and foremost, my thanks go to Dr. Lolin, lecturer of the Department of Chemical Pathology, Prince of Wales Hospital, for her superior guidance and valuable suggestions during the course of my work. Dr. Lolin had tirelessly contributed to the final product in ways too numerous to list. For technical assistance, I am particularly indebted to Dick Chan, Department of Chemical Pathology, The Chinese University of Hong Kong, for collecting the blood specimens, allocating them for biochemical tests and participating in analysing the data and in the graphic work. I would like to thank Professor J R L Masarei, Department of Chemical Pathology, The Chinese University of Hong Kong, for giving me permission to use the facilities in the department and who was always ready to render his assistance. My thanks also go to Dr. Sanderson of the Department of Medicine, Prince of Wales Hospital, who selected the patients in this study. I would also like to thank the colleagues of the Department of Chemical Pathology of The Chinese University of Hong Kong who have given me prompt assistance whenever necessary.
I am grateful to Dr. Tse, the Chief of Service of Department of Pathology, Queen Elizabeth Hospital, who granted me permission to use the instrument in the laboratory. My appreciation is expressed to Mr. P. S. Leung, Scientific Officer of Chemical Pathology Laboratory, Queen Elizabeth Hospital for his support in this study. Last but not least, I thank Miss Joan Ng, Senior Medical Technologist, Chemical Pathology Laboratory, Queen Elizabeth Hospital, for allowing me to work in the laboratory after office hours for my study.
ABSTRACT

A method for measuring total plasma homocysteine was developed, giving linearity up to at least 100 umol/L of homocysteine, a recovery of 101% and a comparable precision to that in published methods.

The method was used for the comparison of pre- and post-methionine homocysteine levels, and the rise in the amino acid after methionine in controls and in patients with documented premature vascular disease, because of recent evidence suggesting that mild homocysteinaemia, i.e. the heterozygote homocysteinaemia, may be similarly important to conventional risk factors in the development of premature vascular disease.

The pre- and post-methionine homocysteine levels and the increase in the amino acid after the load were all significantly higher in patients than controls as was the frequency of post-methionine hyperhomocysteinaemia. Post-methionine hyperhomocysteinaemia was of similar frequency (28%) to that reported in Western patients (20 to 40%) while in controls it was slightly higher (4.8% versus 1 in 70 to 1 in 200). Although cystathionine β-synthase could not be measured, the absence of obvious liver disease and the infrequency of B12 and folate
deficiency in the patient group suggest heterozygosity for homocysteinaemia as the most likely cause.

The comparison of the frequency of hyperhomocysteinaemia and other risk factors suggested that hyperhomocysteinaemia may be primarily an independent risk factor with, at the most, only a mild interaction with hypertriglyceridaemia.
CHAPTER ONE

1.1 Introduction

1.1.1. Sources of homocysteine and origins of deficiency or excess in the human body

Homocysteine is an essential amino acid not present in ingested proteins and is derived from methionine. Methionine is obtained both from the diet and by re-conversion from homocysteine. Both amino acids are metabolized in the liver (1).

Deficiency of homocysteine occurs in protein malnutrition or as a result of a defect in metabolism from methionine (1). Since homocysteine is both reconverted into methionine and metabolised into cystathionine, the amino acid accumulates in inborn or acquired errors involving either pathway (2).
1.1.2 Homocysteine metabolism

In Figure 1.1 is shown a simplified diagram of methionine metabolism (3). Natural protein contains approximately 0.3 to 5.0% methionine. Some dietary methionine is used by the body for tissue protein synthesis, but the majority is utilized through the transsulfuration pathway to form adenosylmethionine, adenosylhomocysteine, homocysteine, cystathionine, α-ketobutyrate, cysteine and their derivatives (4). Homocysteine is an intermediate of the transsulfuration pathway.
TETRAHYDROFOLATE METHYLTRANSFERASE (+methylcobalamin)

S-methyltetrahydrofolate

N, N-dimethylglycine

S-adenosylhomocysteine

5-adenosylmethionine

METHIONINE ADENOSYLTRANSFERASE

S-adenosylhomocysteine

BETAINÉ-HOMOCYSTEINE METHYLTRANSFERASE

methionine

tetrahydrofolate

5-adenosylhomocysteinase

NHS CH₂ CH₂ CH NH₂ CO₂ H

SCH₂ CH₂ CH NH₂ CO₂ H

CH₂ CH NH₂ CO₂ H

cystathionine

HS CH₂ CH NH₂ CO₂ H

cysteine

SO₄²⁻

Fig. 1.1 A simplified diagram of the metabolic pathway of methionine
The first step in the transsulfuration pathway is the synthesis of S-adenosylmethionine, a reaction catalysed by methionine adenosyltransferase (Fig. 1.2) (5).

\[
\text{L-Methionine} + \text{ATP} \xrightarrow{\text{Methionyl adenosyl transferase}} \text{S-Adenosyl-L-methionine}
\]

Fig. 1.2 The synthesis of S-adenosylmethionine from methionine
S-adenosylmethionine is the principal methyl donor in the body. Biologically important compounds that obtain their methyl group from S-adenosylmethionine include: creatine, choline and phosphatidylcholines, methylated DNA and RNA, and epinephrine (4). S-adenosylhomocysteine is formed as an intermediary product in this pathway (Fig. 1.3), and S-adenosylhomocysteine is then hydrolysed to homocysteine (6).

![Chemical reaction diagram](image)

**Fig. 1.3** The conversion of S-adenosylmethionine to homocysteine
Under normal circumstances, only small concentrations of homocysteine are measurable in human plasma and neither homocysteine nor its disulphide homocystine are found in urine because of the effective removal of homocysteine by 4 possible pathways (4,7).

In pathway 1, homocysteine reacts with serine in the presence of cystathionine β-synthase (EC 4.2.1.22) to form cystathionine. This is an irreversible reaction requiring pyridoxal phosphate as a coenzyme (8). Cystathionine is further metabolised to cysteine and d-ketobutyrate by another pyridoxal phosphate-dependent enzyme, cystathionase. The sulfur atom of methionine, transferred into the cysteine molecule through the trans-sulfuration pathway, ends up mainly as inorganic sulfate in the urine (Fig. 1.4) (5).
Fig. 1.4 Formation of sulphate from homocysteine
In pathways 2 and 3, homocysteine is remethylated to form methionine through two different enzymatic reactions. In pathway 2, the methyl group is derived from betaine and is catalysed by betaine homocysteine methyltransferase (EC 2.1.1.5) (Fig. 1.5). The reaction is essentially irreversible (5).

![Diagram of betaine homocysteine methyltransferase reaction]

**Fig. 1.5** Remethylation of homocysteine to L-methionine through the betaine pathway
Pathway 3 requires $N^5$-methyltetrahydrofolate as a methyl donor and methylcobalamin (methyl B$_{12}$) as coenzyme. The enzyme catalyzing this reaction is 5-methyltetrahydrofolate-homocysteine methyl-transferase (EC 2.1.1.13) (Fig. 1.6). This is the only significant metabolic reaction in humans that uses both folate and B$_{12}$ coenzymes (5).

Fig. 1.6 Remethylation of homocysteine through 5-methyltetrahydrofolate pathway

In pathway 4, intracellular homocysteine is released into the extracellular fluid (9).
1.2 Causes of and clinical syndromes in homocysteinaemia

Reduction in homocysteine metabolism through either the transsulfuration (pathway 1) or the 5-methyltetrahydrofolate-dependent sulfur conservation pathway (pathway 3) leads to accumulation of the amino acid with resultant homocysteinaemia and homocystinuria. The biochemical defects concerned include: deficiency in enzymes cystathionine $\beta$-synthase and 5,10-methylene-tetrahydrofolate reductase and defects of cobalamin synthesis (10). Defects in pathways 2 and 4 have not been described.

1.2.1 Deficiency of cystathionine $\beta$-synthase

Cystathionine $\beta$-synthase deficiency is the most frequently encountered cause of hyperhomocysteinaemia (11). More than 350 cases of proved or presumptive cystathionine $\beta$-synthase deficiency have been described. It is inherited as a Mendelian recessive trait with genetic heterogeneity in expression (2). The patients can be divided into two groups depending on their
biochemical response to vitamin B$_6$ (12). In one group there is partial basal enzyme activity which may improve with vitamin B$_6$ administration while in the other there is no enzyme activity and no response to vitamin B$_6$ treatment (13). Vitamin B$_6$-responsive and vitamin-B$_6$-non responsive patients are equally frequent. B$_6$-nonresponsive patients have an earlier onset and more severe manifestation of their disease than do patients responsive to vitamin B$_6$ (7).

1.2.1.1a Homozygous homocysteinaemia

The clinical manifestations in homozygous homocysteinaemia differ significantly from those in the heterozygous state.

In the West, the incidence of this autosomal recessive metabolic disease is 1:8000 - 1:200,000 (14). In the Chinese, the incidence is unknown.

Clinical manifestations
Subjects with this disease are normal at birth, but develop progressively more severe clinical features from
childhood to young adulthood. The eyes and the skeletal, central nervous and vascular systems are mainly involved (14).

Ocular lesions
Ectopia lentis is common (15). The lenticular dislocation takes place between three to ten years of age. Subluxation of the lens is often accompanied by myopia and iridodonesis and, less frequently, by retinal degeneration and detachment, glaucoma and cataracts. The ectopic lentis is secondary to disruption of the zonula fibers (high content of cystine) that connect the lens to the ciliary body. In cystathionine $\beta$-synthase deficiency, these fibers are thickened and broken (14).

Skeletal lesions
Osteoporosis, especially in the spine and long bones, is the most consistent skeletal abnormality. Scoliosis is also frequent, with and without osteoporosis. Other abnormalities include biconcavity of the vertebrae, thought to be due to spinal osteoporosis or to vascular occlusion of the vertebral arteries. The patients are usually tall and the long bones are generally excessively long (1).
Central Nervous System lesions
Developmental delay during the first or second year of life is often the earliest clinical feature with homozygous cystathionine β-synthase deficiency. Mental retardation is common and becomes obvious in middle childhood. However, about 20% of patients have normal or near normal intelligence. Convulsions, hemiparesis, unilateral weakness and spasticity are frequent and thought to be due to cerebrovascular occlusions (1).

Vascular lesion
The life expectancy of homozygous homocysteinaemic patients is rarely above early middle age, predominantly because of premature occlusive vascular disease (7). Cardiovascular, cerebrovascular and peripheral vascular disease and pulmonary emboli are all common, occurring at any age and are the usual cause of death. Thrombi and emboli are commonly encountered at postmortem examination even when they were clinically inapparent. Some other clinical features, such as the malar flash and livedo reticularis, are also thought to be of vascular origin (1).
Other features

Cases with hepatomegaly due to fatty changes, electromyographic evidence of myopathy, hyperinsulinemia with abnormal glucose tolerance and increased levels of growth hormone have also been reported (1).

Diagnosis of homozygous homocysteinaemia

The presence of one or more typical clinical features may lead to a suspicion of cystathionine β-synthase deficiency. The most consistent biochemical findings are homocystinuria, hyperhomocysteinaemia, hypermethioninemia and a markedly reduced concentration of serum/plasma cystine. The confirmatory test is the demonstration of severe reduction in activity of cystathionine β-synthase. The activity of the enzyme can be assayed in liver biopsy specimens, cultured phytohemagglutinin-stimulated lymphocytes, and in cultured skin fibroblasts (2). However, in some patients the cystathionine β-synthase activity is within the control range (1). In such cases the diagnosis remains presumptive and is based on the clinical features and the presence of homocystinuria, hyperhomocysteinaemia and hypermethioninemia.
1.2.1.1b Heterozygous hyperhomocysteinaemia

In the general Western population, the incidence is thought to be 1 in 70 to 1 in 200 or less (16). In patients with early vascular disease, it is 20% to 40% (17-20). The prevalence in the Chinese is unknown.

Clinical manifestations

Heterozygous homocysteinaemia does not give rise to any abnormal somatic features as seen in the homozygous state. However, although the association of premature vascular disease with severe homocystinaemia, such as in the homozygous cystathionine β-synthase deficiency is well known, in recent years an association with mild homocysteinaemia, detected either in the fasting state or after a methionine load, has also been increasingly reported (17-20).
Biochemical abnormalities in heterozygous homocysteinaemia

In the heterozygotes, there is no excessive homocystinuria and in the fasting state the plasma/serum homocysteine concentrations are normal or slightly increased. However, after methionine loading, the homocysteine concentrations increase more than in unaffected subjects, indicating that the activity of cystathionine $\beta$-synthase, in the presence of excess methionine, is inadequate.

Identification of Heterozygotes

1) Cystathionine $\beta$-synthase can be assayed in liver biopsy samples, cultured fibroblasts and phytohemagglutinin-stimulated lymphocytes. These assays detect most heterozygotes as the mean specific activity is less than 50% of the mean control specific activity. However, some obligate heterozygotes have enzyme activity within the control range. Therefore, the above methods can detect some but not all heterozygotes (21) and the methionine loading test may be preferable (19).
2) Methionine loading test

After methionine loading, plasma/serum homocysteine levels in heterozygotes increase more than in 'normal' controls. This serves as a diagnostic test (19). Oral methionine loading is a recent investigative procedure. A physiological substance is used and side effects have not been recorded (19).

However, the reference range for the fasting and post-methionine homocysteine levels and the expected rise in homocysteine after methionine loading in the normal population and in the heterozygotes for homocysteinaemia have not been fully evaluated. In most studies to date, the levels in patients with vascular disease had to be compared with those in control subjects investigated at the same time (19).

Further, not all cases of hyperhomocysteinaemia are due to the heterozygous state, thus finding higher post methionine level than controls only suggests hyperhomocysteinaemia, but does not fully differentiate the heterozygous state from other causes of hyperhomocysteinaemia, such as B₁₂ or folate deficiency, although in the former the homocysteine levels tend to be much higher.
Deficiency of 5,10-methylenetetrahydrofolate reductase is a rarer cause of hyperhomocysteinaemia with only 25 patients, from 20 families, having been reported to date (22). The relationship of this enzyme to homocysteine metabolism is illustrated in Fig. 1.6 (see above). The inheritance pattern appears to be autosomal recessive, but thermal stability studies have suggested possible allelic variations (23).

Clinical features
The usual clinical manifestations include neurological dysfunction with delayed psychomotor development, severe mental retardation and psychiatric symptoms, seizures and peripheral neuropathy (24). Vascular complications are uncommon. These symptoms are quite different from homocysteinaemia due to cystathionine β-synthase deficiency. However, three patients suffering from recurrent strokes and pulmonary emboli have been reported to have 5,10-methylenetetrahydrofolate reductase deficiency, indicating that this type of hyperhomocysteinaemia may also be associated with occlusive vascular disease (22).
Diagnosis of 5,10 methylenetetrahydrofolate reductase deficiency

In patients with homocysteinaemia due to 5,10-methylenetetrahydrofolate reductase deficiency, the characteristic biochemical changes are moderate homocystinuria and homocysteinaemia, with relatively normal level of plasma methionine. The enzyme activity can be readily assessed in hepatocytes, fibroblasts, lymphocytes and leucocytes (1).

1.2.3 Defects of cobalamin synthesis

The folate-dependent methylation of homocysteine (pathway 3) requires methylcobalamin as a cofactor. Deficient synthesis of methylcobalamin results in megaloblastic anaemia, homocysteinaemia and a low or normal concentration of methionine in plasma. Patients with deficient synthesis of both adenosylcobalamin and methylcobalamin have in addition, methylmalonic acidemia due to impaired propionate oxidation (Fig. 1.7) (25).
Fig. 1.7 Reactions catalyzed by cobalamin coenzymes
1.3 Standardised oral methionine load test

Homocysteine is either metabolised to cystathionine, remethylated to methionine or exported from the cells unchanged. The distribution of metabolism between these pathways is determined by the enzyme concentrations and their Km for homocysteine, and the regulating effects of adenosylmethionine. Under normal conditions the metabolising and remethylating pathways are balanced and little unchanged homocysteine is exported from the cells (9).

The homocysteine catabolising enzymes are activated by increased levels of adenosylmethionine in response to raised dietary levels of methionine, resulting in increased conversion to cystathionine. Since raised levels of adenosylmethionine also inhibit methionine synthetase, catabolism rather than reconversion to methionine is favoured. However, more unchanged homocysteine also diffuses out of the cells. In the presence of reduced levels of intracellular methionine, homocysteine is reconverted preferentially to methionine rather than metabolised to cystathionine or allowed to
diffuse out, unless the activity of methionine synthetase is reduced (9).

The effects of low and high intracellular concentrations of methionine on the homocysteine levels in blood form the basis of the standardised oral methionine load test, used in clinical practice to detect hyperhomocysteinaemia. In the fasted state the activity of methionine synthetase is increased and unless the enzyme is deficient or there is cobalamin or folate deficiency, the plasma/serum homocysteine levels are low. At high methionine concentrations, the activity of cystathionine synthase increases, thus limiting the amount of homocysteine diffusing out of the cells unmetabolized. If the activity of the enzyme is reduced, as in homozygous or obligate heterozygotes, the plasma/serum homocysteine levels increase 'abnormally' (9).
1.4 Treatment and prospects for homocysteinaemia

1.4.1 Homozygous homocysteinaemia

Management of patients with homocystinuria due to cystathionine β-synthase deficiency includes dietary measures, administration of pyridoxine, specific drug therapy and treatment of clinical complications. Whenever possible, treatment should begin before clinical complications occur, since many are irreversible. Even before clinical effects are recognizable, tissue damage may occur. Thus maximal benefit is thought to be obtained if treatment is instigated as early as possible in the neonate (14).

Diet

Diets specifically low in methionine and supplemented with L-cystine are designed to reduce the accumulation of methionine, homocysteine and their metabolites. The progressive complications of cystathionine β-synthase deficiency may be prevented or ameliorated with low methionine diets supplemented with cystine (26).
Administration of choline (27), a precursor of the methyl donor betaine, or betaine itself results in lower homocysteine and higher methionine concentrations. In some patients, treatment with folate may help while in others, therapy with folate in combination with vitamin B12 and pyridoxine lowers homocysteine levels with little or no increase in the methionine concentration (28).

Administration of Pyridoxine
In vitamin B6 responsive cystathionine β-synthase deficient patients, treatment with vitamin B6 reduced the incidence of vascular events (29). The apparent efficacy of the treatment with vitamin B6 highlights the importance of identifying hyperhomocysteinaemia in patients with vascular disease particularly if other, more common risk factors are absent.

Specific Drug Therapy
Antithrombotic therapy with dipyridamole has been tried but its efficacy is not certain (30). Avoidance of factors associated with an increased risk of thromboembolism such as the use of oral contraceptives has also been suggested (31).
Treatment of clinical complications

The treatment of clinical complications is the same as when due to other causes. An important exception, however, is the need for avoidance of surgery because of the risk of thromboemboli post-operatively (1).

1.4.2 Heterozygous homocysteinaemia

In some heterozygotes complete normalization of the post-methionine loading homocysteine levels occurs following treatment with Vitamin B₆ at a dose of 250 mg daily. Normalization has also been reported after treatment with betaine or folic acid at a dose of 6 g or 5 mg daily respectively (16,32).

Homocysteine lowering treatment, in particular when started early, was shown to prevent a number of serious clinical sequelae in patients with homozygous homocysteinaemia. The clinical relevance of such therapy in heterozygous arteriosclerotic patients, in terms of prevention of progression of the arteriosclerotic lesions or reduction in the number of recurrent thromboembolic events, has, unlike in homozygous, not yet been
demonstrated. If further studies establish such a beneficial clinical effect, then it will become important to screen patients with premature vascular disease, particularly if the usual risk factors for arteriosclerosis are absent, for heterozygosity for homocysteinaemia (33).

1.5 Pathogenesis of vascular disease in homocystinuria

The precise cellular and molecular mechanisms that might link vascular disease with hyperhomocysteinaemia, whether mild or severe, are not yet certain and various suggestions have been made.

Firstly, thromboembolic tendencies in homocysteinaemia are thought to be consequent on conversion of methionine to homocysteine thiolactone, the latter being the reactive cyclic internal lactone of homocysteine. The free amino groups of low density lipoprotein (LDL) are thiolated by homocysteine thiolactone, causing aggregation and increased uptake of LDL by macrophages and therefore lipid deposition in atheromas. Homocysteine thiolactone, released from homocysteinylated
LDL within the vascular wall, promotes intimal injury, oxidation of cholesterol and unsaturated lipids, platelet aggregation, myointimal hyperplasia, deposition of sulfated glycosaminoglycans, fibrosis and calcification of atherosclerotic plaques (34).

Secondly, hyperhomocystaemia has also been reported to inhibit the expression of thrombomodulin and to inactivate both thrombomodulin and protein C irreversibly, leading to decreased protein C activity. It has been suggested that a synergistic interaction between the effects of the reactive homocysteine metabolites on the vascular endothelium and protein C deficiency may be a reason for premature thromboembolism (35).

Thirdly, excess homocysteine is also thought to initiate coagulation by the tissue factor pathway through a mechanism involving the free thiol group of the amino acid and by tissue factor gene transcription (36).
1.6 Aim of the study

In the Hong Kong population, the incidence of the heterozygous homocysteinaemia is unknown. Thus, the aims of the study are:

(1) To validate a known or establish a modified method for homocysteine measurement in plasma/serum.

(2) To measure homocysteine concentration before and after methionine loading in patients with documented premature vascular disease and in controls of similar age group.

(3) To examine, if there is an interaction between homocysteinaemia and conventional risk factors for vascular disease such as hyperlipidaemia, hypertension and smoking.
CHAPTER TWO

Patient and control selection and the methionine loading test

The following are a set of criteria that the patients and controls had to fulfil to be included in the study.

2.1 Patient's criteria:

1. Either sex
2. Age 60 years or less at the time of diagnosis
3. Myocardial infarction or ischaemia (angina) confirmed by electrocardiography and/or cardiac enzyme changes, and in all, by coronary angiography.
4. As the effect of acute illness on methionine is not well understood, patients were investigated at least 3 months after myocardial infarction or admission for angina.
2.2 Control's criteria:

1. Either sex
2. Age 60 years or less
3. Free of overt disease

2.3 Exclusion criteria for patients and controls:

1. Age above 60 years at the time of the first admission and diagnosis of vascular disease
2. Diabetes mellitus
3. Overt renal, hepatic, thyroid or metabolic disease
4. Acute or severe systemic illness within the previous 3 months
5. Cardiovascular disease not due to atherosclerotic obstruction of coronary vessels
6. Use of nitrous oxide within past 3 months
7. Known cardiomyopathy
8. Psychiatric illness
9. Anti-epileptic medication
10. Pregnancy
11. Chronic alcohol abuse
Hyperlipidemia, smoking and hypertension were not exclusion criteria in our study as part of the study was to examine the interaction of homocysteinaemia with these risk factors. The presence of additional, peripheral or cerebrovascular disorders was not an exclusion criteria either.

2.4 The methionine loading test and additional investigations carried out

1) After an overnight fast (water, black coffee and tea were allowed), the following were noted: age, sex, date of last menstrual period, smoking habit, medication (vitamin supplementation and the contraceptive pill which may alter metabolism and although not exclusion criteria, were recorded), weight and blood pressure.

2) 45 ml of blood was taken for measuring haemoglobin, haematocrit, white cell count, urea, creatinine, sodium, potassium, bilirubin, free T₄ and TSH, total serum and red blood cell folate, pre-load homocysteine, cholesterol and triglycerides
concentrations and the activities of SGOT, GGT and alkaline phosphatase.

3) L-methionine was given in an oral dose of 0.1 g/kg body weight together with a methionine-free breakfast provided by the hospital dietitian. Later on, a methionine-free lunch was also provided. Water, coffee and tea without milk and orange juice were allowed ad lib.

4) Six hours after the load, the blood pressure was measured again and 10 ml of blood was taken for homocysteine measurement.

Blood samples for homocysteine measurement were processed as mentioned in chapter three, section 3.1. The haematological and biochemical tests were performed in the Pathology Unit of Prince of Wales Hospital according to the routine laboratory practice. The haematological and biochemical tests carried out were necessary as a profile of general health. The haematological examination and the B₁₂ and folate concentrations were necessary to exclude megaloblastic
anaemia (the vitamins are cofactors for homocysteine metabolism) and the liver function tests to exclude liver disease (which can cause acquired hyperhomocysteinaemia). The thyroid function tests were carried out to exclude subclinical thyroid disease, the general effects of which may include abnormalities in homocysteine metabolism. Fasting lipids were measured because they are a risk factor for ischaemic heart disease.

It was thought that the prospect of methionine loading, a relatively unpalatable diet following, venepuncture and a several hour stay at the hospital could affect the blood pressure particularly when initially measured. Thus two sets of reading were taken, one just before the first venepuncture and methionine load and the other at the end of the test, when any earlier rise would have been expected to have normalized if the subject was not hypertensive.

2.5 Statistics used for data analyses

The subjects' results were expressed as the mean and standard deviation (SD). The difference between the
fasted and post-methionine homocysteine levels, i.e. the response to methionine was calculated for each patient individually.

The fasting and post-methionine homocysteine levels and the response to methionine between controls and patients were compared using the Mann Whitney test because of the non-Gaussian distribution, before and after removal of the controls whose homocysteine levels were above the mean + 2, 2.5 and 3 SD and recalculating the mean. The other haematological and biochemical indices between the two groups were compared using the Student's $t$ test.

The frequency of hyperhomocysteinaemia in patients and controls was compared using the Chi square Fisher's Exact test. The frequency of hyperlipidaemia, hypertension, smoking, the sex distribution, $B_{12}$ and folate deficiency between controls and patients and between patients with and without hyperhomocysteinaemia was also carried out using the Chi square Fisher's Exact test, but after removing the controls with hyperhomocysteinaemia in the control groups, which were examined separately.
Definition

Abnormal results were defined as those above the recalculated control mean+2.5 SD. Moreover, for the frequency distribution of hyperhomocysteinaemia in controls and patients, the data using the control mean+2 and 3 SD are also shown. An abnormal rise in homocysteine after methionine was termed methionine intolerance or abnormal/pathological methionine tolerance.
CHAPTER THREE

3.1 Sample collection

Total homocysteine concentration in whole blood increases rapidly to a value in excess of 180% of the basal concentration if whole blood is left for several hours prior to processing. Methionine continues to be metabolised to homocysteine in the stored red blood cells after venepuncture if left at ambient temperature. The concentration is also affected by sunlight (UV light). Therefore blood samples after venepuncture were stored on ice, in the dark and usually the plasma was separated from the red cells as soon as possible after collection (37). Heparin and EDTA samples have both been used (38). If plasma/serum are kept at -20°C, they can be stored for up to ten years before analysis (39).

In this study, after venepuncture, 10 ml of blood was aliquoted into a plastic bottle containing heparin (0.2 mg/ml), immediately put on ice in a thermal flask which was tightly shut to exclude sunlight, the sample was transported to the laboratory as soon as possible,
centrifuged (at room temperature) and the separated plasma stored at \(-70^\circ C\) until analysis.

3.2 Analytical methods for homocysteine determination

Urine homocystine measurement

3.2.1 Cyanide nitroprusside test

The cyanide nitroprusside test is an old established screening test for excess homocysteine in urine. Nitroprusside combines with sulphur containing amino acids to produce a red colour after the reduction of disulphide bonds by cyanide (40). Since this test detects most disulphides, it may be positive in other inborn errors where excess disulphide is excreted such as in cystinuria and \(\beta\)- mercaptolactate cystinuria (1). Further, an artifactual form of homocystinuria can occur through bacterial conversion of cystathionine to homocysteine. This has been described in urine contaminated with massive cystathionine (11).

A modified cyanide-nitroprusside test is also available for quantitative measurement of urinary cystine and
homocystine. With the addition of silver nitrate, only homocystine reacts to give a magenta colour (41). However, the lack of sensitivity and the labile nature of the colour has restricted the use of this assay.

Plasma/serum homocysteine measurement

In plasma/serum, almost all homocysteine is in the disulfide form—either bound to albumin (70-80%) or to low-molecular-mass sulfhydryls, mainly cysteine (20-30%). Only traces are found in the free form (42). Usually total homocysteine is measured. Thus, in most reported methods, the analytical steps include reduction to disassociate the amino acid from proteins, protein precipitation, centrifugation and the measurement of the total homocysteine in the supernatant.

3.2.2 Radioenzymic Assays

Total homocysteine in plasma/serum has been measured with a radioenzymatic assay based on the conversion of homocysteine to S[^14]C-adenosylhomocysteine in the presence of [^14]C-adenosine and S-adenosylhomocysteine
hydrolase, after reduction with dithioerythritol. Protein is precipitated with perchloric acid. The radioactive S-[\textsuperscript{14}C]-adenosylhomocysteine is isolated by reversed phase high performance liquid chromatography and quantitated by liquid scintillation counting (43).

A modification of the method where S-[\textsuperscript{14}C]-adenosylhomocysteine is adsorbed on a paper chromatograph and the radioactivity of the isolated spot is counted by a scintillator, has also been described (44).

The radioenzymic techniques are sensitive and specific and are used to measure homocysteine in both blood and urine. The modification based on paper chromatography requires inexpensive equipment. However, this technique involves several tedious manual procedures and cannot be easily automated.

3.2.3 Gas chromatography-Mass spectrometry

In this method, a deuterated internal standard is added to serum followed by 2 mercaptoethanol. After heating at 40°C for 30 minutes, protein is precipitated with
sulfosalicylic acid and the supernatant is partially purified by sequential cation exchange and anion exchange chromatography. After drying, the supernatant is derivatized with N-methyl-N-(tert-butyl dimethylsilyl) trifluoroacetamide. The tert-butyl dimethylsilyl derivatives are separated and quantified by capillary GC-MS with selected ion monitoring (45). Deuterated homocysteine is added as internal standard to correct for the variable recovery through the many steps and the reoxidation of homocysteine during sample processing.

This method offers high sensitivity and specificity and can measure homocysteine in urine as well as in blood. The major disadvantages are the relative high cost of the equipment and the many manual steps involved which cannot be automated easily.

3.2.4 HPLC with Electrochemical detection

After reduction with sodium borohydride (NaBH₄) and protein precipitation with perchloric acid, thiols in plasma/serum, including homocysteine are detected in the
column effluent by a single gold-mercury electrode (9). The chromatographic system can be modified to resolve cysteine, homocysteine and cysteinylglycine. The attractive features of this electrochemical assay include: simple sample processing, short run time, large sample throughput, specificity and the possibility of determination of other thiols such as cysteine.

The major disadvantages are the long equilibration time before the detector becomes stabilized, flow cell contamination and deterioration of the gold-mercury electrode.

3.2.5 HPLC and postcolumn derivatization

Total homocysteine in plasma/serum can be measured with the conventional amino acid analyser. The plasma/serum samples are reduced with dithiotreitol, proteins are precipitated with sulfosalicylic acid and ninhydrin is used as the staining reagent (46). Analysis of homocysteine with a conventional amino acid analyser has several advantages such as simple sample preparation and apparatus which is relatively easy to operate. The main
drawback is the nonselectivity for sulfur containing amino acids, and therefore it needs good chromatographic conditions for separation of peaks.

Another assay using post-column derivatization involves reduction of plasma/serum samples with dithiotreitol and protein precipitation with sulfosalicylic acid. Separation is carried out with reversed phase ion-pair liquid chromatography, post-column derivatization with 4,4'-dithiodipyridine and colorimetric detection of the product, formed between 4,4'-dithiodipyridine and homocysteine, at 324 nm. The detection limit of this method is < 50 nmol/l (47). The method gives high precision and better sensitivity than other HPLC methods owing to the high absorbance coefficient. However, there is significant baseline fluctuation, although this can be minimized by using pulse dampers and keeping 4,4'-dithiodipyridine at 0°C.

3.2.6 Precolumn derivatization, HPLC and fluorescence detection

In this method monobromobimane has been used for determining homocysteine in plasma/serum and sodium
borohydride as a reductant. Excess fluorescent monobromobimane is removed by treating the sample with thiol Sepharose and the sample is then extracted by solid phase extraction (48). However, thiol Sepharose treatment and solid phase extraction are tedious procedures.

Recently, a fully automated method for the determination of plasma homocysteine based on sodium borohydride reduction and derivatization with monobromobimane has been developed (39). Baseline separation of homocysteine, cysteine and cysteinylglycine is obtained by eluting the column with an ammonium formate buffer adjusted to pH 3.65.

Another thiol-specific fluorogenic reagent, ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulphonate (SBD-F) has been used as derivatizing reagent. Thiols derivatized with SBD-F are reported to show high fluorescence, SBD-F itself is non-fluorescent and no fluorescent byproducts are formed during the derivatization procedures. Homocysteine, cysteine and cysteinylglycine adducts are separated by gradient elution from a reversed-phase
column and detected by a fluorescence detector (50). The separation of the eluents can be speeded up from the 16 minutes with gradient elution to 12 minutes with the use of isocratic elution without affecting separation (49). However, the low pH of the mobile phase with isocratic elution increases dissolution of the silica-matrix, thus, shortens the life span of the column. This can be circumvented with the use of a guard column behind the injector. The precision of this method can be improved by including mercaptopropionylglycine as internal standard to correct for inaccurate composition of the assay mixture and/or variable injection volume (50).

The SBD-F based methods are sensitive and specific and there are no interfering reagent peaks in the chromatogram. Disadvantages include low reactivity of SBD-F and prolonged reaction time at a high temperature (60°C, 1 hr) which can destroy some of the thiols being measured but does not affect homocysteine.

Recently, 7-fluorobenzo-2-oxa-1,3 diazole-4-sulfonamide (ABDF) has been introduced as derivatizing agent. It
reacts faster than SBD-F and at a lower temperature (51). Shorter reaction time and lower temperature lead to less destruction of some biological thiols. With an isocratic mobile phase, a 7-8 minutes retention time can be obtained.

Total plasma/serum homocysteine has recently been assayed with precolumn derivatization with o-phthalaldehyde followed by HPLC and fluorescence detection (52 & 53). Plasma/serum is reduced with 2-mercaptoethanol and homocysteine is carboxymethylated with iodoacetate before derivatization with o-phthalaldehyde. Homocysteic acid is used as internal standard. The method gives good sensitivity and precision. The widespread experience with the o-phthalaldehyde techniques and the possible determination of other aminothiols and amino acids (including cysteine and cysteinylglycine) are other attractive features.

3.3 The method used in this study

The method for total homocysteine measurement in this project was based on those of Ubbink et al. (49) and
Jacobsen et al. (48) with some modifications. The method includes reduction of the plasma sample with sodium borohydride (48), protein precipitation with trichloroacetic acid, derivatization with SBD-F, separation by reversed phase HPLC with an isocratic mobile phase (49) and the use of N-2 mercaptopropionyl glycine as internal standard (50).

### 3.3.1 Materials

- DL-Homocysteine,
- SBD-F,
- Trichloroacetic acid,
- Disodium EDTA,
- Sodium borate,
- N-2 mercaptopropionyl glycine,
- Trizma Base and Trizma HCL
  (all from Sigma Chemical Co., St. Louis, USA)
- Sodium borohydride (BDH, Poole, UK)
- Sodium hydroxide (BDH, Poole, UK)
- Ammonium acetate (Merck AG, Darmstadt, Germany)
- Acetonitrile (HPLC grade) (Merck AG, Darmstadt, Germany)
3.3.2 Reagents

0.042g NaBH₄ in 10 ml 50 mM Tris HCL buffer, pH 9.0, to give a final concentration of NaBH₄ of 0.11 mol/L

10 % TCA containing 1 mmol/L EDTA

1.55 mol/L NaOH

0.125 mol/L borate buffer containing 4 mmol/L EDTA, pH 9.5

SBD-F 1 mg/ml in 0.125 mol/L borate buffer without EDTA

400 mmol/L N-2 mercaptopropionyl glycine

3.3.3 Instrumentation

The analysis was performed on a Hewlett Packard HP 1090 Liquid Chromatograph with a HP RP18 5 um 200x4.6 mm column protected by a C18 5 um 20x4 mm id ODS guard column. The fluorescence intensities were measured with excitation at 385 nm and emission at 515 nm using a HP 1049A fluorescence detector. The detector signal was recorded by a HP 3396A integrator.
3.3.4 Sample preparation

3.3.4.1 Reduction

200 ul thawed plasma was pipetted into a 1.5 ml snap-cap Eppendorf tube. 100 ul freshly prepared 0.11 M sodium borohydride in 50 mM Tris-HCL, pH 9.0 was added. The vial was capped, mixed on a vortex mixer for 1 minute and incubated at 30°C for 30 mins. After incubation, 200 ul of cold 10% TCA- 1mM EDTA was added to precipitate the protein. The sample was well mixed on a vortex mixer for 2 minutes and left at room temperature for 10 minutes. The vial was centrifuged at 10,000 rpm for 3 minutes.

3.3.4.2 Derivatization:

100 ul clear supernatant was added to a mixture of 20 ul 1.55 M sodium hydroxide, 250 ul of 0.125 M borate buffer (pH 9.5) containing 4 mM EDTA, 100 ul of SBD-F (1 mg/ml dissolved in borate buffer without EDTA) and 5 ul of 400 uM N-2 mercaptopropionyl glycine, in an Eppendorf tube. The mixture was mixed for 30 seconds on a vortex mixer and then incubated for 1 hour at 60°C with constant
shaking in a water bath, for derivatization of homocysteine to take place. After incubation, the tube was put on ice until analysis. 40 ul of the derivatized sample was injected.

3.3.5 Chromatographic conditions

The mobile phase was 0.1 M ammonium acetate buffer with 5% acetonitrile pH 3.27 (50 ml of acetonitrile in 950 ml of 0.1 M ammonium acetate buffer, pH was adjusted with glacial acetic acid). The flow rate was 1.3 ml/min at a temperature of 43°C.

3.3.6 Standard preparation

Stock standard 1 mM was prepared by adding 0.01352 g of homocysteine powder in 100 ml deionised water and was diluted with deionised water to give working standards with concentrations of 100, 200, 300, 400, 500, 600 and 800 umol/l. 40 ul of a working standard was added to 360 ul of normal pool serum and assayed as patient sample.
3.4 Method Optimization

3.4.1 Choice of reducing agent

3.4.1.1 Dithiotreitol (DTT)

Dithiotreitol (46,47) was first tried as reducing agent. 0.5 ml plasma was added to a mixture of 100 ul 0.2 M sodium tetraborate pH 8.3 (47) and 25 ul 0.8 M dithiotreitol in distilled water, and was incubated at 37°C for 20 mins for derivatization to occur.

The thiol group of dithiotreitol also takes up the fluorogenic dye and in our chromatograph gave late eluting and very large and long peaks which took 20 minutes to return to the baseline. As column switching was not possible on our instrument, DTT was thought not to be a suitable reducing agent and was abandoned. (chromatogram 1).
Chromatogram 1. Use of DTT as reducing agent

3.4.1.2 Sodium borohydride

In the method of Jacobson D. W. et al. (48), sodium borohydride has been used as reducing agent. The
The efficacy of sodium borohydride as a reducing agent had proved good as in samples stored at -25°C for one week, no homocysteine peak was detected when no reduction had taken place, whereas homocysteine was detected when reduction was carried out with a recovery of 101%. Further, sodium borohydride did not form peaks likely to interfere with homocysteine measurements, nor did it prolong the running time (chromatogram 2a and 2b).
Chromatogram 2. Use of NaBH₄ as reducing agent

2a. With reduction

2b. No reduction
3.4.2 Choice of precipitating reagent

In order to avoid binding of protein to the column, protein from which homocysteine had been removed by reduction, had to be precipitated as thoroughly as possible. Methanol, ethanol and acetonitrile had all been tried but the precipitation was incomplete. With the addition of 10% TCA into the supernatant, protein precipitated further. 10% TCA precipitated protein from plasma completely and was used for precipitation. 1 mmol/L EDTA was added to the TCA solution to prevent oxidation of homocysteine (42).

3.4.3 Optimization of chromatographic conditions

3.4.3.1 Flow rate, temperature and organic composition of mobile phase

In order to shorten the analysis time, 6% acetonitrile with a flow rate of 1.5 ml/min at a temperature of 45°C was tried, but this resulted in incomplete resolution of the homocysteine peak (chromatogram 3). The conditions were changed to 5% acetonitrile with a flow rate 1.3
ml/min at a temperature of 43°C. Complete resolution of homocysteine was achieved. It took 8 mins for the completion of HPLC analysis of one sample (chromatogram 4).

Incomplete resolution of homocysteine peak (6% acetonitrile, flow rate 1.5 ml/min, temperature 45°C)
Chromatogram 4. Complete resolution of homocysteine peak (5% acetonitrile, flow rate 1.3 ml/L, temperature 43°C)
3.4.3.2 **pH of the mobile phase**

Complete resolution was achieved with the pH of the mobile phase of 3.27. With more alkaline mobile phases, there was incomplete resolution of the homocysteine peak (chromatogram 5).

**Chromatogram 5. Effect of pH on peak separation**
3.4.4 Confirmation of homocysteine peak

The homocysteine peak was confirmed by comparing the retention time of homocysteine standards with those in plasma samples and also by co-eluting a mixture of standard and sample.

3.5 Analysis of results

The standard curves were plotted as the ratio of the peak height of homocysteine over that of the internal standard versus concentration of homocysteine. The homocysteine concentrations in patient and control plasma samples were determined from the graph.

3.6 Method validation

3.6.1 Linearity

Stock homocysteine standard was diluted to give a series of working standard concentrations as shown in Table 3.1. 10 μl of working standards were added to 90 μl plasma and the analytical procedure was identical to that used in patient and control plasma.
<table>
<thead>
<tr>
<th>Working standard concentration (µmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 200 400 600 800 1000</td>
</tr>
<tr>
<td>Standard (µL)</td>
</tr>
<tr>
<td>100 200 400 600 800 1000</td>
</tr>
<tr>
<td>(1000 µmol/L)</td>
</tr>
<tr>
<td>D.W. (µL)</td>
</tr>
<tr>
<td>900 800 600 400 200 0</td>
</tr>
</tbody>
</table>

D.W. = Deionized water

Table 3.1  Preparation of working standard

In figure 3.1 is shown a typical standard curve. A linear relationship \((r=0.995, y=0.0193x-0.0692)\) was observed between the peak height ratios of homocysteine to 2-mercaptopropionyl glycine and the concentrations of homocysteine standard in human plasma.

The linearity range of this method was up to at least 100 µmol/L. This was higher than the highest expected plasma homocysteine concentration after methionine loading (49).
Fig. 3.1 A typical plasma homocysteine standard curve
\[ y = 0.0193x - 0.0692 \quad r = 0.995 \]
3.6.2 Precision

Precision was done by spiking different concentrations of standard to plasma to give final concentrations of 20, 60 and 80 umol/L respectively and assayed as patient samples.

Table 3.2 shows the results of within-run and between-run imprecision. The within-run coefficient of variation varied from 3.74 to 7.75 per cent and the between-run coefficient of variation varied from 4.24 to 16.6 per cent.
<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>mean (s.d.)</th>
<th>C.V. %</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Within-run</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Std. 20</td>
<td>16</td>
<td>20.74 (1.61)</td>
<td>7.75</td>
</tr>
<tr>
<td>Std. 60</td>
<td>16</td>
<td>54.23 (3.25)</td>
<td>6.00</td>
</tr>
<tr>
<td>Std. 80</td>
<td>16</td>
<td>82.91 (3.10)</td>
<td>3.74</td>
</tr>
<tr>
<td><strong>Between-run</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Std. 20</td>
<td>14</td>
<td>19.73 (3.27)</td>
<td>16.60</td>
</tr>
<tr>
<td>Std. 60</td>
<td>14</td>
<td>53.86 (5.68)</td>
<td>10.55</td>
</tr>
<tr>
<td>Std. 80</td>
<td>7</td>
<td>81.84 (3.39)</td>
<td>4.24</td>
</tr>
</tbody>
</table>

Table 3.2 Total plasma homocysteine imprecision studies

3.6.3 Recovery

Table 3.3 shows the results of recovery studies obtained by spiking plasma standards to obtain the assigned concentrations. For each concentration 10 plasma samples were spiked. Only the mean of the observed values are shown. The recoveries ranged from 90.8% to 109.9%.
<table>
<thead>
<tr>
<th></th>
<th>Std. 20</th>
<th>Std. 60</th>
<th>Std. 80</th>
</tr>
</thead>
<tbody>
<tr>
<td>Observed value</td>
<td>20.53</td>
<td>54.46</td>
<td>87.88</td>
</tr>
<tr>
<td>Expected value</td>
<td>20.00</td>
<td>60.00</td>
<td>80.00</td>
</tr>
<tr>
<td>Recovery %</td>
<td>102.70</td>
<td>90.80</td>
<td>109.90</td>
</tr>
</tbody>
</table>

Table 3.3  Total plasma homocysteine recovery studies
CHAPTER FOUR

Results

4.1 The pre- and post-methionine loading plasma homocysteine concentrations in patients and controls

One control subject had the pre-methionine load plasma homocysteine concentration above the mean plus 2.5 and 3 SD and another had the post-methionine load plasma homocysteine concentration above the mean plus 2.5 and 3 SD of the grouped control data. Thus, the data in patients was compared with that in controls before and after removing the control 'outliers' above 2.5 SD and recalculating the mean.

Table 4.1 shows the pre- and post-methionine loading homocysteine concentrations in plasma of controls and patients and the amount by which amino acid levels increased after the load.

The mean homocysteine concentration was significantly higher in patients than in controls both in the pre- and the post-load samples, as was the difference between the
two, both with and without the two control outliers.

<table>
<thead>
<tr>
<th></th>
<th>Homocysteine concentration (μmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pre-loading mean (SD)</td>
</tr>
<tr>
<td>Patient n=32</td>
<td>16.0 (3.9)</td>
</tr>
<tr>
<td>Control n=21 **</td>
<td>11.6 (3.5)</td>
</tr>
<tr>
<td>p</td>
<td>0.0000</td>
</tr>
<tr>
<td>Patient n=32</td>
<td>16.0 (3.9)</td>
</tr>
<tr>
<td>Control n=23 *</td>
<td>12.4 (4.5)</td>
</tr>
<tr>
<td>p</td>
<td>0.004</td>
</tr>
</tbody>
</table>

* including all controls
** without two controls, one with the fasting hyperhomocysteinaemia above the control mean +2.5 and 3 SD and one with post-methionine hyperhomocysteinaemia above the control mean +2.5 and 3 SD

Table 4.1 The pre- and post-methionine loading plasma homocysteine concentrations and the rise in the homocysteine concentrations after methionine in patients and controls
4.2 The frequency distributions of hyperhomocysteinaemia in patients and controls

4.2.1 The distributions of homocysteinaemia in patients and controls

Figs. 4.1, 4.2 and 4.3 show the distribution of the fasting and post-loading homocysteine concentrations, and the response to methionine in patients and controls. The distributions were not Gaussian.

4.2.2 The frequency distributions of fasting hyperhomocysteinaemia in patients and controls

Table 4.2 shows the number and percentage of patients with pre-load plasma homocysteine levels above the mean + 2, 2.5 or 3 SD of the control group with and without removing the two control subjects with hyperhomocysteinaemia from the group. There was no significant statistical difference in the distribution of homocysteinaemia although a tendency was noted towards more frequent hyperhomocysteinaemia in patients both with and without removing the two controls as described earlier (18.8% versus 4.8% at the mean +2.5SD cutoff point).
Fig. 4.1  The distribution of the fasting plasma homocysteine concentrations in patients and controls
Fig. 4.2 The distribution of the post-methionine loading plasma homocysteine concentrations in patients and controls
Fig. 4.3 The distribution of the methionine tolerance in patients and controls
<table>
<thead>
<tr>
<th>Homocysteine Conc. greater than</th>
<th>Mean+2SD</th>
<th>Mean+2.5SD</th>
<th>Mean+3SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient n=32</td>
<td>7 (21.9%)</td>
<td>6 (18.8%)</td>
<td>2 (6.3%)</td>
</tr>
<tr>
<td>Control n=21</td>
<td>1 (4.8%)(^a)</td>
<td>1 (4.8%)(^b)</td>
<td>0 (0.0%)(^c)</td>
</tr>
<tr>
<td>Patient n=32</td>
<td>4 (12.5%)</td>
<td>0 (0.0%)</td>
<td>0 (0.0%)</td>
</tr>
<tr>
<td>Control n=23</td>
<td>1 (4.3%)(^d)</td>
<td>1 (4.3%)(^e)</td>
<td>1 (4.3%)(^f)</td>
</tr>
</tbody>
</table>

\(a\) \(p = 0.1902\) \(b\) \(p = 0.2908\)  
\(c\) \(p = 0.6665\) \(d\) \(p = 0.5742\)  
\(e\) \(p = 0.8671\) \(f\) \(p = 0.8671\)

**Table 4.2** The frequency of fasting hyperhomocysteinaemia in patients and controls
4.2.3 The frequency distributions of post-methionine hyperhomocysteinaemia in patients and controls

Table 4.3 shows the number and percentage of patients with post-load plasma homocysteine levels above the mean $+ 2$, $2.5$ or $3$ SD of the control group with and without removing the two controls with hyperhomocysteinaemia.

Although there was no statistically significant difference in the distribution of hyperhomocysteinaemia between the two groups when all the controls were included, there was a tendency to a higher homocysteine levels in patients since $25.0$ and $18.8$ % had raised homocysteine levels, compared to $8.7$ and $4.3$ % in controls, at the $2$ and $2.5$ SD cutoff points. On removing the two controls as described earlier, a significant difference was noted in the distribution at the $2$ and $2.5$ SD cutoff values with $40.6$ and $28.1$ % of patients having hyperhomocysteinaemia compared to none in the control group. At the $3$ SD cutoff point $18.8$% of patients had hyperhomocysteinaemia, compared to none in controls, but the difference was not statistically significant.
<table>
<thead>
<tr>
<th></th>
<th>Homocysteine Conc. greater than</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean+2SD</td>
</tr>
<tr>
<td><strong>Patient</strong> n=32</td>
<td>13 (40.6%)</td>
</tr>
<tr>
<td><strong>Control</strong> n=21</td>
<td>0 (0.0%) a</td>
</tr>
<tr>
<td><strong>Patient</strong> n=32</td>
<td>8 (25.0%)</td>
</tr>
<tr>
<td><strong>Control</strong> n=23</td>
<td>2 (8.7%) d</td>
</tr>
</tbody>
</table>

a \( p = 0.0024 \)  \quad b \( p = 0.0218 \)

\[ p = 0.0961 \]  \quad d \( p = 0.2333 \)

\[ p = 0.2417 \]  \quad f \( p = 0.8557 \)

**Table 4.3**  The frequency of post-methionine loading hyperhomocysteinaemia in patients and controls.
4.2.4 The frequency distributions of the abnormal methionine tolerance in patients and controls

Table 4.4 shows the number and percentage of patients with abnormal methionine tolerance (abnormal rise in homocysteine after methionine loading) with and without removing control subjects with hyperhomocysteinaemia from the control group.

There was no significant difference between the results between the groups when all the controls were included, although more patients than controls had abnormal methionine tolerance. On removing the controls as described earlier, the difference between the pre- and post-methionine loading homocysteine concentrations was above the mean plus 2 and 2.5 SD of the control value in 34.4% and 21.9% of patients and in none of the controls. These differences were statistically significant.
<table>
<thead>
<tr>
<th></th>
<th>Homocysteine Conc. greater than</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean+2SD</td>
<td>Mean+2.5SD</td>
<td>Mean+3SD</td>
</tr>
<tr>
<td>Patient n=32</td>
<td>11 (34.4%)</td>
<td>7 (21.9%)</td>
<td>5 (15.6%)</td>
</tr>
<tr>
<td>Control n=21</td>
<td>0 (0.0%)a</td>
<td>0 (0.0%)b</td>
<td>0 (0.0%)c</td>
</tr>
<tr>
<td>Patient n=32</td>
<td>7 (21.9%)</td>
<td>4 (12.5%)</td>
<td>3 (9.4%)</td>
</tr>
<tr>
<td>Control n=23</td>
<td>1 (4.3%)d</td>
<td>1 (4.3%)e</td>
<td>0 (0.0%)f</td>
</tr>
</tbody>
</table>

| a | p = 0.0075 | b | p = 0.0593 |
| c | p = 0.1547 | d | p = 0.1525 |
| e | p = 0.5742 | f | p = 0.3637 |

**Table 4.4**  
Frequency of abnormal methionine tolerance in patients and controls
4.3 The frequency distributions of hypertension and hyperlipidaemia in controls and, including smoking, in patients without and with hyperhomocysteinaemia

In most publications the cutoff value for homocysteinaemia in patients was taken as the mean +2 SD of the mean control level after removing control outliers and recalculating the mean. In this study the cutoff value was taken at above 2.5 SD of the recalculated mean in controls, and was thus stricter and included 6 (18.8%) patients with fasting, 9 (28.12%) patients with post-methionine load hyperhomocysteinaemia, 7 (21.9%) patients with methionine intolerance and one control (4.8%) with fasting hyperhomocysteinaemia.

4.3.1. The frequency distribution of hypertension and hyperlipidaemia in patients and controls

Table 4.5 shows the frequency distribution of hypertension and hyperlipidaemia in patients and in controls. There was a preponderance of males in both groups. This could not be avoided because all subjects who fulfilled the entry criteria and agreed to the methionine loading test were accepted, irrespective of
the gender, and males predominated. There was no significant statistical difference in the distribution of hyperlipidaemia or hypertension between the groups. However, in both controls and patients there was a high (but similar) frequency of hypercholesterolaemia. Further, twice more patients had raised triglycerides than controls. Hypertension, was, though, twice more frequent in controls.

<table>
<thead>
<tr>
<th></th>
<th>Patients</th>
<th></th>
<th>Controls</th>
<th></th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N/Total</td>
<td>%</td>
<td>N/Total</td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>Hypertension</td>
<td>7/32</td>
<td>21.9</td>
<td>9/21</td>
<td>42.9</td>
<td>0.1863</td>
</tr>
<tr>
<td>Hypercholesterol</td>
<td>21/32</td>
<td>65.6</td>
<td>13/21</td>
<td>61.9</td>
<td>0.9868</td>
</tr>
<tr>
<td>Hypertrig.</td>
<td>9/32</td>
<td>28.1</td>
<td>3/21</td>
<td>14.3</td>
<td>0.3998</td>
</tr>
<tr>
<td>Sex M</td>
<td>27/32</td>
<td>84.4</td>
<td>17/21</td>
<td>81.0</td>
<td>0.9606</td>
</tr>
<tr>
<td>Sex F</td>
<td>5/32</td>
<td>15.6</td>
<td>4/21</td>
<td>19.0</td>
<td>0.9606</td>
</tr>
<tr>
<td>Pre-load hyperhomocyst</td>
<td>6/32</td>
<td>18.8</td>
<td>1/21</td>
<td>4.8</td>
<td>0.2908</td>
</tr>
<tr>
<td>Post-load hyperhomocyst</td>
<td>9/32</td>
<td>28.1</td>
<td>0/21</td>
<td>0</td>
<td>0.0218</td>
</tr>
<tr>
<td>Hyperhomocyst. between pre and post</td>
<td>7/32</td>
<td>21.9</td>
<td>0/21</td>
<td>0</td>
<td>0.0593</td>
</tr>
</tbody>
</table>

Table 4.5 The frequency distribution of hypertension and hyperlipidaemia and the gender distribution in patients and controls
4.3.2 The frequency distributions of hyperlipidaemia, hypertension, smoking and gender in patients with vascular disease with and without hyperhomocysteinaemia

Table 4.6 shows the frequency distribution of hyperlipidaemia, hypertension and smoking, as well as the gender distribution in patients with and without post-methionine hyperhomocysteinaemia. There was no significant difference in the distribution of hypertension or hyperlipidaemia although raised triglycerides were more frequent in patients with hyperhomocysteinaemia. Smoking was significantly more frequent in hyperhomocysteinaemics. Further all patients with hyperhomocysteinaemia who were smokers, were also males.

Note -- at the time of the study, the data on the smoking habits in controls was not thought accurate and is, thus, not included but will be in follow up studies on this thesis.
<table>
<thead>
<tr>
<th></th>
<th>Patients with vascular disease</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>hyper-homocysteinaemia</td>
</tr>
<tr>
<td></td>
<td>normo-homocysteinaemia</td>
</tr>
<tr>
<td>N/Total</td>
<td>%</td>
</tr>
<tr>
<td>------------------------</td>
<td>------------------------------------------------------------</td>
</tr>
<tr>
<td>Chol. 7/9 77.8</td>
<td>14/23 60.9 0.6231</td>
</tr>
<tr>
<td>Trig. 4/9 44.4</td>
<td>5/23 21.7 0.3969</td>
</tr>
<tr>
<td>H.T. 2/9 22.2</td>
<td>5/23 21.7 0.6557</td>
</tr>
<tr>
<td>Smoking 9/9 100.0</td>
<td>11/23 47.8 0.0195</td>
</tr>
<tr>
<td>Sex M 9/9 100.0</td>
<td>18/23 78.3 0.3264</td>
</tr>
<tr>
<td>Sex F 0/9 0.0</td>
<td>5/23 21.7 0.3264</td>
</tr>
</tbody>
</table>

Table 4.6 The frequency distribution of hyperlipidaemia, hypertension, smoking and gender in patients with vascular disease
4.4 The comparison of the age, haematological and biochemical indices and the blood pressure between the patients and controls

4.4.1 The comparison of the patients' age at presentation and plasma lipids following recovery from the acute episode with those in controls at the time of methionine loading

Since some patients were tested several years after the original presentation with vascular diseases and had been on lipid or hypertension lowering treatment, a comparison was made, where possible, between the age at first presentation, the fasting blood lipids after the acute episode but before any treatment was instigated, in patients, and in controls during methionine loading. The blood pressure indices were not compared because of their variability.
Table 4.7 shows the comparison of the age and the lipid concentrations in the patients at first presentation and in controls during the methionine loading test. There was no difference in the concentration of cholesterol but there was in the concentration of triglycerides, which, together with the age, were slightly higher in patients.

<table>
<thead>
<tr>
<th></th>
<th>patients</th>
<th>controls</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mean (SD)</td>
<td>n</td>
<td>mean (SD)</td>
</tr>
<tr>
<td>Age/years</td>
<td>50.62(5.7)</td>
<td>32</td>
<td>44.2(6.7)</td>
</tr>
<tr>
<td>Chol.(mmol/L)</td>
<td>5.87(1.3)</td>
<td>25</td>
<td>5.51(0.89)</td>
</tr>
<tr>
<td>Trig.(mmol/L)</td>
<td>1.77(0.8)</td>
<td>21</td>
<td>1.26(0.7)</td>
</tr>
</tbody>
</table>

Table 4.7  Age and lipid concentrations in patients and controls
4.4.2 The comparison of the age at presentation and the plasma lipids in patients with and without hyperhomocysteinaemia

<table>
<thead>
<tr>
<th>Patients with vascular disease</th>
<th>hyperhomocysteinaemia</th>
<th>normohomocysteinaemia</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>mean (SD) n</td>
<td>mean (SD) n</td>
<td>p</td>
<td></td>
</tr>
<tr>
<td>Age/years</td>
<td>53.9 (5.9) 9</td>
<td>55.9 (7.2) 23</td>
<td>0.4608</td>
</tr>
<tr>
<td>Chol. (mmol/L)</td>
<td>6.20 (1.8) 9</td>
<td>5.7 (1.0) 23</td>
<td>0.3200</td>
</tr>
<tr>
<td>Trig. (mmol/L)</td>
<td>2.20 (2.4) 9</td>
<td>1.4 (0.8) 23</td>
<td>0.1440</td>
</tr>
</tbody>
</table>

Table 4.8 The age and plasma lipids concentrations in patients at presentation

The age and plasma lipids concentrations at presentation in patients with and without hyperhomocysteinaemia are shown in Table 4.8. There were no significant differences between the two groups.
4.4.3 The comparison of the $\text{B}_12$, serum folate and RBC folate in patients and controls at the time of presentation

The $\text{B}_12$, serum folate and red blood cell folate at the time of methionine loading are shown in Table 4.9. There were no significance differences between the two groups.

<table>
<thead>
<tr>
<th></th>
<th>patient</th>
<th>control</th>
<th>$p$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\text{B}_12$ (pg/ml)</td>
<td>329±121</td>
<td>339±86</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>serum folate (nmol/L)</td>
<td>26.9±9.6</td>
<td>21.65±9.7</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>RBC folate (nmol/L)</td>
<td>657±242</td>
<td>538±273</td>
<td>&gt;0.05</td>
</tr>
</tbody>
</table>

Table 4.9 The $\text{B}_12$, serum folate and red blood cell folate between patients and controls

The other haematological and biochemical indices are not shown, as there were no significant differences between the patients and controls. However, 2 patients had borderline raised bilirubin and alanine amino transferase
activities, which were, thought not to be significant enough to affect homocysteine metabolism, and which were only noted in subjects with unremarkable pre- and post-methionine load plasma homocysteine levels.

4.4.4. The comparison of the B₁₂, serum folate and RBC folate levels in patients with and without hyperhomocysteinaemia and in controls

<table>
<thead>
<tr>
<th>RBC folate reduced</th>
<th>Controls</th>
<th>Patients</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>% of all</td>
<td>n</td>
</tr>
<tr>
<td>All Subjects</td>
<td>8</td>
<td>38.1</td>
<td>5</td>
</tr>
<tr>
<td>Subjects with raised fasting homocysteine levels</td>
<td>1</td>
<td>4.8</td>
<td>2</td>
</tr>
<tr>
<td>Subjects with raised post-load homocysteine levels</td>
<td>2</td>
<td>9.5</td>
<td>2</td>
</tr>
</tbody>
</table>

Table 4.10 The frequency distribution of low and normal RBC folate concentrations in controls and patients with 'normal' and raised homocysteine levels
Table 4.10 shows the frequency distribution of low and normal RBC folate concentrations in controls and patients with 'normal' and raised homocysteine levels. Only one control, with 'normal' homocysteine levels, had a serum B₁₂ level below the lower limit of the reference range. The serum folate was normal in all subjects. Eight controls had low RBC folate levels which were associated with 'normal' homocysteine levels in five, a raised fasting homocysteine level in one and an abnormal response to methionine in two. Five patients had reduced RBC folate levels, of which in two the fasting homocysteine levels was raised and in two the response to methionine was abnormal. On statistical analysis, there were no differences in the frequency of low RBC folate in controls and patients nor in patients with normal and high homocysteine levels.
CHAPTER FIVE

Discussion

HPLC method

The method we used for homocysteine measurement involves a number of manual preparation steps such as sample reduction, protein precipitation and derivatization. These procedures introduced inherent random errors affecting the precision of the assay. Further, the internal standard used, 2-mercaptopropionyl glycine gave broad, late eluting internal standard peaks which made quantitation difficult. Birta et al. (50) obtained sharp, well separate peaks with the same internal standard, but their separation conditions and sample preparation steps were different and could not be incorporated in this study. Other workers even measured homocysteine without an internal standard (49).

The assay had acceptable within-run precision, with the coefficient of variation varying from 3.74% to 7.75%. The between-run precision was poorer, with coefficient of variation varying from 4.24% to 16.6%. Although the within-batch imprecision was comparable to those in published methods (49, 50), the between batch was
slightly poorer. However, for the purpose of this study all data, including occasional outliers, was included. If a better internal standard could have been obtained, the imprecision may have been improved.

The method had good linearity from 0 to at least 100 μmol/L. Linear regression analysis yielded $y = 0.0193x - 0.0692$ where $y$ was the peak height ratio of homocysteine to internal standard, $x$ the concentration of homocysteine and the correlation coefficient 0.995. In heterozygous homocysteinaemia, after methionine loading, the homocysteine concentration seldom increases to more than 100 μmol/L. Therefore the linearity range obtained in this study was thought acceptable for homocysteine measurements to identify heterozygotes.

The recovery of homocysteine, added to serum samples was 101.1% (S.D. = 9.7). This compared favourably with the other report using the same SBD-F (ammonium 7-fluorobenzo-2-oxa-1,3 diazole-4-sulphonate) reagent (104.3±5.4%) (51).
Homocysteinaemia in patients and controls

Hyperhomocysteinaemia is usually due to cystathionine $\beta$-synthase deficiency. Homozygous homocysteinaemia is rare, but may, in the heterozygous state affect up to 1 in 70 to 1 in 200 general population (19). Cystathionine $\beta$-synthase deficiency blocks the transsulfuration pathway from methionine to cysteine, resulting in homocysteinaemia. Defects in vitamin B$_{12}$ and folate dependent remethylation of homocysteine constitute other rare causes of inherited hyperhomocysteinaemia (18). Liver disease and drugs which affect the metabolism of methionine are also aetiological factors for hyperhomocysteinaemia (1). Fasting homocysteine levels are reported raised in homozygous homocysteinaemia, in defects in vitamin B$_{12}$ and folate metabolism but are normal with vitamin B$_{6}$ deficiency and in heterozygotes homocysteinaemia (19). However, after methionine loading, cobalamin deficient patients and patients with methylenetetrahydrofolate reductase deficiency are reported to have normal increases in plasma homocysteine levels, whereas patients who are homozygous or obligate heterozygotes for cystathionine $\beta$-synthase deficiency
show an abnormal response (9). Thus, by measuring the fasting and post-methionine loading homocysteine concentrations, one should be able to differentiate whether the cause of hyperhomocysteinaemia is due to defects in vitamin metabolism or due to cystathionine β-synthase insufficiency. In our study, therefore, the fasting and post-methionine loading homocysteine concentration were measured.

Boers and co-worker (19) measured the post-methionine loading free homocysteine levels in post-menopausal women and observed them to be higher than in pre-menopausal women, while later Lars Brattström (17) found no such difference in the total plasma homocysteine levels and suggested that measurement of the total homocysteine concentration would better discriminate between heterozygotes for homocystinuria and normal subjects, especially in women. Therefore, in our study, where both men and women were included, we measured total homocysteine concentrations.

The association of hyperhomocysteinaemia with atherosclerosis and predisposition to venous and arterial
thromboembolism in homozygous hyperhomocysteinaemia is well known and is thought to be due to raised levels of homocysteine (20). Increased risk, for vascular disease is suggested also in heterozygotes (18). In 1985, Boers et al. (19) showed that a large proportion of patients with cerebral or peripheral occlusive vascular disease responded to methionine loading with abnormally high homocysteine concentrations, which were within the same range as found in obligate heterozygotes for homocystinuria due to cystathionine β-synthase deficiency. As a low activity of the enzyme was also found in the cultured skin fibroblasts from all their patients with an abnormal response to methionine, Boers et al. (19) considered them to be heterozygotes for cystathionine β-synthase deficiency and suggested that the methionine loading test could detect most heterozygotes.

Since the reference range for normal homocysteine levels is not fully established yet, in most studies where homocysteine was measured in patients with vascular disease, the amino acid was also measured in controls, and various cutoff values were used to distinguish
hyperhomocysteinaemia from normohomocysteinaemia. Boers and Andersson (17) first removed control outliers with homocysteine concentrations in the fasting state or an after loading which exceed the mean + 3 SD, recalculated the mean and used the new mean + 2 SD as their cutoff values. In our study we also used the control mean + 3 SD to remove the control outliers (the same patients were outliers at 2.5 and 3 SD) but after recalculating the mean, we took the new mean + 2.5 SD as the cutoff value, thus slightly stricter criteria than in the study of Boers and Andersson. Based on this, six out of 32 patients had fasting hyperhomocysteinaemia, giving the incidence of 18.8%, while in controls the incidence (one subject) was 4.8%. The frequency distribution between the two groups was thought not statistically different. In Boers' study, fasting homocysteinaemia was found only in two out of twenty premenopausal heterozygote patients giving a 10% incidence, but no significant difference was found in other subjects.

Although the frequency distribution of fasting hyperhomocysteinaemia was not statistically different between the two groups, we found that the fasting homocysteine
concentration was significantly higher in the patient groups (16.0 ± 3.9 umol/L) than in the controls (11.6 ± 3.5 umol/L). This is different from a previous report where no differences were found between patients and controls (19).

The incidence of hyperhomocysteinaemia after methionine loading (with post-methionine homocysteine concentration greater than 45.9 umol/L and after two of the control outliers were excluded) was 28.1% in patients and 0% in controls. This frequency distribution was significantly different between the groups and similar to that found in Boers' study, where an incidence of 28% in a group of 50 Western patients was found. Thus, in this first study in the Chinese population, the incidence of hyperhomocysteinaemia in patients with vascular disease appears to be the same as that in the Western population.

In the control group there was one subject with fasting hyperhomocysteinaemia and one with post-methionine hyperhomocysteinaemia above 3 SD before removing them as outliers, giving an incidence of 4.3% for each. Further, after removing the outliers, there was still one control
with a fasting homocysteine concentration above 2.5 SD (thus total 8.7%). This was higher than previously reported (0% for fasting and 2.5% for post-methionine loading (19).

Most studies measure the pre- and post-methionine load concentrations (17,19,48). We also compared the difference between pre- and post-methionine loading homocysteine concentrations because the fasting state level depends on a number of factors such as the vitamin status while the post-load state must depend at least partly on the fasting level. Thus it was thought that the difference between the two levels may better reflect the adequacy of enzyme activity than the post-load level alone.

As with the post-methionine homocysteine concentration, methionine intolerance was significantly different both as frequency distribution and as actual levels, between the patients and controls. However, although fewer patients had methionine intolerance than had raised post-methionine levels, the discriminating value of this index was less than was hoped. It had been expected that in
patients with raised post-methionine homocysteine levels and the 'normal' difference between the pre- and post-load levels, raised fasting levels and vitamin deficiencies would be obtained. This was not, however, usually the case.

No subject had reduced plasma folate. The B12 was reduced in one control only, who had normal homocysteine levels. In addition, twice as many controls had reduced RBC folate levels (38.1%) as patients (15.6%), and in both groups, this was associated with raised fasted homocysteine levels only in the minority. Thus, although a larger proportion of patients than controls had raised fasting homocysteine levels, this did not appear to be because of B12 or folate deficiency. Therefore, in patients with raised fasting homocysteine levels, heterozygous hyperhomocysteinaemia was more likely although the diagnosis of the heterozygous state should be confirmed by measuring the enzyme, at least, until the reference range for the three indices used in the studies is established with a larger sample population.

There was no statistically significant difference in the
occurrence of hypertension and hyperlipidaemia between patients and controls, nor between patients with 'normal' and raised post-methionine homocysteine levels. However, the incidence of hypercholesterolaemia was high in both patients and controls, hypertension twice more frequent in controls and raised triglycerides twice more common in patients. Raised triglycerides were also twice more frequent in patients with than without hyperhomocysteinaemia.

However, on comparison of the actual levels, though both the cholesterol and triglycerides concentration were higher in patients with hyperhomocysteinaemia than in controls, only the latter was highly significant, suggesting that hyperhomocysteinaemia may be mainly an independent risk factor, although it may interact slightly with hypertriglyceridaemia, in the pathogenesis of vascular disease.

In conclusion, the results of this study showed that there is a similarly high incidence of mild hyperhomocysteinaemia in patients with premature occlusive
vascular disease in the Chinese as in other populations and that hyperhomocysteinaemia may be an important risk factor for premature occlusive peripheral arterial or cerebrovascular disease. This deserves attention. If further studies establish that homocysteine lowering treatment may prevent the progression of the arteriosclerotic lesions or to prevent the occurrence of premature occlusive vascular disease, particularly where there is an absence of other risk factors for atherosclerosis, investigation for mild hyperhomocysteinaemia may become as important as detection of hypertension or hyperlipidaemia in the prevention of premature vascular coronary artery disease.
REFERENCES


53. Hyland K, Bottiglieri T. Measurement of total plasma and cerebrospinal fluid homocysteine by