EMERGING RISK FACTORS AND OUTCOME PREDICTORS OF CORONARY ARTERY DISEASE IN A SRI LANKAN POPULATION

BY

PORUTHOTAGE PRADEEP RASIKA PERERA

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PORUTHOTAGE PRADEEP RASIKKA PERERA

Thesis submitted to the University of Sri Jayewardenepura for the award of the Degree of Doctor of Philosophy in Biochemistry on 30th January 2009.
DECLARATION BY THE CANDIDATE

The work in this thesis was carried out by me under the supervision of Professor Hemantha Peiris (Head of the Department of Biochemistry, Faculty of Medical Sciences, University of Sri Jayewardenepura), Professor Lal Chandrasena (Professor of Biochemistry, Department of Biochemistry, Faculty of Medicine, University of Kelaniya) and Dr. J. Indrakumar (Senior Lecturer, Department of Medicine, Faculty of Medical Sciences, University of Sri Jayewardenepura) and a report on this has not been submitted in whole or in part to any University or any other institution for another Degree/Diploma.

Poruthotage Pradeep Rasika Perera

30.01.2009

Date
DECLARATION BY THE SUPERVISORS

We certify that the above statement by the candidate is true and that this thesis is suitable for submission to the University for the purpose of evaluation.

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(Supervisor)

Date: 30th January 2009
I dedicate this thesis to
my parents, my wife Chandana
and my daughters
Pavithri, Kaveetha and Dilini.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>List of Tables</td>
<td>xiv</td>
</tr>
<tr>
<td>List of Figures</td>
<td>xvii</td>
</tr>
<tr>
<td>List of Plates</td>
<td>xviii</td>
</tr>
<tr>
<td>Abbreviations</td>
<td>xix</td>
</tr>
<tr>
<td>Acknowledgement</td>
<td>xxii</td>
</tr>
<tr>
<td>Abstract</td>
<td>xxv</td>
</tr>
</tbody>
</table>

## 1. INTRODUCTION

### 1.1 General Introduction

### 1.2 Cardiovascular risk factors

#### 1.2.1 Non-modifiable risk factors

- 1.2.1.1 Age
- 1.2.1.2 Gender
- 1.2.1.3 Heredity / family history
- 1.2.1.4 Ethnicity

#### 1.2.2 Modifiable risk factors

- 1.2.2.1 Hypertension
1.2.2.2 Hypercholesterolaemia and hyperlipidemia

i. Elevated total cholesterol

ii. Elevated Low-Density Lipoprotein cholesterol

(LDL-cholesterol)

iii. Low High Density lipoprotein Cholesterol

(HDL-cholesterol)

1.2.2.3 Effect of tobacco smoking

1.2.2.4 Overweight and obesity

1.2.2.5 Diabetes mellitus

1.2.2.6 Effect of alcohol

1.2.2.7 Infrequent exercise/ Physical inactivity

1.3 Traditional vs. Novel coronary risk factors

1.4 Justification

1.5 Objectives

1.5.1 General Objectives

1.5.2 Specific Objectives

2. LITERATURE REVIEW

2.1 Homocysteine and CAD

2.1.1 Homocysteine metabolism

2.1.2 Homocysteine in plasma/Serum
2.1.3 Factors affecting plasma (serum) homocysteine levels

2.1.3.1 Metabolic derangements affecting plasma (serum) homocysteine levels

2.1.3.2 Genetic mutations affecting plasma (serum) homocysteine levels

2.1.3.3 Vitamin deficiencies affecting plasma (serum) homocysteine levels

2.1.4 Other contributory factors affecting homocysteine levels

2.1.5 Homocysteine and its association with coronary artery disease

2.1.5.1 Homocysteine as a risk factor for CAD

2.1.5.2 Mechanisms responsible for atherosclerosis and CAD in hyperhomocysteinaemia

2.2 Apolipoproteins and CAD

2.2.1 Apolipoprotein A and apolipoprotein B

2.2.2 Apolipoprotein A-I and apolipoprotein B in atherosclerosis and CAD

2.2.2.1 Lipids and their role in atherosclerosis

2.2.2.2 Assessment of lipid related risk of cardiovascular disease

2.2.2.3 Apolipoprotein A-I and apolipoprotein B in the assessment of risk of cardiovascular disease

2.3 Glutathione peroxidase and CAD

2.3.1 Glutathione peroxidase
2.3.2 Glutathione peroxidase in atherosclerosis and CAD

2.4 Assessment of severity of coronary ischaemia

3. MATERIALS AND METHODS

3.1 Chemicals and reagents

3.1.1 Water

3.1.2 Chemicals

3.1.3 Special chemicals/Reagents

3.2 Methods

3.2.1 Selection of study subjects

3.2.1.1 Selection of study subjects for study 1 - Association of Hyperhomocysteinaemia with coronary artery disease in a Sri Lankan population.

   i. Cases

   ii. Controls

3.2.2 Selection of study subjects for study 2 - Association of Homocysteine, Vitamin B_{12}, Folate, Apolipoprotein A-I, Apolipoprotein B, and glutathione peroxidase in CABG patients

3.3 Collection of blood samples

3.3.1 Study 1:

3.3.2 Study 2
3.4 Data Collection

3.4.1 Study 1

3.4.2 Study 2:

3.5 Height, weight and Body Mass Index

3.6 Method development and analysis of homocysteine concentration by high performance liquid chromatography in study 1

3.6.1 Preparation and assay of plasma samples for homocysteine:

3.6.2 Apparatus and chromatographic conditions

3.6.3 Chromatogram patterns obtained with different mobile phase methanol concentrations

3.6.4 Comparison of the internal standards

3.6.5 Validation of assay method

3.6.6 Performance characteristics of the Homocysteine assay method

3.6.7 Calculation of homocysteine concentration

3.6.8 Quality control

3.7 Quantitative analysis of Vitamin B₁₂

3.7.1 Vitamin B₁₂ assay procedure

3.7.2 Reaction principle

3.7.3 Calibration

3.7.4 Quality control
3.8 Quantitative analysis of folic acid

3.8.1 Folic acid assay procedure
3.8.2 Reaction principle
3.8.3 Calibration
3.8.4 Quality control

3.9 Quantitative analysis of homocysteine in study 2

3.9.1 Homocysteine assay procedure
3.9.2 Biological principle of the procedure
3.9.3 Calibration
3.9.4 Quality control

3.10 Quantitative analysis of apolipoprotein A-I

3.10.1 Apolipoprotein A-I assay procedure
3.10.2 Reaction principle
3.10.3 Calibration
3.10.4 Quality control

3.11 Quantitative analysis of apolipoprotein B

3.11.1 Apolipoprotein B assay
3.11.2 Reaction principle
3.11.3 Calibration
3.11.4 Quality control
3.12 Quantitative analysis of glutathione peroxidase activity

3.12.1 Glutathione peroxidase assay procedure

3.12.2 Reaction principle

3.12.3 Calibration

3.12.4 Quality control

3.13 Genotyping to detect MTHFR C677T and A1298C polymorphisms

3.13.1 Polymerase chain reaction / restriction fragment length polymorphism

3.13.2 DNA extraction

3.13.3 Amplification of DNA segments of interest to detect MTHFR C677T and A1298C polymorphisms

3.13.3.1 Principle of the PCR procedure

3.13.3.2 PCR amplification of DNA for MTHFR C677T polymorphism analysis

i. PCR amplification of genomic DNA segment to analyze MTHFR C677T polymorphism using DMD 48 as internal control

ii. PCR amplification of genomic DNA segment to analyze MTHFR C677T polymorphism and amplification of bacteriophage λ DNA segment to use as internal control.

a). PCR amplification of genomic DNA segment to analyze MTHFR C677T:
b). Preparation of primers to generate 150bp bacteriophage λ fragment

c). PCR amplification of bacteriophage λ DNA segment
to use as an internal control

3.13.3.3 PCR amplification of DNA for MTHFR A1298C polymorphism analysis

3.13.4 Restriction endonuclease digestion of amplified DNA segments to detect MTHFR C677T and A1298C polymorphisms

3.13.4.1 Restriction endonuclease digestion of amplified DNA segments with Hinf I to detect MTHFR C677T polymorphism

i). Restriction endonuclease digestion of amplified DNA segments with Hinf I to detect MTHFR C677T polymorphism using DMD as internal control

ii. Restriction endonuclease digestion of amplified DNA segments with Hinf I to detect MTHFR C677T polymorphism using bacteriophage λ 150bp PCR product as internal control.

3.13.4.2 Restriction endonuclease digestion of amplified DNA segments with Mbo II to detect MTHFR A1298C polymorphism
3.13.5 Visualization of DNA fragments with agarose gel electrophoresis

3.13.5.1 Preparation of the agarose gel

3.13.5.2 Electrophoresis of prepared DNA samples using the prepared agarose gels.

3.13.6 Quality control

3.14 Calculation of severity of ischaemia in study 2

3.14.1 Calculation of vessel score

3.14.2 Calculation of stenosis score

3.14.3 Calculation of extent score

3.15 Data processing and analysis

3.16 Ethical considerations

4. RESULTS

4.1 Study I

4.1.1 Characteristics of study subjects

4.1.2 Association between Hyperhomocysteinaemia and CAD

4.1.3 Association between hyperhomocysteinaemia and CAD in the young and adults over 50 years of age

4.1.4 Association of risk of CAD in persons with two or less traditional risk factors for the development of CAD and with patients with 3 or more traditional risk factors
4.2 Study II

4.2.1 Characteristics of study subjects

4.2.1.1 General characteristics of the study subjects

4.2.1.2 Biochemical and anthropometric characteristics of the study population.

4.2.2 Association of vitamin B\textsubscript{12} and folate with Homocysteine concentrations in patients with coronary artery Disease.

4.2.3 MTHFR C677T/A1298C polymorphism analysis:

4.2.3.1 Comparison of the internal controls and results of agarose gel electrophoresis in MTHFR C677T polymorphism analysis

4.2.3.2 MTHFR A1298C polymorphism analysis using agarose gel electrophoresis

4.2.4 Association between Hyperhomocysteinaemia and MTHFR gene polymorphisms (A1298C, C677T polymorphism):

4.2.5 Severity of coronary artery disease in the study population

4.2.6 Association between vitamin B\textsubscript{12}, Folate, and Homocysteine concentrations with the severity of coronary artery disease.

4.2.7 Association of Apolipoprotein A-I, Apolipoprotein B and Glutathione peroxidase with the severity of coronary artery disease

4.2.8 Association of age with Apolipoprotein A-I, Apolipoprotein B, glutathione peroxidase concentrations in coronary artery disease patients
5. DISCUSSION

5.1 Association between Hyperhomocysteinaemia and CAD

5.1.1 High performance liquid chromatography in the assay of Homocysteine

5.1.2 Association between Hyperhomocysteinaemia and coronary artery disease

5.2 Association between hyperhomocysteinaemia and CAD in the young (age ≤ 50 years) compared to the elderly (age > 50 years).

5.3 Association between Hyperhomocysteinaemia and the number of risk factors (having two or less traditional risk factors compared to 3 or more traditional risk factors) among coronary artery disease patients.

5.4 Deficiency vitamin/s (vitamin B12 and folate) giving rise to hyperhomocysteinaemia in patients with coronary artery disease.

5.5 Association between Hyperhomocysteinaemia and MTHFR gene mutations (A1298C, C677T polymorphism) using restriction fragment length polymorphism analysis

5.5.1 Use of bacteriophage λ DNA as an internal control in restriction enzyme digestion in the MTHFR C677T gene mutation analysis by RFLP agarose gel electrophoresis

5.5.2 Prevalence of MTHFR C677T polymorphism in the study sample

5.5.3 Prevalence of MTHFR A1298C polymorphism in the study sample
5.5.4 Association of MTHFR C677T gene mutations (A1298C, C677T polymorphisms) with homocysteine concentrations in CAD patients in the study sample.

5.6 Association between vitamin B12, Folate, and Homocysteine levels with the severity of coronary artery disease.

5.7 Association of Apolipoprotein A-I, Apolipoprotein B and Glutathione peroxidase with severity of coronary artery disease.

5.8 Influence of age on the association of apolipoprotein A-I, apolipoprotein B and glutathione peroxidase concentrations with coronary artery disease patients.

6. CONCLUSIONS

7. REFERENCES

8. APPENDICES

Appendix 1: List of Publications and Communications from Thesis
Appendix 2: Questionnaire used in study 1
Appendix 3: Questionnaire/Data sheet used in study 2
Appendix 4: Ethical clearance for study 1
Appendix 5: Ethical clearance for study 2 (excluding genetic analysis)
Appendix 6: Ethical clearance for the genetic analysis in study 2
Appendix 7: Information leaflet used in study 1(English)
Appendix 8: Information leaflet used in study 1(Sinhala)
List of Tables

Table 3.1  Comparison of retention times for homocysteine and internal standard (cystamine dihydrochloride) after 3 months storage at -20 °C.  

Table 3.2  PCR primers used for the PCR/RFLP genotyping assays to genotype the MTHFR C677T polymorphism and the initial internal control DMD 48.  

Table 3.3  PCR primers developed for amplification of selected bacteriophage λ 154 bp segment.  

Table 3.4  PCR primers used for the PCR/RFLP genotyping assays to genotype the MTHFR A1298C polymorphism.  

Table 4.1  Prevalence characteristics of the subjects in study 1.  

Table 4.2  Weight, height and BMI of cases and controls in study 1.  

Table 4.3  Homocysteine concentrations of the subjects in the study.  

Table 4.4  Correlation between plasma homocysteine concentration and age in the study population.  

Table 4.5  Conditional logistic regression analysis of confounding variables.  

Table 4.6  Association between hyperhomocysteinaemia and CAD by age group.
| Table 4.7 | Frequency distribution of number of traditional risk factors in CAD patients and controls | 121 |
| Table 4.8 | Association between the number of traditional risk factors and development of CAD. | 122 |
| Table 4.9 | Association between homocysteine levels and number of traditional risk factors in CAD patients. | 122 |
| Table 4.10 | Personal characteristics of subjects in study 2. | 124 |
| Table 4.11 | Biochemical and anthropometric characteristics of subjects in study 2. | 127 |
| Table 4.12 | Association of vitamin B$_{12}$ and folate concentrations to homocysteine concentrations. | 130 |
| Table 4.13 | Significance of the association between vitamin B$_{12}$ and folate with normal homocysteine levels and hyperhomocysteinaemia at different cut off values. | 130 |
| Table 4.14 | Genotype frequencies for MTHFR A1298C and C677T polymorphisms of the study sample (n=79). | 135 |
| Table 4.15 | Relationship between MTHFR C677T genotype and homocysteine, folate and vitamin B$_{12}$ concentrations. | 137 |
| Table 4.16 | Relationship between A1298C genotype and homocysteine, folate and vitamin B$_{12}$ concentrations. | 138 |
Table 4.17  Relationship between MTHFR A1298C /C677T genotype combinations and homocysteine concentrations.

Table 4.18  Severity of ischaemia in the study sample.

Table 4.19  Association of vitamin B₁₂, Folate, and Homocysteine concentrations to severity of CAD.

Table 4.20  Association of apolipoprotein A-I, apolipoprotein B and glutathione peroxidase to severity of CAD.

Table 4.21  Influence of age on the association of apolipoprotein A-I, apolipoprotein B, glutathione peroxidase on coronary artery disease.
LIST OF FIGURES

Figure 2.1 Schematic diagram of the Homocysteine metabolic pathway in humans. 20
Figure 2.2 Structure of Homocysteine and its oxidized forms in plasma. 23
Figure 3.1 Chromatogram of a representative plasma sample measured at a methanol concentration of 30 ml/L. 71
Figure 3.2 Chromatogram of a representative plasma sample measured at a methanol concentration of 12 ml/L. 71
Figure 3.3 Chromatogram of Homocysteine standard (70 μmol/L) and Internal standard Mercapto-propionyl-glycine (200 μmol/L). 73
Figure 3.4 Chromatogram of Homocysteine standard (70 μmol/L) and internal standard Cystamine dihydrochloride (200 μmol/L). 73
Figure 3.5 Homocysteine standard curve. 75
Figure 4.1 Distribution of serum homocysteine with age in normal subjects of study 1. 114
Figure 4.2 Distribution of serum homocysteine with age in CAD patients of study 1. 114
Figure 4.3 Distribution of serum homocysteine with age in CAD patients of study 2. 128
LIST OF PLATES

Plate 4.1  MTHFR 677C>T genotyping: The pattern created by electrophoresing the Hinf I digested PCR products of samples of the three genotypes and the DMD 48 internal control on a 2% agarose gel.

Plate 4.2  MTHFR 677C>T genotyping: The pattern created by electrophoresing the Hinf I digested PCR products of samples of the three genotypes and the 150bp bacteriophage λ DNA internal control on a 2% agarose gel.

Plate 4.3  MTHFR A1298C genotyping: The pattern created by electrophoresing the Mbo II digested PCR products of samples of the three genotypes on a 2% agarose gel.
### ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
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<tr>
<td>Apo-A</td>
<td>Apolipoprotein A</td>
</tr>
<tr>
<td>Apo B</td>
<td>Apolipoprotein B</td>
</tr>
<tr>
<td>BMI</td>
<td>Body Mass Index</td>
</tr>
<tr>
<td>CABG</td>
<td>Coronary artery bypasses grafting</td>
</tr>
<tr>
<td>CAD</td>
<td>Coronary Artery Disease</td>
</tr>
<tr>
<td>CHD</td>
<td>Coronary Heart Disease</td>
</tr>
<tr>
<td>CPK</td>
<td>Creatine phospho kinase</td>
</tr>
<tr>
<td>CV</td>
<td>Coefficient of variation</td>
</tr>
<tr>
<td>CVD</td>
<td>Coronary Vascular Disease</td>
</tr>
<tr>
<td>dATP</td>
<td>deoxy Adenosine Triphosphate</td>
</tr>
<tr>
<td>dCTP</td>
<td>deoxy Cytosine Triphosphate</td>
</tr>
<tr>
<td>dGTP</td>
<td>deoxy Guanosine Triphosphate</td>
</tr>
<tr>
<td>DMD</td>
<td>Duchene’s Muscular Dystrophy</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxy ribonucleic acid</td>
</tr>
<tr>
<td>dTTP</td>
<td>deoxy Thymidine Triphosphate</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>FPIA</td>
<td>Fluorescence Polarization Immunoassay</td>
</tr>
<tr>
<td>GNMT</td>
<td>Glycine N-methyltransferase</td>
</tr>
<tr>
<td>GPX</td>
<td>Glutathione Peroxidase 1</td>
</tr>
<tr>
<td>Hb</td>
<td>Haemoglobin</td>
</tr>
<tr>
<td>HDL</td>
<td>High-density lipoprotein cholesterol</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
</tr>
<tr>
<td>ICD</td>
<td>International Classification of Diseases</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>IDL</td>
<td>Intermediate-density lipoprotein</td>
</tr>
<tr>
<td>IHD</td>
<td>Ischaemic Heart Disease</td>
</tr>
<tr>
<td>LCAT</td>
<td>Lecithin cholesterol acyl transferase</td>
</tr>
<tr>
<td>LDL</td>
<td>Low-density lipoprotein</td>
</tr>
<tr>
<td>Lp(a)</td>
<td>Lipoprotein (a)</td>
</tr>
<tr>
<td>MI</td>
<td>Myocardial infarction</td>
</tr>
<tr>
<td>MTHFR</td>
<td>Methylene tetrahydrofolate reductase</td>
</tr>
<tr>
<td>n</td>
<td>Number</td>
</tr>
<tr>
<td>NEB</td>
<td>New England Biolabs</td>
</tr>
<tr>
<td>OR</td>
<td>Odds ratio</td>
</tr>
<tr>
<td>PAR</td>
<td>Population attributable risks</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<tr>
<td>PLP</td>
<td>Pyridoxal-5'-phosphate</td>
</tr>
<tr>
<td>RBC</td>
<td>Red blood cell</td>
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<tr>
<td>RFLP</td>
<td>Restriction Fragment Length Polymorphism</td>
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<tr>
<td>RNAse</td>
<td>Ribonuclease</td>
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<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>RP-HPLC</td>
<td>Reversed phase HPLC</td>
</tr>
<tr>
<td>RV</td>
<td>Reaction vessel</td>
</tr>
<tr>
<td>SAH</td>
<td>S-adenosylhomocysteine</td>
</tr>
<tr>
<td>SAM</td>
<td>S-adenosyl methionine</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>sd LDL</td>
<td>Small dense LDL</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
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<td>--------------</td>
<td>------------------------------------</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris acetate EDTA</td>
</tr>
<tr>
<td>TBP</td>
<td>Tri-butyl phosphine</td>
</tr>
<tr>
<td>TC</td>
<td>Total cholesterol</td>
</tr>
<tr>
<td>TCEP</td>
<td><em>Tris</em>(2-carboxyethyl)phosphine</td>
</tr>
<tr>
<td>TG</td>
<td>Triglyceride</td>
</tr>
<tr>
<td>SBD-F</td>
<td>7-Fluorobenzofurazan-4-sulfonic acid Ammonium salt</td>
</tr>
<tr>
<td>U.K.</td>
<td>United Kingdom</td>
</tr>
<tr>
<td>U.S.A.</td>
<td>United States of America</td>
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<tr>
<td>UV</td>
<td>ultra violet</td>
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<tr>
<td>VLDL-C</td>
<td>Very Low-density lipoprotein cholesterol</td>
</tr>
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<td>WHO</td>
<td>World Heath Organization</td>
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Emerging risk factors and outcome predictors of coronary artery disease (CAD) in a Sri Lankan population

PORUTHOTAGE PRADEEP RASIKA PERERA

ABSTRACT

Introduction: Coronary Artery Disease (CAD) is the number one killer disease in Sri Lanka. Apart from the conventional risk factors for CAD, a few new risk factors have been identified. Of these fasting hyperhomocysteinaemia (HHcy), elevated apolipoprotein B, decreased apolipoprotein A-I and the deficiency of the cardiovascular associated antioxidant Glutathione Peroxidase (GPx) have been reported to be significantly associated with CAD.

Objectives: This study was designed to determine a). the association between HHcy and CAD and whether this association was age dependent; b). the likely causes of variations in homocysteine levels [mainly changes in serum vitamin B\textsubscript{12} and folate status, and methylenetetrahydrofolate reductase (MTHFR) A1298C and C677T gene polymorphisms], and c). the association of novel risk factors such as folate, vitamin B\textsubscript{12}, apolipoprotein A-I, apolipoprotein B, GPx and HHcy in relation to severity of Coronary Artery Disease (CAD).

Methods: A case control study was conducted using 221 subjects with diagnosed acute coronary syndromes and 221 age and sex matched controls to assess the association between HHcy and CAD. The associations between severity of CAD and the other risk factors and folate and vitamin B\textsubscript{12} with homocysteine were also assessed in 79 subjects with diagnosed CAD.

Results: Results revealed that there was a significant association ($p = 0.002$) between HHcy and CAD. Furthermore, a significant association ($p=0.02$) was observed between...
HHcy and CAD in young patients but not in subjects over 50 years of age. HHcy was found to be a significant predictor of CAD after controlling for hypertension and hypercholesterolaemia (adjusted odds ratio 2.411). The vitamin B\textsubscript{12} and folate levels showed a significantly ($p < 0.01$) negative correlation with serum homocysteine concentrations. There was no significant association ($p>0.05$) between MTHFR C677T and A1298C polymorphisms and homocysteine levels.

Serum homocysteine and folate levels were not significantly related to the severity of coronary artery disease. However, the serum vitamin B\textsubscript{12} concentrations showed a significant negative correlation with severity of ischaemia when assessed by the vessel score ($p <0.05$) and the extent score ($p < 0.01$). Apolipoprotein A-1 (inversely) and apolipoprotein B/A-I ratio showed a significant correlation ($p<0.01$) with the stenosis and extent scores but not with the vessel score whilst the apolipoprotein B levels correlated significantly only with the vessel score ($p<0.05$). GPx showed a significant inverse correlation ($p<0.001$) with the vessel, the stenosis and the extent scores. This has not been reported in the literature before.

Conclusions: Hyperhomocysteinaemia is an independent risk factor for CAD and its association is more in the young compared to elderly subjects. While MTHFR gene polymorphisms were not associated with homocysteine concentrations, a decrease in serum concentrations of either vitamin B\textsubscript{12} or folate was associated with higher homocysteine concentrations. GPx, Apolipoprotein B/A-1 ratio and Apolipoprotein A-1 are better predictors of severity of CAD than apolipoprotein B and homocysteine and they may have a value in assessing the severity of CAD in the future.
1. INTRODUCTION

1.1 General Introduction

Ischaemic heart disease (IHD) is the leading cause of death in the world accounting for nearly 12.5% of deaths worldwide. By the year 2020, it is estimated that the number of people dying of cardiovascular diseases will surpass the number of deaths due to all types of infectious diseases combined (1) and ischaemic heart disease is projected to remain as the leading cause of death in the world even by year 2030 (2). The importance of cardiovascular disease as a cause of death and disability is not restricted to developed countries as death and disability due to cardiovascular disease is increasing rapidly in the developing world (3). With rapid urbanization and increase in population in the developing countries, the trends in food habits and the way of living are changing. Thus the popular perception that many risk factors of non-communicable diseases, when compared to communicable disease, are more prevalent in high income than low income populations and in the developed world than the developing countries, is rapidly changing (1).

The mortality trends in Sri Lanka are also changing rapidly from that observed in the developing world, which was the norm for Sri Lanka previously, to what is observed in the developed world. In Sri Lanka deaths due to communicable diseases are on the increase and deaths due to non-communicable diseases on the rise. In this respect mortality due to cardiovascular related diseases as a non-communicable disease takes prominence, accounting for the highest mortality rates among ICD (International Classification of Diseases) classified diseases (4).
1.2 Cardiovascular risk factors

A cardiovascular risk factor is any factor that is associated with an increased risk of developing cardiovascular disease. The concept of risk factors for ischaemic heart disease/cardiovascular disease has evolved over the past 60 years and new risk factors are periodically added as new insights into the disease process leading to cardiovascular disease are made. Traditionally, cardiovascular risk factors have been divided into two main categories, non-modifiable risk factors and modifiable risk factors.

1.2.1 Non-modifiable risk factors

1.2.1.1 Age

The risk of having an acute coronary event such as myocardial infarction or death due to myocardial infarction increases with age. Age remains one of the strongest predictors of cardiovascular disease in epidemiological studies and it has been found that this association holds true for both males and females (5).

1.2.1.2 Gender

Traditionally coronary heart disease has been considered a disease of men. But coronary heart disease is the leading cause of death in both men and women (1, 6). Epidemiological studies have shown that middle aged men are 2 to 5 times more likely to develop coronary heart disease than women of similar age, and life time risk of developing coronary heart disease at age 40 is 50% for men and 33% for women (7). The predominant factor thought to be responsible for this difference is the protective role played by oestrogen in females (8). This is supported by the fact that the risk of coronary heart disease in women rises rapidly after menopause with the cessation of
production of oestrogens by the ovaries. But results from some studies have queried the validity of this association especially in respect of older women (9, 10). Moreover in Europe, overall figures indicate that more women die from cardiovascular disease than men—specifically, cardiovascular disease is believed to be responsible for 45% of deaths among females and 38% of deaths among males, and in the United States, by the age of 60 years, one in four women as well as one in four men, die of coronary heart disease (11).

1.2.1.3 Heredity / family history

First degree relatives of patients with premature myocardial infarction (before 55 years in males and before 60 years in females) have been found to have a two fold increase in the risk of developing premature myocardial infarction. Studies have found that more than one third of the admissions for premature myocardial infarction could still be prevented by screening and treating first degree relatives of patients with premature myocardial infarction (MI) (12).

1.2.1.4 Ethnicity

Epidemiological studies have found that people with different ethnic backgrounds have different death rates even if they live in the same country. For example South Asians living in the United Kingdom (people from India, Pakistan and Sri Lanka) have a higher death rate from premature coronary heart disease compared to the general population. West Africans and people from the Caribbean have a much lower death rate from premature coronary heart disease compared to the U.K. population (13-15).
1.2.2 Modifiable risk factors

1.2.2.1 Hypertension

High blood pressure or hypertension has been known as a risk factor for coronary heart disease for a long period of time. Worldwide, high blood pressure is estimated to cause 7.1 million deaths, amounting to about 13% of the total deaths and globally 49% of ischaemic heart disease are attributable to suboptimal blood pressure (systolic >115 mmHg), with little variation due to sex (3). In adults aged 40-69 years, a 10 mmHg rise in diastolic blood pressure or a 20 mmHg rise in systolic blood pressure has been found to increase the risk of death due to ischaemic heart disease two fold (16). It is also observed that 22% of myocardial infarctions in Europe are associated with a history of high blood pressure and those with hypertension had almost twice the risk of developing myocardial infarction (17). Similarly a 10 mmHg lowering of the systolic blood pressure or a 5 mmHg lowering of diastolic blood pressure lowers the risk of death from ischaemic heart disease by 30% throughout middle age. Even at older ages this lowering of risk from CAD remains with only a very slight reduction in the percentage decrease when compared to middle age. It is also noted that throughout middle and old age usual blood pressure is strongly and directly related to vascular and overall mortality. This association does not show any evidence of a threshold down to a blood pressure of at least 115/75 mmHg (16). In persons older than 50 years, systolic blood pressure of more than 140 mmHg is a much more important cardiovascular disease risk factor than diastolic blood pressure of more than 90 mmHg (18).

1.2.2.2 Hypercholesterolaemia and hyperlipidemia

i. Elevated total cholesterol
Coronary heart disease risk is related to blood cholesterol levels with risk of coronary heart disease rising with elevation of total blood cholesterol levels. In Western Europe 45% of myocardial infarctions are due to abnormal blood lipid parameters (17). High cholesterol is estimated to cause 56% of global ischaemic heart disease amounting to about 4.4 million deaths (7.9% of total deaths) worldwide. In most regions, the proportion of deaths in females attributable to cholesterol is slightly higher than that for men (3). Lowering of blood cholesterol levels has been found to decrease the risk of coronary heart disease. A 10% reduction in serum cholesterol level attained at age 40 years, lowers the risk of ischaemic heart disease by 50% whereas a 10% reduction of serum cholesterol begun at age of 70 years will give only a 20% reduction in risk of ischaemic heart disease (19).

ii. Elevated Low-Density Lipoprotein cholesterol (LDL-cholesterol)

Epidemiological studies have implicated LDL-cholesterol as an important risk factor for coronary heart disease. In men and women free of coronary heart disease, LDL-cholesterol levels have been found to be directly related to the rate of new-onset coronary heart disease (20-23). Persons with established coronary heart disease having high LDL-cholesterol are also liable to get recurrent coronary events compared to coronary heart disease patients with LDL-cholesterol in the desired range (24, 25). Studies involving different populations reveal that a positive relationship between serum cholesterol levels and the development of first or subsequent attacks of coronary heart disease is observed over a broad range of LDL-cholesterol levels (22).
Absence or a very low risk of clinical coronary heart disease is found only in populations that maintain very low levels of serum cholesterol, e.g., LDL-cholesterol levels of <100 mg/dL (or total-cholesterol <150 mg/dL) throughout life (19, 26, 27).

iii. Low High Density lipoprotein Cholesterol (HDL-cholesterol)

Epidemiological data support an independent inverse association of high density lipoprotein cholesterol levels and risk of coronary heart disease. Risk of coronary heart disease decreases by 2-3% for each 1 mg/dL increment in HDL-cholesterol level (28-31). In prospective studies HDL-cholesterol has been found to be the lipid risk factor most highly correlated with cardiovascular risk (32). HDL-cholesterol levels have been found to have a greater impact on coronary heart disease risk in women compared to men (33). Therapeutic lifestyle changes have been advocated by the American Heart Association for men having HDL-cholesterol levels below 40 mg/dL and women having HDL-cholesterol levels below 50 mg/dL (34).

1.2.2.3 Effect of tobacco smoking

Smoking is a major contributor not only to coronary heart disease but to stroke and peripheral vascular disease as well (34, 35). Overall mortality from coronary heart disease is 60% higher in smokers compared to non-smokers (36) and evidence from the Framingham Heart Study shows that the risk of sudden death increases more than ten fold in men and almost five-fold in women who smoke. In fact smoking is the number one risk factor for sudden cardiac death and peripheral vascular disease (37-39). World Health Organization (WHO) research estimates that over 20% of cardiovascular diseases are due to smoking when the whole world population is taken into account (3).
But fortunately, the risk of heart disease begins to decline rapidly as soon as smoking is stopped even when individuals are heavy long term smokers. Ultimately their levels of coronary heart disease risk, but not other risks like risk of lung cancer, can be reduced to almost the same level as that of people who have never smoked (40).

Unfortunately the risk of coronary heart disease is not restricted to persons who smoke but is attributable to passive smokers as well. Regular exposure to passive smoking has been found to increase the risk of coronary heart disease by 25% (41, 42).

1.2.2.4 Overweight and obesity

People who have excess body fat are more likely to develop heart disease even if they have no other risk factors. Obesity is not only a risk factor for coronary heart disease (43, 44) but it is also a risk factor for cerebrovascular disease, hypertension, hyperlipidemia, diabetes mellitus, and impaired glucose tolerance (43). The prevalence of overweight and obesity is commonly assessed using Body Mass Index (BMI) with a strong correlation to body fat content. WHO criteria define overweight as a BMI of at least 25 kg/m\(^2\) and obesity as a BMI of at least 30 kg/m\(^2\). The risks of disease in all populations increase progressively from BMI levels of 22 kg/m\(^2\). Globally 21% of CAD is attributable to a BMI of over 21 kg/m\(^2\) (3). The way the weight distribution has occurred also plays a part in overweight and obese subjects. Male pattern obesity (android obesity or central obesity) is characterized by excess fat being found primarily in the abdominal area ("beer belly" or apple shape) while female pattern obesity or gynecoid obesity is characterized by excess fat being deposited mainly around the hips and buttocks (pear shape). Cardiovascular risk is more significantly associated with android obesity and those with android obesity have twice the risk of myocardial
infarction compared to non-obese (17). Although many overweight and obese persons
find it difficult to lose weight, it has been found that even a modest reduction in weight
by 5 kg in obese subjects can lead to a significant reduction in coronary heart disease
risk (45).

1.2.2.5 Diabetes mellitus

Patients with type 2 diabetes have a markedly increased risk for coronary heart
disease (46). This association has been suggested to be stronger in women than in men
(33), thus eliminating or substantially decreasing the advantage being female, especially
in middle age (47). Patients with type 2 diabetes mellitus are also at a two-fold greater
risk of developing a fatal coronary event when compared to non-diabetic patients (48)
and diabetic patients without previous myocardial infarction have as high a risk of
myocardial infarction as non-diabetic patients with previous myocardial infarction (49).
The life expectancy free of cardiovascular disease in diabetic men and women, 50 years
and older have been found to be on average 7.8 and 8.4 years less than in non diabetic
subjects (50).

1.2.2.6 Effect of alcohol

Moderate alcohol intake is associated with reduced cardiovascular events in a
number of population studies. This association is found mainly with moderate intake of
red wine but it has also been associated with some other alcoholic beverages too (51-
55). On the other hand high alcohol intake (typically ≥3 drinks or ≥45 g ethanol per
day) is associated with elevated blood pressure and consequent rise in cardiovascular
disease risk in a number of studies (56, 57). Reduction in alcohol consumption has been
found to lower the blood pressure in normotensive and hypertensive men (58, 59). Based on the overall epidemiological data regarding alcohol consumption and risk of coronary heart disease, the American Heart Association has recommended that, if alcoholic beverages are consumed, they should be limited to the equivalent of 2 drinks (30 g ethanol) per day for men and 1 drink per day for women (55). Unlike other potentially beneficial dietary substances, alcohol is not recommended as a cardio-protective substrate (60).

1.2.2.7 Infrequent exercise/Physical inactivity

According to the World Health Report of 2002, the global estimate for physical inactivity among adults is 17% worldwide (3). An inactive lifestyle is a risk factor for coronary heart disease and regular moderate to vigorous physical activity helps prevent heart and blood vessel disease. Globally, physical inactivity is estimated to cause 22% of CAD (3) and in the developed world physical inactivity is reported to be the cause of 20% of CHD (61). In general, physical activity improves glucose metabolism, reduces body fat and lowers blood pressure; these are the main ways in which it is thought to reduce the risk of cardiovascular diseases. At least 30 minutes of moderate physical activity on most if not all days per week is recommended to decrease the risk of cardiovascular disease (60). But unfortunately when assessing the adequacy of physical activity there is no internationally accepted definition or measure of physical activity (3) that can be used in research and clinical practice.
1.3 Traditional vs. Novel coronary risk factors

Association between these modifiable and non-modifiable traditional risk factors and coronary artery disease have been studied extensively in the past. In the more developed countries five of these modifiable risk factors cause at least one-sixth of their total disease burden(3). At the same time in the most industrialized countries of North America, Europe and the Asian Pacific, at least one-third of all disease burden is caused by tobacco, alcohol, blood pressure, cholesterol and obesity(3). Furthermore, more than three-quarters of cardiovascular disease results from tobacco use, high blood pressure or cholesterol, or a combination of these. Overall, cholesterol causes more than 4 million premature deaths a year, tobacco causes almost 5 million, and blood pressure causes 7 million (3).

In the past 40 years more research has been undertaken to understand the disease processes leading to coronary artery disease and new insights have been made regarding atherosclerosis and its implications on the development of coronary artery disease. With this additional knowledge, some new coronary risk factors associated with development of coronary artery disease have been recognized. Some of these new coronary risk factors have been postulated to be superior to the traditional modifiable risk factors, especially in prediction of coronary artery disease and in management of high risk individuals. These newly identified risk factors are now widely referred to as novel or non-traditional coronary risk factors to distinguish them from the traditional risk factors like diabetes, hypertension and hypercholesterolemia. These novel coronary risk factors, which are not included in the Framingham Risk scores (62) include elevated serum homocysteine, elevated serum apolipoproteins B and decreased apolipoprotein A, elevated serum lipoprotein(a), elevated small dense LDL particles, elevated fibrinogen
and elevated inflammatory markers like C-reactive protein (63). Apart from these, decreased glutathione peroxidase activity and reduction in some other antioxidants have also been identified as new risk factors for coronary artery disease (64).
1.4 Justification

IHD is the major non-communicable disease in Sri Lanka. Yet research data on risk factors for coronary artery disease in Sri Lanka is scanty especially regarding novel coronary risk factors which are now being given prominence especially in the young, as risk factors for CAD (65).

Many patients develop atherosclerosis in the absence of conventional risk factors. Studies have shown that conventional risk factors predict less than one half of future cardiovascular events (66, 67). Furthermore, evidence shows that conventional risk factors may not have the same effect in different ethnic groups in whom the other novel risk factors may play a significant role (68). Hence research work on (a). coronary risk factors for different ethnic populations and on (b). novel coronary risk factors to predict the unexplained 50% of future coronary events, may be of value.

According to the available literature, no work has been reported in Sri Lanka on the association between hyperhomocysteinaemia and CAD comparing the young and the elderly CAD patients. On the other hand according to the latest hospital statistics, incidence of CAD in the young is increasing (4). Furthermore vitamin B₁₂ and folate deficiency is thought to be an important and a common cause of anaemia in Sri Lanka and deficiencies of these would contribute to hyperhomocysteinaemia.

Retrospective and prospective case control studies have shown an association between plasma homocysteine and CAD. These studies have also found an association between homocysteine and vitamin B₁₂ and folate, as these vitamins play a vital role in homocysteine metabolism (69-73).

Hence the present study attempted to investigate the association between homocysteine and the risk of IHD /CAD and the association of homocysteine with
vitamin B₁₂ and folate in Sri Lankan CAD patients. Furthermore, this study was also designed to assess the association of homocysteine concentrations with different genotypes of the methylenetetrahydrofolate reductase polymorphisms (A1298C and C677T). These polymorphisms have been reported to affect the homocysteine levels, as subjects with these genotypes are reported to develop hyperhomocysteinaemia, especially in low folate states (74).

As homocysteine is believed to exert its effects primarily through a mechanism involving oxidative damage (75), the relationship between the antioxidant enzyme glutathione peroxidase, which is the most potent enzyme involved in antioxidant protection against CAD (76), was also investigated in this study.

Prospective studies have been conducted in many parts of the world to assess the association between hyperhomocysteinaemia and CAD, and the effect of vitamin B₁₂ / folate supplementation to lower homocysteine levels in order to decrease the risk of CHD (70, 77-79). Thus in this study, if an association between vitamin B₁₂ and folate with homocysteine levels is found, especially in the young, vitamin B₁₂ / folate supplementation maybe of value in reducing the risk of CAD in these Sri Lankans. The importance of this lies in the fact that this is an easy and cheap way of preventing or reducing the incidence of CAD and its consequent mortality in the population at risk by the simple means of supplementing with vitamin B₁₂ and folate to the population at risk.

Apolipoprotein A-I and apolipoprotein B are now increasingly recognized as novel risk factors as well as risk predictors for CHD (80, 81). There are no reports on the association of these novel risk factors and CHD in Sri Lanka. Hence an attempt was made in this study to investigate whether these risk factors correlate with CAD in Sri Lanka. This was with the aim of improving the current knowledge regarding these risk factors.
factors and CAD and this may further facilitate broad based studies to assess the clinical applicability in Sri Lankans.

Therefore the overall aim of this study was to investigate (i) the association between homocysteine and CAD, (ii) the vitamin deficiencies & MTHFR gene polymorphisms that could lead to hyperhomocysteinaemia, and (iii) the correlation between homocysteine, vitamin B₁₂, folate, MTHFR gene polymorphisms, atherogenic apolipoproteins (B and A-I) and glutathione peroxidase concentrations with severity CAD.
1.5 Objectives

The present study was conducted in two parts:

Study 1: A case control study to determine general objective 1 and the specific objectives 1-3.

Study 2. An analytical study to determine general objectives 2-4 and the specific objectives 4-8.

1.5.1 General Objectives:
1. To identify the association between Hyperhomocysteinaemia and Coronary Artery Disease (CAD) among patients with an acute coronary syndrome.
2. To assess the vitamin B\textsubscript{12} and folate status of CAD patients and their association with hyperhomocysteinaemia.
3. To determine the association of Apolipoprotein A-I, Apolipoprotein B, and Glutathione peroxidase with CAD.
4. To determine the association between Hyperhomocysteinaemia and MTHFR gene polymorphisms (A1298C, C677T polymorphism)

1.5.2 Specific Objectives:
1. To determine the association between Hyperhomocysteinaemia and CAD among patients with an acute coronary syndrome.
2. To determine whether hyperhomocysteinaemia is associated with a higher risk of CAD in the young (age \leq 50 years) compared to the elderly (age > 50 years).
3. To identify the association between Hyperhomocysteinaemia and CAD among CAD patients who have more than two traditional risk factors compared to CAD patients who have two or less than two traditional risk factors among patients with an acute coronary syndrome.

4. To identify the vitamin deficiencies (vitamin B\textsubscript{12} and folate) associated with hyperhomocysteinaemia in patients with CAD.

5. To identify the association between Homocysteine concentrations and MTHFR gene polymorphisms (A1298C, C677T polymorphisms).

6. To determine whether there is an association between, vitamin B\textsubscript{12}, Folate, and Homocysteine levels with the severity of CAD.

7. To determine whether there is an association of Apolipoprotein A-I, Apolipoprotein B and Glutathione peroxidase with severity of CAD.

8. To determine the association of age with Apolipoprotein A-I, Apolipoprotein B, and Glutathione peroxidase concentrations in CAD patients.
2. LITERATURE REVIEW

2.1 Homocysteine and CAD

2.1.1 Homocysteine metabolism

Homocysteine is a sulfur containing non-protein–forming amino acid synthesized from the metabolism of the essential amino acid, methionine. High dietary consumption of methionine, predominantly found in meats and dairy products could result in overproduction of homocysteine. Homocysteine is subsequently metabolized in the body through either the remethylation pathway which occurs in all tissues or through the transsulfuration pathway which occurs only in liver, kidney, small intestine and pancreas (82). Thus homocysteine is a metabolic intermediate that occupies a critical branch-point in methionine metabolism.

Homocysteine can acquire a methyl group from N-5-methyltetrahydrofolate or from betaine in a reaction catalyzed by the enzymes methionine synthase or betaine-homocysteine methyltransferase respectively to form methionine in the remethylation pathway (83). In its reaction with N-5-methyltetrahydrofolate, which occurs in all tissues, homocysteine requires vitamin B\textsubscript{12} as a cofactor, compared to its reaction with betaine which is not dependent on vitamin B\textsubscript{12} (84). Methionine can then be activated by ATP to form S-adenosyl methionine (SAM) in a reaction catalysed by methionine–adenosyltransferase (SAM synthetase). In mammals SAM serves primarily as a universal methyl donor for a variety of acceptors in many biological methylation reactions. Many mammalian S-adenosylmethionine-dependent methyl transferases act on SAM to produce S-adenosylhomocysteine (SAH) and a variety of methylated products like nucleic acids, proteins and lipids etc. SAH is subsequently hydrolysed to...
adenosine and homocysteine. The formed homocysteine can again undergo remethylation to methionine or transsulfuration (85).

Both methionine and cysteine are sulfur containing amino acids, and in humans all sulfur found in these two amino acids must be obtained from the diet as they are unable to incorporate inorganic sulfur into amino acids. The transsulfuration pathway allows the transfer of sulfur from methionine to cysteine, and the presence of the transsulfuration pathway ensures that only methionine and not cysteine is essential in the human diet (85). In the transsulfuration pathway, which occurs only in liver, kidney, small intestine and pancreas (82), cystathionine-β-synthase (CBS) catalyses the condensation of homocysteine and serine. This step requires the coenzyme form of vitamin B₆, pyridoxal phosphate (PLP) and the formed cystathionine is hydrolyzed by a second PLP containing enzyme, γ-cystathionase to form the sulfur containing amino acid cysteine and α-ketobutyrate. Thus in addition to synthesis of cysteine, the transsulfuration pathway plays a role in the catabolism of excess homocysteine which is not required for methyl transfer (84). As methionine catabolism by other routes is not significant the transsulfuration pathway is, with rare exceptions the only route of methionine and homocysteine elimination from the body (86).

The two pathways of homocysteine metabolism are nutritionally regulated. When the intake of labile methyl groups (methionine and choline) is modified, de novo synthesis of methionine methyl group is affected. Organisms which are in a state of metabolic equilibrium eliminate methionine at the same rate it enters the body from the diet; this is achieved by the equilibrium between the transsulfuration rate and the methionine intake. Remethylation represents a mechanism by which methyl groups
from diverse sources are channelled into SAM-mediated transmethylation reactions via homocysteine reconversion to methionine (Figure 2.1).

Intracellular SAM concentration plays a major role in regulating these two independent but intersecting metabolic pathways. When dietary methionine is high SAM synthetase will rapidly convert the methionine to SAM. Thus in high methionine states there is an increase in the intracellular concentration of SAM which activates cystathionine-β-synthase leading to elimination of excess methionine. The increased SAM will also act as an allosteric inhibitor of methylenetetrahydrofolate reductase (MTHFR), which supplies N-5-methyltetrahydrofolate to the enzymes that remethylate homocysteine (87), limiting the unnecessary synthesis of methionine in a state in which methionine is already abundant. Thus in a state where methionine intake is high, homocysteine transsulfuration is promoted over remethylation, which is consistent with the reduced need for de novo methionine synthesis seen in a situation where dietary supply of methionine is high. When the dietary methionine supply is low, the resulting low methionine state will decrease the concentration of intracellular SAM. Thus the SAM concentration is insufficient to activate cystathionine-β-synthase which decreases the rate of homocysteine catabolism. Low intracellular SAM, being insufficient for the inhibition of MTHFR, will give rise to an increase in production of N-5-methyltetrahydrofolate. This rise in N-5-methyltetrahydrofolate is associated with conservation of SAM and an increase in the availability of substrate for homocysteine remethylation (88).
Figure 2.1. Schematic diagram of the Homocysteine metabolic pathway in humans.


SAM, S-adenosyl methionine; THF, Tetrahydrofolate; PLP, Pyridoxal phosphate).
The coordinated regulation of intracellular homocysteine concentration by the remethylation and transsulfuration pathways is also achieved by regulation of intracellular SAM itself. This second mechanism of homocysteine metabolism involves control over the synthesis of SAM in the liver. In the liver, the synthesis of SAM is catalyzed by two enzymes which have similar immunological properties but differ in their activities (89, 90). These two enzymes function optimally at two different methionine concentrations. Of these, one enzyme, a tetramer of high molecular weight has a high affinity for methionine and functions at normal physiological conditions. The second, which is a dimer of low molecular weight, has a low affinity for methionine and functions under conditions of high methionine intake. As with synthesis, utilization of SAM is also regulated by a reaction in which a methyl group is transferred from SAM to the amino acid glycine, giving rise to sarcosine in a reaction catalyzed by glycine N-methyltransferase (GNMT). GNMT is found in high concentrations in the liver and it is inhibited by N-5-methyltetrahydrofolate polyglutamates (91). These mechanisms guarantee a sufficient methionine influx for essential transmethylation reactions as well as an adequate supply of methionine when necessary (84).

2.1.2 Homocysteine in plasma/serum

Homocysteine is found in plasma as homocysteine, homocystine and as homocysteine mixed disulphides (Figure 2.2). In healthy individuals ≤ 1% of the total homocysteine in the blood stream is found as free reduced homocysteine. The rest of the circulating homocysteine is in the oxidized forms of homocysteine and mixed homocysteine disulfides. Homocysteine is formed by the auto-oxidation of two homocysteine molecules in the presence of an electron acceptor such as molecular
oxygen to form disulfide bonds. Homocysteine accounts for 5-15% of total circulation homocysteine. Homocysteine can also oxidize with other thiols such as cysteine and glutathione to form mixed disulphides (Figure 2.2) and these mixed disulphides account for another 5-15% of the total circulating homocysteine. The majority of the total circulating homocysteine, accounting for >70% is bound to proteins in the circulation (85).

The sum of reduced and oxidized forms of homocysteine in the circulation is known as plasma (serum) total homocysteine (85).

In this thesis the terms total homocysteine/ serum homocysteine/ plasma homocysteine refers to the sum of reduced and oxidized forms of homocysteine made up of homocysteine, homocystine, homocysteine mixed disulphides and protein bound homocysteine unless otherwise stated.
Figure 2.2 Structure of Homocysteine and its oxidized forms in plasma
2.1.3 Factors affecting plasma (serum) homocysteine levels

2.1.3.1 Metabolic derangements affecting plasma (serum) homocysteine levels

Cellular export mechanisms help in maintaining low intracellular concentrations of the potentially cytotoxic amino acid homocysteine. This gives rise to the small amount of homocysteine normally found in plasma. However, if excess homocysteine is formed, this cellular export, while preventing toxicity to the cell, exposes the vascular tissue to the possible harmful effects of excess homocysteine. Excess homocysteine concentrations in plasma, in a person whose kidney functions are normal, indicate that the normal metabolic pathway of homocysteine metabolism may get interrupted and subsequently lead to accumulation of homocysteine inside the cell. Excess homocysteine levels in plasma, hyperhomocysteinaemia, can be due to a genetic defect in the enzymes involved with the homocysteine metabolic pathway (92, 93) or due to a nutritional deficiency of one or more of the vitamins that are involved in the metabolic pathway (82, 94). Depending on the extent to which the genetic defect or nutritional deficiency affects the regulation of the remethylation and transsulfuration pathways of homocysteine metabolism, the severity and the type of hyperhomocysteinaemia can vary among individuals.

Defective synthesis of N-5-methyltetrahydrofolate, defective homocysteine remethylation and defective homocysteine transsulfuration can lead to hyperhomocysteinaemia.

N-5-methyltetrahydrofolate synthesis is the first reaction that is specifically concerned with synthesis of methionine. A decrease in N-5-methyltetrahydrofolate concentrations in cells would lead to a decrease in the synthesis of methionine and this is usually due to a defect in the enzyme methylenetetrahydrofolate reductase (MTHFR).
or as a consequence of folate deficiency. This diverts homocysteine to the
transsulfuration pathway from the remethylation pathway. But the transsulfuration
pathway is also affected due to the decrease in methionine synthesis giving rise to a
decrease in intracellular concentration of SAM leading to failure of activation of
cystathionine-β-synthase. Secondly the decrease in N-5-methyltetrahydrofolate allows
GNMT to be fully active, leading to a further decrease of intracellular concentration of
SAM which gives rise to an increase in synthesis of homocysteine as a consequence of
glycine methylation. These two mechanisms make the transsulfuration pathway
ineffective by increasing the homocysteine concentration along with a SAM
concentration too low to activate cystathionine-β-synthase which is the starting point of
the transsulfuration pathway, thus resulting in accumulation of homocysteine in blood
causing hyperhomocysteinaemia.

Defective homocysteine remethylation could also lead to
hyperhomocysteinaemia. This is usually due to defects in enzymes involved in the
synthesis of methyl cobalamin or due to a deficiency of vitamin B₁₂. The methyl trap
hypothesis predicts that N-5-methyltetrahydrofolate will accumulate when
remethylation is impaired (95). This is due to the fact that the enzyme reaction catalyzed
by MTHFR is irreversible in vivo. Thus once N-5-methyltetrahydrofolate is formed, the
only way it can be recycled in the cell is by being converted to tetrahydrofolate by the
vitamin B₁₂ dependent enzyme methionine synthase. In vitamin B₁₂ deficiency this
reaction takes place at a decreased rate leading to an accumulation of N-5-
methyltetrahydrofolate giving rise to a pseudo folate deficiency in cells. Thus, despite
the decreased rate of SAM synthesis, the intracellular SAM concentration is less
affected as its utilization in glycine methylation is inhibited by the accumulated N-5-
methyltetrahydrofolate. As a consequence less homocysteine will be synthesized from
SAM and there will be some activation of cystathionine-\(\beta\)-synthase by SAM. Thus,
homocysteinaemia due to remethylation defects may not be as severe as in defects of N-
5-methyltetrahydrofolate synthesis as transsulfuration may be somewhat active in
catabolism of homocysteine. Yet, hyperhomocysteinaemia will be present as
homocysteine metabolism will be disrupted to a significant extent even in defective
homocysteine remethylation (84, 86).

Hyperhomocysteinaemia could also result from defective homocysteine
transsulfuration. The transsulfuration pathway can be defective due to homozygous or
heterozygous cystathionine-\(\beta\)-synthase defect or due to vitamin B\(_6\) deficiency. In
homozygous cystathionine-\(\beta\)-synthase deficiency, the transsulfuration pathway gets
severely impaired leading to a diversion of homocysteine towards the remethylation
pathway. This leads to an increase in the production of methionine and consequently an
increase in the concentration of intracellular SAM. As SAM concentration increases it
will feedback inhibit MTHFR, and at this point the remethylation pathway will also get
inhibited. Thus both pathways of homocysteine metabolism will be impaired leading to
severe hyperhomocysteinaemia. But in heterozygous cystathionine-\(\beta\)-synthase
deficiency or in B\(_6\) deficiency, the transsulfuration pathway is only mildly impaired.
This is due to the remethylation pathway being fully functional and the residual activity
of the transsulfuration pathway being sufficient to maintain normal homocysteine levels
in situations where the homocysteine concentration is low, as in the fasting state.
However despite the lack of hyperhomocysteinaemia under fasting conditions, persons
with heterozygous cystathionine-\(\beta\)-synthase deficiency or B\(_6\) deficiency will show signs
of disruption of homocysteine metabolism in situations where the homocysteine burden
is high as in non-fasting conditions especially if there is a significant intake of dietary methionine. This rise in dietary methionine will give rise to an increase in intracellular SAM which will inhibit N-5-methylytetrahydrofolate with a consequent depression of the use of homocysteine by the remethylation pathway. Furthermore it will give rise to a highly active GNMT because of the low concentration of N-5-methylytetrahydrofolate, leading to increased synthesis of homocysteine through glycine methylation. Along with the primary impairment of transsulfuration due to heterozygous cystathionine-β-synthase deficiency or B₆ deficiency this would lead to the impairment of homocysteine metabolic pathways to metabolise homocysteine after a methionine load, but to a lesser extent in the fasting state (84, 87).

Thus severe hyperhomocysteinaemia is due to deficiencies of cystathionine-β-synthase, MTHFR, or due to deficiency in enzymes of vitamin B₁₂ metabolism (96). The mild to moderate hyperhomocysteinaemia observed under fasting conditions reflects impaired homocysteine methylation and is due to folate, vitamin B₁₂ or moderate enzyme defects like the thermolabile variant of MTHFR (88, 97-99). Post-methionine load hyperhomocysteinaemia, showing an abnormal increase in total homocysteine after a methionine load, reflects impaired homocysteine transsulfuration, usually due to heterozygous cystathionine-β-synthase defects or due to vitamin B₆ deficiency.

As far as these two metabolic pathways are concerned, an impairment of the remethylation pathway, even if it is mild, would lead to a greatly increased plasma homocysteine concentration under fasting conditions whereas a mild impairment in the transsulfuration pathway will give rise to only a very slight increase in fasting plasma homocysteine levels.
2.1.3.2 Genetic mutations affecting plasma (serum) homocysteine levels

Fourteen rare mutations of MTHFR have been found and they are associated with severe MTHFR deficiency and hyperhomocysteinaemia (100, 101). Among the genetic defects which can give rise to mild to moderate hyperhomocysteinaemia due to defects in the MTHFR gene, two common single nucleotide polymorphisms have been reported, namely C677T and A1298C that lead to altered amino acids (102). Out of these the most important genetic determinant identified so far is the MTHFR C677T polymorphism (103). This thermo labile variant of the MTHFR plays a significant role in giving rise to mild elevations in fasting homocysteine concentrations. The C677T single nucleotide polymorphism results in a common C to T transition in the MTHFR gene coding sequence, resulting in substitution of alanine to valine residue within the N-terminal catalytic domain (104). The variant MTHFR has reduced activity at 37 °C (50% of activity compared to controls) and marked thermo lability at higher temperatures (46 °C) (105). The variant also shows a lower specific activity compared to the normal enzyme (96). This single nucleotide polymorphism has been reported to explain 4-9% of the variation in plasma total homocysteine levels (106-108).

The A1298C single nucleotide polymorphism leads to a glutamate to alanine substitution within the S-adenosylmethionine (SAM) regulatory domain of the enzyme due to an Adenine (A) to Cytosine (C) transversion (109). In vitro studies have shown that MTHFR A1298C polymorphism leads to a decrease in the enzyme activity, although the exact molecular mechanism is not known (102). Although the A1298C polymorphism is associated with decreased MTHFR activity, neither the homozygous nor the heterozygous genotypes for this mutation are associated with higher blood homocysteine level, or a lowered plasma folate concentration (109) in contrast to
individuals with homozygous C677T allele (109, 110). A possible explanation for this discrepancy is that the C677T polymorphism found in exon 4 affects directly on the N-terminal catalytic domain of MTHFR protein (111, 112) whereas the A1298C mutation is in the C-terminal region and could only influence the enzymes allosteric regulation through the S-adenosylmethionine binding site (111, 112). Furthermore there appears to be an interaction between these common mutations, because heterozygosity for both mutations is associated with lower enzyme activity than heterozygosity alone for either mutation, resulting in increased blood homocysteine levels and reduced plasma folate levels (111). When compared to these two mutations, other genetic MTHFR variants studied have shown weak and inconsistent effects (113).

2.1.3.3 Vitamin deficiencies affecting plasma (serum) homocysteine levels

The relationship between homocysteine and vitamin status was first reported by Kang et al. (1988) who found an inverse relationship between homocysteine levels and plasma folate concentrations (96). Later studies have confirmed the inverse association between homocysteine and plasma folate and in addition they have found an inverse association between vitamin B₁₂ concentration and homocysteine levels, as well as the efficacy of folate and vitamin B₁₂ supplementation in lowering plasma homocysteine concentration (71, 78, 98, 114-122). These recent studies have revealed that folate and vitamin B₁₂, but not vitamin B₆ supplementation, lowers fasting plasma homocysteine. But post-methionine load plasma homocysteine was lowered by supplementation with vitamin B₆.
2.1.4 Other contributory factors affecting homocysteine levels

Vitamin status and genetic factors are not the only determinants of plasma homocysteine levels. The other determinants include age, sex, renal function, alcohol consumption, smoking, caffeinated coffee consumption, regular physical activity, some clinical conditions and drugs (123). Total homocysteine levels have been found to increase with age and this may partly be due to differences in vitamin status, influence of sex hormones (124, 125) and the physiological decline in renal function with age (126). Men have higher levels of plasma homocysteine than women (125, 127). Plasma homocysteine levels increase after menopause (125, 128) and this may explain the age related increase of plasma homocysteine seen in women compared with men (124). Renal function is an important and strong determinant of plasma homocysteine levels (129-134) and this is possibly related to clearance of plasma homocysteine by renal metabolism (135) rather than the minor part played by the kidney in urinary excretion of homocysteine (136). There have been conflicting reports on the association between alcohol consumption and plasma homocysteine levels in the past. Some studies have found an inverse relationship between plasma homocysteine levels and alcohol consumption while others have found an increase in homocysteine levels with alcohol consumption. The current consensus agreement regarding alcohol and homocysteine is that chronic, high ethanol consumption is associated with elevated plasma homocysteine levels, possibly through its effect on vitamin status (137) and even consumption of ethanol in moderate amounts are associated with high homocysteine levels (138) although not to the same level as with chronic high ethanol consumption. Smoking increases the plasma homocysteine concentrations (124, 139-143) and coffee too gives a similar picture (139, 144). Regular physical activity has been found to decrease plasma
homocysteine levels (124, 145). The effect of smoking, coffee consumption, physical activity and vitamin status on plasma homocysteine levels have been shown to be more pronounced in women than in men (124, 127, 144).

Increased plasma homocysteine is observed in renal failure (134, 146) and the severity of hyperhomocysteinaemia depends on the severity of renal failure (146). The reason for the increase in plasma homocysteine in renal failure is due to reduced clearance of homocysteine from plasma (136). Other clinical conditions like thyroid disease can have an impact on the plasma homocysteine concentrations. Hypothyroidism gives rise to an elevation in plasma homocysteine levels whereas a decrease is observed in hyperthyroid patients (147). Pernicious anaemia, leukaemia and carcinoma (breast, ovarian, pancreatic) are the other clinical conditions that have been associated with an increase in plasma homocysteine levels (75). Hyperhomocysteinaemia is induced by drugs like methotrexate (87, 148, 149), nitrous oxide (150, 151) phenytoin, theophyllin, and trimethoprim (75). Hormone replacement therapy has been found to decrease plasma homocysteine levels in postmenopausal women (152, 153).

2.1.5 Homocysteine and its association with Coronary Artery Disease

2.1.5.1 Homocysteine as a risk factor for CAD

In 1969, McCully was the first researcher to describe the vascular pathology associated with homocysteine in homocysteinuric patients (154). He described that thromboembolic disease was a characteristic feature of homocysteinuria independent of the site of metabolic defect. This finding which suggested homocysteine to be the causative agent responsible for thromboembolic disease led to the homocysteine theory
of atherosclerosis and that moderately elevated plasma homocysteine may be one of the possible cardiovascular risk factors in the general population (155).

In a landmark study involving 25 patients aged under 50 with angiographically proven coronary artery disease and 22 control patients, Wilcken et al. (1976) were the first to report that patients having coronary artery disease have abnormal homocysteine metabolism (156). Thereafter studies carried in the past have confirmed that homocysteine is one of the risk factors for coronary artery disease. These studies have further postulated that moderately elevated plasma homocysteine is an independent risk factor for coronary artery disease (157). In a meta-analysis published in 1995 covering 27 studies and over 4000 patients with occlusive vascular (cardiovascular, peripheral and cerebrovascular) disease Boushey et al. (1995) showed that homocysteine is an independent, graded risk factor for atherosclerotic disease in the coronary, cerebral, and peripheral vessels. This study further revealed that a 5µM increase in total plasma homocysteine level increases the risk of CHD by 60% and 80% in men and women respectively (158).

Furthermore, a review of literature on homocysteine and cardiovascular morbidity or mortality has demonstrated that an elevated plasma homocysteine level both predicts and precedes the occurrence of cardiovascular disease. Researchers have postulated that the joint effect of an elevated plasma homocysteine level with conventional risk factors like smoking and hypertension may confer a particularly higher risk and that homocysteine seems to be a particularly strong predictor of cardiovascular mortality (123). Evidence to the association between elevated plasma homocysteine and cardiovascular mortality was further strengthened by a cohort study carried out with angiographically confirmed coronary artery disease patients who were
followed up for 4.6 years in which a strong graded relationship was observed between plasma homocysteine levels at baseline and mortality even after adjustment for potential confounders (159).

On the contrary, another meta-analysis involving a total of 5073 patients with coronary heart disease events revealed that an elevation in serum homocysteine is at most a modest independent predictor of coronary heart disease in healthy populations. This meta-analysis, performed with the objective of assessing the relationship of homocysteine concentrations with vascular disease risk, found stronger associations between homocysteine and vascular disease in retrospective studies involving patients who presented with cardiovascular disease than in prospective studies involving individuals who had no history of cardiovascular disease when blood was collected. After adjustment for known cardiovascular risk factors and regression dilution bias in the prospective studies, they found that a 25% lower homocysteine concentration compared to normal (amounting to a decrease of about 3 \mu mol/L) was associated with an 11% lower risk of coronary artery disease (160).

Nygard et al. (1997) indicated that elevated plasma homocysteine levels are more predictive of fatal than non-fatal vascular events. They found that when death due to cardiovascular diseases was used as the end point in the analysis of their study group in comparison with non fatal myocardial infarction, the association between homocysteine and cardiovascular mortality was stronger in the group with the end point of death due to cardiovascular disease (159). In addition Al-Obaidi et al. (2002) also found a similar picture where relationship between homocysteine and mortality in patients with angiographically determined coronary artery disease was stronger than those presenting with non fatal acute coronary syndromes (161).
However, some prospective studies have not found a significant association between homocysteine and cardiovascular events. A study conducted by Alfthan et al. (1994) using 7424 men and women aged 40-64 who were followed up for a period of 9 years found that there was no association between plasma homocysteine concentration and acute myocardial infarction. The authors suggested that the lack of association between serum homocysteine and atherosclerotic disease may be due to the exceptionally low gene frequency predisposing to hyperhomocysteinaemia in Finland (162). In a 3.3 year follow up of a sample of middle aged men and women [Atherosclerosis Risk in Communities (ARIC) study], age, race, and field center-adjusted coronary heart disease incidence had a positive association (P<0.05) with plasma homocysteine in women but not in men. However, after accounting for other risk factors, Folsom et al. (1998) found that even in men the association between homocysteine and coronary heart disease risk became non significant (163). Furthermore a case-control 3 year follow-up study conducted in the United States Ridker et al. (1999) demonstrated that homocysteine was a risk factor for cardiovascular disease among postmenopausal women (164).

In 2001, Knekt et al. reported on a study of interest examining the association between homocysteine and major coronary events. In their prospective case control study where both women with coronary heart disease and women free of heart disease at base line were followed up for a period of 13 years, it was found that there was a significant association between plasma homocysteine and coronary artery disease in women with prevalent heart disease at base line. However a similar association was not observed among women free of heart disease at baseline (165). But these findings may not give the correct picture as the follow-up period may not be adequate to get a clearer
understanding as indicated by recent reviews which have demonstrated that most of the previous research has been based on relatively short-term follow-up periods, from 1.4 to 13 years (123, 166).

In a recent study where the follow-up period was longer, the association between homocysteine and coronary heart disease was found to be more significant. Zylberstein et al. (2004) reporting on a 24 year follow up of the population study of women in Gothenburg, involving 1368 women aged 38, 46, 50, 54 and 60 years at the time of recruitment who were free of previous acute myocardial infarction at baseline, found that homocysteine concentrations in excess of 14.2 μmol/L in middle-aged women is an independent risk factor for future myocardial infarction and in particular, mortality due to myocardial infarction. This study is of importance compared to other studies as this illustrates the importance of long-term assessment of the effect of homocysteine levels on cardiovascular morbidity and mortality, especially in women (167).

2.1.5.2 Mechanisms responsible for atherosclerosis and CAD in hyperhomocysteinaemia

Different mechanisms are responsible for arterial and venous thromboembolic disease, and these include abnormalities in platelet function to disorders of coagulation, lipid abnormalities and endothelial dysfunction. Numerous studies have been conducted to ascertain mechanisms responsible for atherosclerosis in hyperhomocysteinaemia. Most of the earlier studies have explored the mechanisms of homocysteine induced atherosclerosis and these studies have found that the endothelial damage, platelet activation and altered thrombus formation to be the reasons for vascular disease in hyperhomocysteinaemia (168, 169). Some earlier studies have reported on patients with
severe homocystinuria who had serum homocysteine levels as high as 200-400 μmol/L or in animal models where they have infused homocysteine concentrations in the range of 1-10 mmol/L. However these concentrations are higher than even in the most severe cases of hyperhomocysteinaemia as seen in severe homocystinuria. In mild hyperhomocysteinaemia, the levels of homocysteine are on average 30% higher than normal, as compared to the above pathological and experimental conditions used in animal studies (84). Thus the possibility that the observed effects were due to non-specific reactivity of the sulphydryl group of homocysteine could not be ruled out. This was further supported by studies where effects similar to homocysteine have been found when other thiol containing compounds like cysteine and mercaptoethanol have been used (170-174). Thus the inferences made on the results of these studies have been questioned on the basis that these may not give the true picture on the mechanisms of how mild to moderate hyperhomocysteinaemia induce atherosclerosis.

Recent and some earlier studies conducted to explore the mechanisms of homocysteine induced atherosclerosis have been on patients with mild to moderate hyperhomocysteinaemia or on animal models having similar homocysteine concentrations. These studies have provided possible explanations to the mechanisms involved in atherogenesis in individuals with mild to moderate hyperhomocysteinaemia. One of the mechanisms by which homocysteine induce vascular disease is by modulating vasodilatation through lowering the endothelium-dependent vasodilation (175) and this has been corroborated in animal models too (176). Endothelium dependent vasodilatation is a function of the relaxing action of nitric oxide on the blood vessel and elevated homocysteine levels have been found to interfere with and decrease the nitric oxide levels in blood vessels (177-180). The reason for this reduction in nitric oxide levels is not fully understood, but it is thought to be related to the increased production of superoxide anions by the endothelial cells in response to homocysteine. This overproduction of superoxide anions can lead to the inactivation of nitric oxide, thereby reducing its vasodilatory effects. Therefore, the reduction in nitric oxide levels can contribute to the development of atherosclerosis by impairing the ability of the endothelium to relax and dilate blood vessels. The exact mechanisms by which homocysteine induces atherosclerosis are still under investigation, and further research is needed to fully understand the role of homocysteine in the development of this disease.
oxide concentration was neither due to decreased activity of endothelial nitric oxide synthase nor due to decreased activity of nitric oxide synthetase 3 transcription suggesting that the decrease in nitric oxide levels is not entirely due to decreased synthesis but due to the inability to sustain production of S-nitrosithiols (compounds with potent vasodilatory and antiplatelet effects) owing to a progressive imbalance between the production of nitric oxide by progressively dysfunctional endothelial cells and the levels of homocysteine (172). The same study found that prolonged exposure of endothelial cells to homocysteine for more than 3 hours can result in decreased endothelium derived relaxing factor thus altering the normal functions of the endothelium (172). In addition homocysteine has been found to decrease the levels of glutathione peroxidase and it is thought that in an environment where antioxidants (including GPx) are not active or lacking, elevated homocysteine being a prooxidant that produce H$_2$O$_2$ (181), leaves NO at a greater susceptibility to oxidative inactivation (178).

Vascular endothelial cells, which play an active role in the physiological processes of vessel tone regulation and vascular permeability, form a border separating deeper layers of the blood vessel wall and cellular interstitial space from the blood and circulating cells. Damage or dysfunction of endothelial cells may reduce the effectiveness of the endothelium to act as a selectively permeable barrier to plasma components, including cholesterol-rich lipoprotein remnants. This process is involved in the etiology of atherosclerosis and it has been found that one mechanism by which homocysteine induces vascular disease is by promoting endothelial dysfunction.

In vitro studies have shown that homocysteine has the ability to directly damage blood vessel endothelial cells in a concentration dependent manner (182). It has also
been noted that homocysteine induced cell damage can be prevented by the addition of catalase, suggesting that homocysteine induced hydrogen peroxide generation is an important intermediary step in this process (183) and presence of copper has been found to be essential for the generation of hydrogen peroxide for the cell damage observed in these in vitro studies (181).

In a study to assess the effect of homocysteine on the growth of both vascular smooth muscle cells and endothelial cells at concentrations similar to those observed in clinical studies, Tsai et al. (1994) found that homocysteine has growth-promoting effects on vascular smooth muscle cells, together with an inhibitory effect on endothelial cell growth both important in explaining homocysteine induced atherosclerosis (173). Other studies have also found that homocysteine induces endothelial dysfunction in vivo (184-186). It is also noted that homocysteine can modify the adhesive properties of endothelium (187), promote endothelial neutrophil interactions (188), and enhance monocyte adhesion to endothelial cells (189), all involved in promoting atherosclerosis.

Several studies have found that homocysteine plays a significant role in development of a prothrombotic environment in the vasculature (190) by enhancing platelet activation and aggregation (187, 191-193), increasing thromboxane biosynthesis (191-193), suppressing activity of the anticoagulant heparan sulfate (171), reducing the binding of the principle fibrinolytic activator tissue plasminogen activator to the endothelial cells (194), activating endogenous factor V of the clotting pathway (195) and by reducing protein C activation.
2.2 Apolipoproteins and CAD

2.2.1 Apolipoprotein A and apolipoprotein B

Apolipoprotein B (apo B) is a large amphipathic protein which exists in two forms, apo B-48 and apo B-100. Apo B proteins are referred according to the percentage of apo B-100 that they represent in terms of the amino acid sequence. Apo B-48 shares some amino acid determinants with apo B-100. Thus apo B-48 represents the N-terminal 48% of apoB-100 (196) and it is synthesized in the intestine. Dietary triacylglycerols and free cholesterol absorbed from the gut lumen complexes with apoB-48 to form chylomicron particles which are metabolized in the circulation and in the liver. Apolipoprotein B-100 which is synthesized in the liver is present in low-density lipoprotein cholesterol (LDL-C), very low density lipoprotein (VLDL), intermediate density lipoprotein(IDL) and lipoprotein(a) (Lpa) (197). In cholesterol absorption, apo B is essential for the binding of LDL particles to the LDL receptor, allowing the cell to internalize LDL. Although clinical assays measure both apo B-100 and apo B-48, apo B-100 is the major apolipoprotein in plasma compared to the small amounts of Apolipoprotein B-48 found even in the postprandial state. Thus most often Apolipoprotein B is synonymous with apo B-100 unless specifically referring to studies focusing on apo B-48.

Apolipoprotein A also exists in two forms, namely apo A-I and apo A-II. Apo A-I is the major apolipoprotein found in HDL-C. It is postulated that expression of apo A-I is largely responsible for the levels of HDL-C in plasma. Apo A-I is involved in removal of excess cholesterol from tissues and incorporating into HDL for reverse transport to the liver by acting as an essential cofactor for the enzyme lecithin cholesterol acyl transferase (LCAT) (198). Thus its main function is to pick-up and
transfer excess cholesterol from peripheral tissues back to the liver, the only organ capable of excreting it in significant quantities (in bile).

Apolipoprotein A-I reflects the athero-protective mechanism of lipid metabolism as apolipoprotein A-I is not found in the atherogenic apolipoprotein B containing particles. Apart from the anti-atherogenic properties (199), apolipoprotein A-I has anti-inflammatory and anti-oxidant properties (198, 200-202) which help in its cardio-protective role as inflammation and oxidation are believed to be important processes in initiation and progression of atherosclerosis and vascular disease.

There is evidence from research studies that apoA-II plays a key role in HDL metabolism (203). Increased apoA-II deteriorates two major antiatherogenic properties of HDL, namely protection against oxidative modification of LDL and reverse cholesterol transport (204) although the role of Apo A-II in reverse cholesterol transport compared to Apo A-I is unclear (205). Nevertheless, studies performed in genetically modified mice show a positive correlation between apo A-II and plasma free fatty acid and triglyceride levels, while its deficiency has been associated with increased atherosclerosis and insulin hypersensitivity (204). Over expression of mouse apoA-II in transgenic mice has been found to promote development of atherosclerosis on both normal and atherogenic diets despite causing significant elevations in plasma HDL cholesterol levels (206). Apo A-II also counteracts several beneficial effects of apo A-I in humans, including diminishing the latter’s atheroprotective and anti-inflammatory roles (207, 208). The apo A-I antagonist ability of apo A-II might be a consequence of its higher affinity for lipids which permits it to displace apo A-I from HDL (209-211). Displacement of apo A-I from HDL affects the interaction of the particles with proteins.
involved in exchange of neutral lipid, lipolysis of triglycerides, and HDL remodeling (209, 212).

2.2.2 Apolipoprotein A-I and apolipoprotein B in atherosclerosis and CAD
2.2.2.1 Lipids and their role in atherosclerosis

Several epidemiological studies have reported that apolipoprotein A-I is both an important risk predictor, and low apolipoprotein A-I a risk factor, for myocardial infarction (198, 199, 201, 213-216). The postulated protective effect of HDL cholesterol and apolipoprotein A-I has not been found to be true in all studies as found by Corsetti et al. (2006) and Asztalos et al. (2002) in their studies. Asztalos et al. (2002) found that HDL subpopulation profile of low levels of α-1 and α-2 and high levels of α-3 was associated with increased risk of cardiovascular disease events (217-219). In addition, Corsetti et al. (2002) have reported that in non-diabetic post-infarction patients with high levels of C-reactive protein and total cholesterol levels, elevated HDL levels would further contribute to an increase in the risk of recurrent myocardial infarctions. Therefore it was postulated that in these patients oxidative or any other modulatory mechanism could change the apolipoprotein from being athero-protective to atherogenic (220). Thus patho-physiologically all HDL sub-particles and all cholesterol in HDL particles cannot be considered to act the same (217, 218). Evidence against high HDL cholesterol and apolipoprotein A-I levels being atheroprotective is scarce and in the majority of studies, high apolipoprotein A-I concentrations have been found to be protective against vascular disease (197, 202).

There are three competing hypotheses that have been put forward to explain the events that initiate atherosclerosis. The “response-to-injury” hypothesis proposes that
the initial event in atherosclerosis is injury to endothelial and smooth muscles (221), and the “Oxidation” hypothesis emphasizes the significance of the oxidative modification of LDL in the subsequent atherogenic processes such as recruitment of macrophages and other inflammatory cells to the site of lesion (222). The third, ‘response-to-retention’ hypothesis postulates that the initiating event in atherosclerosis is the retention and accumulation of LDL and other atherogenic lipoproteins in the arterial wall (223). The retained lipoproteins directly or indirectly provoke all of the known pathological features of early lesions such as lipoprotein oxidation, monocyte migration into the arterial wall, cytokine production and smooth muscle proliferation (223).

There is increasing evidence from recent studies to support the response-to-retention hypothesis as the most correct of the three hypotheses. The response-to-retention hypothesis proposes that the apo-B containing lipoproteins’ ability to promote atherogenicity depends on four main factors:

(i) The plasma concentration of atherogenic lipoproteins.

(ii) The difference between the arterial wall influx and efflux of atherogenic lipoproteins (retention of atherogenic lipoproteins in the artery wall). Two proteoglycan binding sites in apo B-100 (site A at residues 3148-3158 and site B at residues 3359-3369), which are important for retaining the lipoprotein in the intima of the artery, have been identified (224). These basic amino acid sequences in the apo B-100 sites bind the negatively charged sulphate groups on the proteoglycans (223, 225), thus ensuring retention within the intima of the arterial wall. Experimental evidence suggests that the second of these two sites, site B, is the main site on apo B-100 that interacts with proteoglycans (225).
(iii) The modification of the protein and lipid moieties of the retained lipoproteins. The composition of the core lipids in LDL influences their ability to interact with arterial wall proteoglycans. Cholesterol enriched LDL has increased affinity for both glycosaminoglycans and the LDL receptor in the arterial wall. Modification of LDL in the subendothelium also exposes other proteoglycan binding sites. These interactions with proteoglycans induce structural alterations of the LDL that expose glycosaminoglycan binding sites, which may contribute to the intramural retention of LDL after the initial interaction with the primary binding site.

(iv) The inflammatory response to the modified retained lipoproteins. Evidence for this comes from epidemiological studies which have shown that elevated levels of apo-B containing lipoproteins in humans are required to increase the incidence of atherosclerosis and cardiovascular disease (226). Other risk factors have the ability to accelerate the atherosclerotic process, but in the absence of dyslipidemia, their contribution to atherogenesis is minimal (227). At the same time nonatherosclerotic arterial lesions due to rare causes could occur without altering lipid deposition but they can be easily distinguished from lesions that are due to atherosclerosis (227). Evidence for apo-B containing lipoproteins being required for atherogenesis also comes from animal studies where there are no animal models found in which atherosclerosis can be induced without altering lipoprotein profiles (223).

Thus the presence of apo B in atherogenic particles leads to the entrapment of these lipoproteins in the arterial wall leading to atherosclerosis. Apo B-100 also aids in the stabilization, and allows transport of triglycerides and cholesterol in plasma VLDL, IDL, large buoyant LDL and small dense LDL molecules and it also acts as the ligand
for the apo B and Apo B, E receptors which facilitate the liver and the peripheral tissues in the uptake of cholesterol (197, 198).

Approximately 90% of the Apo B is found in LDL (228, 229). Normal or a low LDL-C along with an increased level of apo B may indicate that there is an increase in the number of small dense LDL (sd-LDL) particles in plasma. This is highly significant as sd-LDL particles are considered more atherogenic than large buoyant LDL molecules. sd-LDL is easily internalized into the sub-intimal space where they get adhered to proteoglycans in the matrix (230), and are oxidized thus increasing the risk of atherothrombosis (231). sd-LDL also has the ability to promote inflammation thus aiding the growth of atherogenic plaques (197, 198, 232). Although not to the extent of sd-LDL, other apo B containing lipid particles like VLDL and IDL too can stimulate atherogenesis. This is by stimulation of inflammatory reactions and by inhibition of the fibrinolytic system leading to atherothrombosis (198).

2.2.2.2 Assessment of lipid related risk of cardiovascular disease

Lipid related risk of cardiovascular disease is usually assessed by measurement of high-density lipoprotein cholesterol (HDL-C), non-HDL-C as well as triglyceride (TG) levels and lipid ratios such as total cholesterol (TC) /HDL-C and LDL-C/HDL-C. Out of these, LDL-C is recognized as the primary lipid risk factor for ischaemic heart disease. These lipid parameters are extensively used in assessment of CV risk as proposed by several major guidelines (233, 234). Of these LDL-C and HDL-C are the ones most clinicians use in risk evaluation as it is cumbersome to remember the recommended guidelines/cut off values by National Cholesterol Education Programme 2004 (NCEP -2004) Adult Treatment Panel III (ATP- III) (235) for all parameters and
ratios that come under different risk categories related to cardiovascular disease. There is indisputable evidence for the positive relationship between the plasma levels of LDL-C and the risk of coronary heart disease and the fact that reduction of LDL-C reduces clinical cardiovascular events (236). But new evidence based on multiple cell animal biological studies, epidemiological studies and clinical trials, has led to what is now referred to as the atherogenic lipoprotein particle paradigm, which postulates that the total number of atherogenic particles is a more important determinant of the risk of vascular disease than any of the conventional lipid measures (237). The importance of this lies in the fact that the number of lipid particles within any of the lipoprotein fractions determines the likelihood that the given type of lipoprotein type would tend to lodge within an arterial wall. In using the lipid parameters that are used currently in assessing risk of atherothrombosis, it is assumed that the risk due to a given lipoprotein fraction is equal to the concentration of that particular type of lipid concentration in plasma. Thus triacylglycerols are the estimate of the risk due to VLDL, LDL cholesterol the estimate of the risk due to LDL, and non-HDL cholesterol the estimate of the combined risk of VLDL, IDL, LDL and Lp(a). But the lipid levels do not automatically equal the level of lipoprotein particles because the lipid composition of the main atherogenic lipoprotein fractions differs amongst individuals. In comparison to this, each VLDL, intermediate density lipoprotein(IDL), LDL and lipoprotein(a) lipoprotein particle contains one molecule of apo B-100 (237, 238) therefore the total apo B value indicates the total number of potentially atherogenic lipoproteins (80). The lipid composition of VLDL, IDL and LDL differs substantially and as each contains one molecule of apo B, it is evident that there is no precise relationship between the number of particles within a given lipid fraction and the lipid concentration of the specific
lipoprotein fraction. Thus the best indicator currently available to estimate the atherogenic particle number is the measurement of total plasma apo B, which represent the atherogenic burden of lipoprotein (237).

2.2.2.3 Apolipoprotein A-I and apolipoprotein B in the assessment of risk of cardiovascular disease

Previous studies have shown evidence for the positive relationship between the plasma levels of LDL-C and the risk of coronary heart disease. However, a series of newer prospective epidemiological studies involving both LDL and apolipoprotein B have shown apolipoprotein B to be superior to LDL in predicting the risk of vascular disease including coronary heart disease. This is in comparison to earlier studies analyzing the predictive value of apolipoprotein in prediction of myocardial infarction showing mixed results and this has been attributed to the fact that methods, procedures and population based reference intervals have not been standardized. But with WHO-International Federation of Clinical Chemistry standardized reference materials for measurement of apolipoprotein B and apolipoprotein A-I (239, 240) and results from population based studies using standardized techniques being available (241-245)), uniformity in apolipoprotein assaying methods and reference intervals are observed now.

Recent studies support the new concept of evaluating cardiovascular risk by using apolipoproteins and especially the apolipoprotein B/A-I ratio as a simple integrated index of risk assessment when compared to all lipid parameters and also the use of apolipoprotein B and apolipoprotein B/A-I ratio as a target for lipid-lowering therapy.
A cohort study conducted for 5 years by Lamarche et. al (1996) using 2155 men aged 45-76 indicated that the baseline apolipoprotein B levels were strongly associated with onset of coronary heart disease. This association was independent of co-variables such as age, smoking, systolic blood pressure and diabetes mellitus and the predictive effect of apolipoprotein B remained significant even after controlling for triglycerides, HDL-cholesterol and total/HDL cholesterol ratio. After stepwise logistical regression analysis apolipoprotein B was found to be the strongest correlate among the metabolic variables. Furthermore, multivariate analysis indicated that apolipoprotein A-I was also protective, but not as strongly as apoB (246). The 13 year follow-up of the same study sample revealed that plasma apolipoprotein B levels were an independent risk factor for IHD in multivariate analysis even after taking into account variables related to the LDL size phenotype. Plasma Apolipoprotein levels were found to modify the short term risk of IHD attributable to the small dense LDL phenotype suggesting that information on both LDL size and LDL particle number as suggested by the Apolipoprotein B levels are important in giving a more accurate view of a patient's risk of IHD over a given period of time (247).

In a different study (Talmud et al. 2002) involving middle aged men free of coronary heart disease at baseline, 163 subjects out of a total of 2508 suffered a coronary heart disease event (acute myocardial infarction, coronary artery surgery or ECG evidence of silent myocardial infarction) during a follow-up period of 6 years. In this study, total cholesterol, triglycerides, apolipoprotein B, apolipoprotein B/A-I ratio, LDL-Cholesterol and HDL-Cholesterol were all observed to be predictors of significant risk. The apolipoprotein B/A-I ratio was associated with the strongest effects on relative risk (RR). Apolipoprotein B and TGs in combination added risk information over and
above lifestyle factors when compared to apolipoprotein B and HDL cholesterol levels of the individual (248).

In another cohort study, involving 6605 men and women (Gotto et al. 2000) the Air Force/Texas Coronary Atherosclerosis Prevention Study (AFCAPS/TexCAPS), the relationship between baseline and on-treatment lipid parameters and first acute major coronary events following treatment with lovastatin in comparison to a placebo was studied. Results from this randomized double blind case control study revealed that apolipoprotein B is the single most significant and consistent lipid measurement predictive of risk, both at baseline and on treatment with lovastatin. In regression analysis the predictive power was only slightly improved by the incorporation of apolipoprotein A-I to form the apolipoprotein B/A-I ratio. Apolipoprotein B/A-I ratio was found to be the best discriminator of baseline risk. The apolipoprotein B/A-I ratio was able to identify not only the group at highest risk of an acute major coronary event, but also the group with the lowest risk. Depending on these findings the authors have suggested that it maybe of more value to use apolipoprotein B rather than LDL-cholesterol to assess the on-treatment effect of reducing the atherogenic burden. This study further suggested that apolipoproteins B and A-I should be considered in risk assessment and, treatment goals based on apolipoprotein B and /or apolipoprotein B/A-I ratio be further explored in populations considered to be at average risk, as apolipoprotein B was found to be a more accurate predictor of acute major coronary events than LDL-cholesterol (249).

However, some studies have failed to find a significant association between apolipoprotein B and coronary heart disease events, thus questioning the rationale for advocating apolipoprotein B and/ or apolipoprotein A-I as predictors of CHD events. In
1997 Cremer et al. presented data on a prospective study which was conducted in 5790 male subjects with the aim of evaluating the independent impact of lipoproteins, apolipoproteins, fibrinogen and family history on myocardial infarction compared to the accepted major coronary risk factors of that time, namely cholesterol, hypertension and smoking. Results from this Goettingen Risk Incidence and Prevalence Study (GRIPS) show LDL-cholesterol to be the strongest predictor of myocardial infarction after multivariate regression model for the prediction of MI risk. Family history of MI, Lp(a), age, smoking, systolic blood pressure, HDL-cholesterol (Inversely related) and plasma glucose were found to be other additional predictors of MI. Apolipoproteins B and A-I along with the total/HDL-cholesterol ratio, LDL/HDL-cholesterol, and Apolipoprotein B/A-I ratio were found to be less effective predictors of MI as they did not contribute independently to the prediction of MI risk. HDL-cholesterol was found to be a better predictor of MI risk than apolipoprotein A-I (250). These results, highlighting the association between the traditional lipid parameters and risk of coronary heart disease are in accordance with another prospective study, the Atherosclerosis Risk in Communities (ARIC) study. The results of this latter study did not show a significant predictive power of apolipoprotein B and apolipoprotein A-I for coronary heart disease when they were considered together with LDL-cholesterol, HDL-cholesterol and triglycerides although apolipoprotein B and apolipoprotein A-I were strongly identified as predictive of coronary heart disease when considered alone. ARIC study showed a strong association between total cholesterol, LDL-cholesterol and triglycerides with increased risk of coronary heart disease, and of HDL-cholesterol with a decreased risk (251). However, findings from this study has been questioned on the basis of not using a standardized Apolipoprotein B assay method, and the estimated error of the assay was
17% which was considered to be significantly higher than the accepted value of 5% (252).

The association between apolipoprotein B concentrations and coronary events in subjects who were on treatment following a previous episode of a CHD event has also been reported. The Long-term Intervention with Pravastatin in Ischaemic Disease (LIPID) trial was conducted to assess baseline and on-study lipid levels as independent risk factors for subsequent coronary heart disease events in subjects with a history of acute myocardial infarction or a diagnosis of unstable angina. Another objective of this LIPID trial was to assess the effect of pravastatin on lipid concentrations and coronary heart disease events according to baseline lipid levels and the proportion of the treatment effect explained by the effects of pravastatin on lipid levels. Results indicated that HDL cholesterol and total/HDL-cholesterol ratio were significant predictors of coronary heart disease events. Apolipoprotein B levels were found to be a stronger predictor of risk of coronary heart disease events than LDL-cholesterol and the best single predictor of the treatment effect explained by the effects of pravastatin on lipid levels. Clinical relevance of this lies in the fact that apolipoprotein B signifies the number of lipoprotein particles which are associated with vascular disease as described (253).

Furthermore, the Thrombogenic Factors and Recurrent Coronary Events (Thrombo) study on post infarct patients with and without metabolic syndrome also demonstrated that the apolipoprotein B concentrations are significantly associated with risk for recurrent coronary events in metabolic syndrome patients. However, the other lipid parameters like triglycerides, total cholesterol, HDL-cholesterol, Lipoprotein (a),
and apolipoprotein A-I were found to be poorly associated with risk for recurrent coronary events in the study sample (254).

The Apolipoprotein–related MOrtality RISk (AMORIS) study conducted using 175,553 individuals followed up for a period of 5 years with the aim of assessing the predictive power of apo B, Apo A-I and apo B/Apo A-I ratio revealed that values for apo B and apo B/apo A-I ratio were strongly and positively related to an increased risk of fatal myocardial infarction in both men and women whereas apo A-I was shown to be protective. Similar observations were also seen even after adjusting for age in univariate analysis and for age, total cholesterol and triglycerides in multivariate analysis. More importantly in both sexes apo B was found to be a strong predictor of risk in multivariate analysis than LDL-C. The authors suggested that although LDL-C and HDL-C were known risk factors, apo B, apo A-I and Apo B/Apo A-I ratio should also be considered as highly predictive in evaluating cardiac risk. They also suggested that apo B and apo A-I might be of greatest value in diagnosis and treatment of men and women who have common lipid abnormalities with normal or low concentrations of LDL-cholesterol. Furthermore, when compared to total cholesterol, which lost its predictive power in people over 70 years of age, apo B, apo A-I and apo B/apo A-I remained as an important risk predictor in old people even beyond the age of 70 (80).

Current knowledge about cardiovascular risk factors and prevention of cardiovascular disease is mainly derived from studies conducted with populations of European origin. Thus these findings cannot be directly applied to populations of non-European origin as this may give a false interpretation. Previous studies also suggest that risk factors like abnormal lipid parameters and even hypertension may vary between populations. Along with this, even if the association of risk factors were
similar, the prevalence of these risk factors may vary from one population to another
giving rise to different population attributable risks (PAR). The INTERHEART study
involving 15,152 cases with acute myocardial infarction and 14,820 controls from 52
countries representing all inhabited continents of the world found that collectively nine
potentially modifiable risk factors, namely smoking, raised apolipoprotein B/A-I ratio,
history of hypertension, diabetes, abdominal obesity, psychosocial factors, lack of daily
consumption of fruits and vegetables, not having regular physical activity and regular
alcohol consumption, accounted for 90% of the PAR in men and 94% in women. The
study also revealed that five risk factors, namely smoking, lipid abnormalities
(apolipoprotein B/A-I ratio), hypertension, diabetes and obesity, which a large
proportion of the study sample had, accounted for about 80% of the PAR. This study
further suggested that in view of the overlap in the effect of these nine risk factors, most
of the PAR could be accounted for by a combination of various factors, as long as they
included smoking and the apolipoprotein B/A-I ratio (PAR for the combination being
66.8% [99% confidence interval 62.8- 70.6]). Both smoking and apolipoprotein B/A-I
ratios showed a graded relation with the odds of myocardial infarction without a
threshold or a plateau in dose response. This graded relationship was consistent with the
previously described AMORIS study. Apolipoprotein B/A-I ratio was found to be the
most important risk factor in all geographic regions in this study and the authors were of
the view that a substantial modification of its population distribution is important for
worldwide reduction of myocardial infarction (17).
2.3 Glutathione peroxidase and CAD

2.3.1 Glutathione peroxidase

Glutathione peroxidase (GPx) is a selenocysteine-containing enzyme which serves an important role in the cellular defence against oxidant stress (255) by utilizing reduced glutathione (GSH) to reduce hydrogen peroxide to water and lipid peroxides to their corresponding alcohols. There are four GPx’s which contain selenocysteine at the active site (256). GPx-1, also referred to as cytosolic or cellular GPx, was first described by Mills et al. in 1957 and its function was hypothesized to be protection of red blood cells from haemolysis induced by oxidants (257). The tetrameric protein GPx-1 is the most abundant and the major intracellular form of GPx (256). GPx-1 can metabolize hydrogen peroxide and other organic peroxides like cholesterol and long chain fatty acid peroxides (256). GPx-1 does not have the ability to metabolize fatty acid hydroperoxides in phospholipids unless accompanied by phospholipase A2 activity which is needed to release the fatty acids from phospholipids for GPx-1 to act upon (258). Though it can metabolize a range of peroxide substrates, GPx-1 is very specific for glutathione as reducing substrate. Thus GPx-1 activity is generally discussed in parallel with glutathione reductase activity, which maintains a constant supply of reduced glutathione (GSH) from oxidised glutathione (GSSG) for enzyme activity.

The second type of GPx, which occurs in the cytosol is referred to as GPx-2 or gastrointestinal glutathione peroxidase. GPx-2 mRNA is mainly found in the gastrointestinal tract in the rat, hence the name, but in humans the mRNA is found in liver and large intestine but not in other organs. GPx-2 which is also tetrameric has approximately 65% amino acid sequence identity and 60% nucleotide sequence identity with GPx-1 and have similar substrate specificity to GPx-1 in that they reduce hydrogen
peroxide or fatty acid hydroperoxides rapidly but not phospholipid hydroperoxides (259). Finding that plasma GPx activity had similar substrate specificity to GPx-1 but plasma GPx did not react with antibodies to GPx-1 (260) led to the discovery of plasma GPx or GPx-3. GPx-3 is a tetrameric glycoprotein enzyme (261) seen in plasma and based on presence of mRNA or immunological identification of the protein it has been found that it is predominantly seen in the kidney. It is also found in the heart, placenta, lung, gastrointestinal cells and the thyroid gland. The exact role played by GPx-3 is unknown (262) and it is thought to act as an extracellular antioxidant. Phospholipid hydroperoxide GPx or GPx-4 is structurally different to the other three forms of glutathione peroxidases in that it is a monomer in contrast to the tetrameric structure of other GPx’s (256). Compared to GPx-1 it can react with phospholipids hydroperoxide as substrate and it can also use hydrogen peroxide and other lipid hydroperoxides as substrate.

Hydrogen peroxide has the ability to form the highly reactive toxic oxygen species hydroxyl radical (·OH). This can cause lipid peroxidation and formation of hydroxide anion (OH\(^-\)) which in turn promote alkaline tissue damage, a process that is offset in part by catalase and GPx-1 dependent reduction to H\(_2\)O. Elevated levels of peroxides are accompanied by an increase in peroxyl radicals which has the ability to inactivate nitric oxide through formation of lipid peroxinitrites (263). Although the exact mechanism of how these peroxyl radicals are formed is not known, theoretically deficiency of GPx-1 can lead to an increase in reactive oxygen species and a decrease in the bioavailability of nitric oxide.
2.3.2 Glutathione peroxidase in atherosclerosis and CAD

Many studies have been carried out to assess the association of free radicals and oxidative stress in atherogenic processes and it has been found that oxidant-mediated LDL oxidation and vascular injury are crucial events in atherogenesis (264, 265). The endogenous antioxidant capacity of arterial tissues seems relevant in atherosclerosis because, given the strong antioxidant properties of plasma, LDL oxidation may occur in sequestered areas of the arterial wall, where a low antioxidant potential and/or a high prooxidant activity could be operative (265-267). But recent studies have shown that human atherosclerotic plaques are equipped with a surprisingly high content of low-molecular-weight antioxidants, such as vitamin E, and ascorbate, despite the occurrence of lipid oxidation in the atheromatous plaques (268). Enzymatic antioxidant defenses have been found to play a decisive part in limiting atheromatous plaque formation (269) and glutathione-related enzymatic systems have been found to play an important role in biomolecular antioxidant protection especially in vascular cells (255, 270-272).

In a study using carotid atherosclerotic plaques, Lapenna et al. (1998) found that GPx activity is often absent in atherosclerotic plaques (269) and this has been attributed to inactivation of GPx by oxidant species (273-276). Previous studies have found that GPx is particularly susceptible to inactivation by myeloperoxidase-derived hypochlorous acid (274) and researchers have found hypochlorous acid modified proteins and myeloperoxidase as an oxidative catalyst in human atherosclerotic lesions (277, 278). Another mechanism for the decrease in GPx activity in vascular disease has been attributed to products of lipid peroxidation in vascular wall inactivating GPx (279). It has been reported that specific antioxidant enzyme inactivation, especially involving GPx, occurs in atherosclerotic lesions which have to be considered as a pro-oxidant
environment with lipoperoxide burden in vascular tissue (266-268, 277, 278, 280). Peroxides, especially in the presence of redox active transition metals, are cytotoxic to vascular cells (266) and researchers have found that redox active metals like copper and iron are present in a catalytically active form in human atherosclerotic plaques (272).

GPx-1 is the key antioxidant enzyme of the mammalian cell and is essential for the removal of inorganic and organic peroxides (255). Along with this it has been found that the other two main antioxidant enzymes found in the cells, namely catalase and superoxide dismutase do not play an important role in human vascular cells. It has been reported that catalase activity is lacking in human vascular cells (281) and superoxide dismutase has been found to be poorly effective in safeguarding the cells in the human vasculature from damage caused by oxidants (255, 282). Thus any derangement in the GPx antioxidant activity would give rise to serious consequences leading to significant weakening of the antioxidant status in atherosclerotic tissues and oxidative damage to the vasculature even though the concentration of low molecular weight antioxidants like ascorbate and vitamin E are adequate (268). This would lead to a more severe expression of atherosclerosis in susceptible humans (269). Some have challenged the notion that specific deficiency of GPx-1 increases susceptibility to atherosclerosis. One example is where de Haan et al. (2006) did not find any association between lack of the antioxidant GPx-1 and an increase in atherosclerosis in GPx-1 deficient mice who were fed a high fat diet for 12 – 20 weeks. They found that expression of antioxidant genes were mostly unaltered and concluded that according to the results of their study, a specific deficiency in GPx-1 is not accompanied by an increase in markers of oxidative damage or increased atherosclerosis in a murine model of high fat diet induced atherogenesis (283).
On the other hand studies done on cell lines have shown that over expression of GPx-1 can protect cells against reactive oxidant species, for example, damage caused by hydrogen peroxide and lipid hydroperoxides and redox cycling drugs like paraquat (284, 285).

The hypothesis of cellular antioxidant enzymes constituting a cellular defense against acute stress has been investigated by several researchers. A study conducted by Yoshida et al. (1996) using transgenic mice who were over-expressing GPx-1 found that after subjecting the hearts of these mice to 30 minutes of ischaemia and 20 minutes of reperfusion to induce myocardial ischaemia/reperfusion injury, the cellular damage due to ischaemia in the heart is decreased in transgenic mice who had a four fold increase in heart GPx-1 compared to non-transgenic controls who had normal GPx-1 activities. They also found that transgenic mice hearts had significantly improved recovery of contractile force and rate of contraction compared to controls and the infarct size was lower in transgenic mice compared to controls. Release of creatine kinase from the transgenic mice were also found to be significantly lower than the control groups and the authors were of the view that increased GPx-1 expression renders the heart more resistant to myocardial ischaemia reperfusion injury (286). They further reported that in another similar experiment involving glutathione peroxidase knockout mice, the GPx-1 knockout mice had significantly low force of contraction and increased infarct size compared to nontransgenic controls. The transgenic mice also had increased release of creatine kinase compared to controls. These findings suggest that GPx-1 plays an important role in myocardial protection from ischaemia reperfusion injury (287). In another experiment conducted to assess the effect of cerebral ischaemia/ reperfusion injury on GPx-1 knockout mice compared to wild-type controls, the authors reported
that Gpx-1 knockout mouse brains had increased infarct size and exacerbated apoptosis compared to the wild type controls (288), thus highlighting the importance of GPx-1 in rendering tissues resistant to ischaemia-reperfusion injuries, not only in the cardiac muscle but in neural cells as well.

Studies performed on GPx-1 knockout mice have also found that GPx-1 to be protective against virus-induced myocarditis (289), endotoxaemia (290) and pro-oxidant induced neurotoxicity (291, 292) whilst some studies have not found any association between reactive oxygen species induced oxidative stress and GPx-1 in in-vitro and cell line studies (293, 294).
2.4 Assessment of severity of coronary ischaemia

Severity of ischaemia can be assessed by different methods, both invasive and non-invasive. These include the invasive coronary angiography, cardiac positron emission tomography (PET) perfusion imagery and non-invasive methods like the Doppler measurement of blood flow (295, 296).

Out of these one of the widely used methods of assessing severity of coronary atheroma is by utilizing coronary angiograms. Although there are different ways of quantitating angiographic severity of coronary atheroma, methods that are of greatest clinical use may not provide the best reflection of the atherosclerotic process (297). Two of the most used methods in assessing the severity of coronary atheroma by use of coronary angiograms are the vessel score and the stenosis score as initially described by Gensini, which has been modified by Gensini himself in 1983 (298) and subsequently by several authors (299). While the vessel score places emphasis on the number of vessels showing significant stenosis only, the stenosis score places emphasis on the severity of stenosis while including some measure on the extent of CHD. Thus both the vessel and the stenosis scores are of use in clinical practice as they place more emphasis on the severity of luminal narrowing. Plaque rupture and arterial thrombosis are two episodic events that strongly influence the angiographic severity of stenosis and these two processes underlie the clinical presentation of myocardial infarction and unstable angina (300-302). It has been argued that this may not be related to the same risk factors as the atherosclerotic process and that studies designed to examine the relationship between risk factors and coronary stenosis should, in addition to vessel and stenosis score, include an estimate of the extent of coronary atherosclerosis (297). The extent score developed by Sullivan et al (1990), which gives an indication about the proportion
of the coronary arterial tree involved by angiographically detectable atheroma, fulfils this need in assessing extent of coronary atherosclerosis (297).
3. MATERIALS AND METHODS

3.1 Chemicals and reagents

3.1.1 Water

Distilled water was used in all experiments except in the case of High Performance Liquid Chromatography (HPLC) where double distilled water was used.

3.1.2 Chemicals

Chemicals used for experiments were of analytical grade except for HPLC and gene analysis where HPLC and molecular biology grade were used.

3.1.3 Special chemicals/Reagents

L- homocysteine, cystamine dihydrochloride, N- (2-mercapto propionyl) glycine, Phosphate-buffered saline (PBS; pH 7.4), Tris(2-carboxyethyl)phosphine (TCEP), 7-Fluorobenzofurazan-4-sulfonic acid Ammonium salt (SBD-F) and EDTA were purchased from Sigma-Aldrich Corporation, U.S.A. Acetic acid and Methanol were purchased from Fischer scientific U.K. and all the other chemicals used in HPLC analysis were from BDH U.K. Folic acid and vitamin B12 assay kits were from Diagnostic Products Corporation, U.S.A. Konelab® apolipoprotein A-I and apolipoprotein B assay kits were from Thermo Electron Corporation, U.K. Ransel® Glutathione peroxidase assay kits were from Randox Laboratories Limited, United Kingdom. Axsym® Homocysteine assay kits were from Axis-Shield, United Kingdom. Molecular biology enzymes and reagents were from Sigma-Aldrich Corporation, U.S.A. and Promega Corporation, U.S.A.
3.2 Methods

3.2.1 Selection of subjects

3.2.1.1 Selection of study subjects for study 1 - Association of Hyperhomocysteinaemia with coronary artery disease in a Sri Lankan population.

i. Cases

221 patients diagnosed as having acute coronary syndrome were recruited as cases for the study. The diagnosis of an acute coronary syndrome was based on the presence of two out of the three clinical, biochemical and electrocardiogram criteria given below as recommended by the World Health Organization (WHO) (303):

Clinical:

Highly suggestive symptoms - substernal chest pain on exertion or at rest,

Biochemical: Elevation of cardiac enzymes/markers

- cardiac troponin I or T or
- creatine phosphokinase, and
- CPK-MB fraction elevation to suggestive range,

Electrocardiographic changes: Characteristic features of cardiac ischaemia on an ECG taken at rest or during treadmill exercise (showing T inversions / ST depression of > 2mm or ST elevation of ≥1mm).

Exclusion criteria for cases were evidence of chronic renal failure.

ii. Controls

221 apparently healthy persons matched for age (± 1 year) and sex with the cases were recruited from general surgical wards of Colombo South Teaching Hospital, Kalubowila, Sri Lanka. Exclusion criteria for controls were history of coronary artery
disease or symptoms suggestive of coronary artery disease, evidence of chronic renal failure, cerebrovascular accidents, taking medication which has been found to affect homocysteine levels (ex. Methotrexate), past history of deep vein thrombosis and peripheral vascular disease.

3.2.2 Selection of study subjects for study 2 - Association of Homocysteine, Vitamin B\textsubscript{12}, Folate, Apolipoprotein A-I, Apolipoprotein B, and glutathione peroxidase in CABG patients

79 patients awaiting coronary artery bypass grafting with angiographically confirmed coronary artery disease were selected for the study. Exclusion criteria for the subjects were evidence of chronic renal failure, cerebrovascular accidents, taking medication which has been found to affect homocysteine levels (ex. Methotrexate), past history of deep vein thrombosis and peripheral vascular disease.

3.3 Collection of blood samples

3.3.1 Study 1:

Blood samples for homocysteine assay were obtained from the participants who had undergone fasting for 12-14 hours. 2 ml of Blood was collected by venipuncture under aseptic conditions into pre-chilled K\textsubscript{3} EDTA containing Vacutainer\textsuperscript{®} tubes and immediately placed in ice. Plasma was separated within one hour of collection after centrifuging at 3000 rpm for 10 minutes. Samples were assayed immediately or stored for a maximum of 1 month at -20 °C pending analysis.
3.3.2 Study 2:

Blood samples were obtained from participants awaiting coronary artery bypass grafting who had undergone fasting for 12 hours. Venous blood was collected from a central venous catheter immediately prior to anaesthesia.

Aliquots of 2 ml of blood were transferred carefully into different vials containing heparin and K₃ EDTA as anticoagulant and also into plain tubes without an anticoagulant for analysis of Glutathione peroxidase, homocysteine, vitamin B₁₂, folate, Apolipoprotein A-I, apolipoprotein B and MTHFR gene analysis as indicated below.

- Heparin - Glutathione peroxidase
- K₃ EDTA - MTHFR gene analysis
- Plain tubes - Homocysteine, Folate, vitamin B₁₂, Apolipoprotein A-I and Apolipoprotein B

Blood collected to plain Vacutainer® tubes were placed in a dark container and allowed to clot and once the clot had formed, serum was separated by centrifuging at 3000 rpm for 10 minutes and stored at -20 °C until analysis (all samples were analyzed within one week for apolipoprotein A-I, apolipoprotein B, homocysteine, folate and vitamin B₁₂). Blood collected to K₃ EDTA and heparinized tubes were assayed immediately for the full blood count and glutathione peroxidase activity respectively and remaining K₃ EDTA blood was stored at -20 °C for MTHFR gene polymorphism analysis.
3.4 Data collection

3.4.1 Study 1:

A questionnaire was administered to all subjects recruited to the study (Appendix 2). The researcher was kept unaware to the fact whether the blood samples were from a case or a control subject until all samples were collected. To facilitate this process, recruitment of patients, filling of the questionnaire and collection of blood samples were performed by a qualified medical officer after being fully briefed by the researcher to facilitate blinding. The questionnaire filling was piloted by the researcher and the medical officer recruiting the patients in 10 patients with an ACS (who were not enrolled to the study) prior to commencement of the study.

Summary of data collected and definitions

1. Age (to the nearest completed year)
2. Sex – Male/Female
3. Weight – in kg
4. Height in meters
5. Smoking- Current smokers were defined as individuals who smoked any tobacco in the previous 12 months and included those who had stopped smoking within the past year.

   Former/Non smokers were defined as individuals who had not smoked any tobacco at any stage and those who had quit more than a year earlier (17).

6. Alcohol intake - Regular alcohol use was defined as consumption of any form of alcohol three or more times a week (17).
7. Past history of – Hypertension (Self reported systolic blood pressure ≥ 140 mmHg, diastolic blood pressure ≥ 90 mmHg (18), or taking antihypertensive medication)
   - Diabetes
   - High cholesterol (Self reported High-Cholesterol, Cholesterol level > 240mg/dl (32), or taking Lipid-lowering medications)
   - Chronic renal failure
   - Cerebrovascular disease
   - Deep vein thrombosis
   - Peripheral vascular disease
   - Parental history of myocardial infarction before 45 years

8. Whether involved in regular physical activities - Regularly involved in walking, cycling, gardening, jogging, football, vigorous swimming or exercising for 4 hours or more a week (17).

9. Taking regular vitamin supplementations - Taking multivitamin or vitamin B complex on 3 or more days per week.

3.4.2 Study 2:

A questionnaire/data sheet was administered to each subject recruited to the study (Appendix 3). The questionnaire/data sheet filling was piloted on 10 patients awaiting coronary artery bypass grafting (who were not enrolled for the study) before commencement of the study.
Summary of data collected and definitions

1. Age (to the nearest completed year)
2. Sex – Male/Female
3. Weight – in kg
4. Height - meters
5. Smoking- Current smokers were defined as individuals who smoked any tobacco in the previous 12 months and included those who had stopped smoking within the past year.
   Former/Non smokers were defined as individuals who had not smoked any tobacco at any stage and those who had quit more than a year earlier (17).
6. Alcohol intake - Regular alcohol use was defined as consumption of any form of alcohol three or more times a week (17).
7. Past history of – Hypertension (Self reported systolic blood pressure ≥140 mmHg, diastolic blood pressure ≥90 mmHg, or taking antihypertensive medication)
   - Diabetes
   - High cholesterol (Self reported High-Cholesterol, Cholesterol level > 240mg/dl (32), or taking Lipid-lowering medications)
   - Chronic renal failure
   - Cerebrovascular disease
   - Deep vein thrombosis
   - Peripheral vascular disease
- Parental history of myocardial infarction before 45 years

8. Whether involved in regular physical activities - Regularly involved in walking, cycling, gardening, jogging, football, vigorous swimming or exercising for 4 hours or more a week (17).

9. Taking regular vitamin supplementations - Taking multivitamin or vitamin B complex on 3 or more days per week.

10. Serum creatinine levels

11. Blood urea concentration

12. Coronary angiogram findings

3.5 Height weight and Body Mass Index

Heights and weights of the subjects were recorded using standard procedures. All subjects were weighed using an analytical digital balance (Chyo MW 150K, Chyo Balance Company, Kyoto, Japan) with an accuracy of 0.1 kg and their standing height was measured using a standard measuring tape. The body mass index (BMI) was calculated using height and weight measurements to determine the nutritional status of subjects as

\[ BMI = \frac{\text{Weight (kg)}}{[\text{Height (m)}]^2} \]
3.6 Method development and analysis of homocysteine concentration by high performance liquid chromatography in study 1:

Plasma homocysteine was measured by Reversed Phase High Performance Liquid Chromatography (RP-HPLC) using a method described by Pfeiffer et al. in 1999 with modifications (304).

3.6.1 Preparation and assay of plasma samples for homocysteine

Plasma sample (50 µl) was mixed with 25 µl of internal standard [IS; Cystamine dihydrochloride 100 µmol/L or N-(2-mercaptopropionyl) glycine 200 µmol/L dissolved in phosphate buffered saline (PBS, pH 7.4)] and 25 µl of PBS, pH 7.4 and incubated with 10 µl of 100 g/l Tris(2-carboxyethyl)phosphine (TCEP) for 30 minutes at room temperature to reduce disulfides and release protein bound thiols. 90 µl of 100 g/l trichloro acetic acid containing 1 mmol/L EDTA was added for deproteinization. Precipitated proteins were removed by centrifugation at 13000 rpm for 10 minutes and 50 µl of the supernatant was added to a vial containing 10 µl of 1.55 mol/L NaOH; 125 µl of 0.125 mol/L borate buffer containing 4 mmol/L EDTA, pH 9.5; and 50 µl of 1 g/l 7-Fluorobenzofurazan-4-sulfonic acid Ammonium salt (SBD-F) in the borate buffer. The sample was incubated for 30 minutes at 60 °C. After deproteinization, the samples were cooled and protected from direct light until injection into the column.

3.6.2 Apparatus and chromatographic conditions

Plasma homocysteine was measured by RP-HPLC using a Waters 515 solvent delivery pump and a Waters 474 scanning fluorescence detector (Waters technologies Corp. U.S.A). Chromatograms were obtained by way of a Chromjet® CH1 integrator.
from Thermo Electra Corporation U.S.A. Aminothiols were separated on a Supelco®
LC18-DB analytical column 150x 4.6 mm ID packed with 3 μm particles with a
Supelguard® LC18-DB 2 cm guard column (Sigma-Aldrich U.S.A.). 10 μL of prepared
sample was injected into the column and 0.1 mol/L acetic acid – acetate buffer (pH 5.5),
containing 30 ml/L methanol was used as the mobile phase at a flow rate of 0.7 ml/min.
The fluorescence intensities were measured with excitation at 385 nm and emission at
515 nm. All analyses were performed at room temperature.

3.6.3 Chromatogram patterns obtained with different mobile phase methanol
concentrations

According to the chromatographic conditions used for the total Hcy assay, it was
found that homocysteine and the internal standard were not clearly baseline separated
from other aminothiols (Figure 3.1).

Lowering the methanol concentration of the mobile phase stepwise from 30
ml/L to 12 ml/L methanol in acetic acid – acetate buffer (pH 5.5), showed that with the
decrease in the level of methanol, a good baseline separation of the aminothiols and the
internal standard could be achieved (Figure 4.4).

No interfering peaks were observed between the peaks for the aminothiols and
the internal standard. It was also observed that the plasma samples without the internal
standard showed no cystamine peak.
Figure 3.1 Chromatogram of a representative plasma sample measured at a methanol concentration of 30 ml/L.

Figure 3.2 Chromatogram of a representative plasma sample measured at a methanol concentration of 12 ml/L.
3.6.4 Comparison of the internal standards:

When N- (2-mercaptopropionyl) glycine was used as the internal standard (IS), the retention time for the IS was 25 minutes (Figure 3.3), whereas when cystamine dihydrochloride was used the retention time was much shorter at 10 minutes (Figure 3.4). Therefore it was decided to use cystamine dihydrochloride as the internal standard in the assay of total plasma homocysteine.

3.6.5 Validation of assay method

L-homocystine calibrators (0-150 μmol/L free thiol) were prepared by adding known L-homocystine concentrations to PBS (pH 7.4). Initially the two internal standards, Cystamine dihydrochloride and N- (2-mercaptopropionyl) Glycine were run separately and the selected internal standard was then added to all samples.
Figure 3.3: Chromatogram of Homocysteine standard (70 μmol/L) and Internal standard Mercapto-propionyl-glycine (200 μmol/L).

Figure 3.4: Chromatogram of Homocysteine standard (70 μmol/L) and internal standard Cystamine dihydrochloride (200 μmol/L).
3.6.6 Performance characteristics of the Homocysteine assay method:

The analyses of homocysteine standards prepared in PBS at pH 7.4, produced a linear curve up to 150 μmol /l with a coefficient correlation of 0.994 and a 0.25 μmol/L lower limit of detection for homocysteine (Figure 3.5).

When selected plasma samples and internal standard was kept at -20°C for 3 months and analyzed to assess the effect of storage of samples, it was observed that there was no significant change in the retention times of total Hcy and the internal standard (Table 4.1).

3.6.7 Calculation of homocysteine concentration

The selected internal standard viz cystamine dihydrochloride (100 μmol/L) were run15 times according to the method described above using 25 μl of distilled water in place of sample. The mean peak area for the internal standard was used to get the corrected peak area for all samples according to the following formula.

\[
\text{Corrected homocysteine peak area} = \frac{\text{Peak area for internal standard}}{\text{Peak area for homocysteine}} \times \text{Mean peak area}
\]

Serial dilutions of homocysteine standard were chromatographed according to the method described above using 25 μl volumes of the prepared standard diluents in triplicate to plot the standard curve.
Figure 3.5 Homocysteine standard curve

Table 3.1. Comparison of retention times for homocysteine and internal standard (cystamine dihydrochloride) after 3 months storage at -20 °C.

<table>
<thead>
<tr>
<th></th>
<th>Total Homocysteine</th>
<th>Internal standard</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Beginning</td>
<td>3 months later</td>
</tr>
<tr>
<td>Mean</td>
<td>5.34</td>
<td>5.92</td>
</tr>
<tr>
<td>SD</td>
<td>0.10</td>
<td>0.15</td>
</tr>
<tr>
<td>CV</td>
<td>1.96</td>
<td>2.49</td>
</tr>
</tbody>
</table>
Corrected homocysteine peak area was calculated using the mean internal standard as described above. The homocysteine concentration of the samples was calculated using the formula obtained from the standard curve as follows.

\[
\text{Homocysteine concentration} = \frac{\text{Corrected Hcy peak area} - 30608}{35325}
\]

(Equation obtained: \( y = 35325x + 30608 \))

3.6.8 Quality control

All samples were run with internal standard and the peak areas were corrected accordingly before calculation of results. Known concentrations of homocysteine were run (prepared homocysteine standards) each week and analyzed to ensure that the values were within ± 5% of the expected value.

3.7 Quantitative analysis of Vitamin B\textsubscript{12}

3.7.1 Vitamin B\textsubscript{12} assay procedure

Solid phase competitive chemiluminescent enzyme immunoassay principle was used to assay the concentration of vitamin B\textsubscript{12} using an IMMULITE\textsuperscript{®} auto analyzer as follows:

1. 200 µl of serum was added to tubes containing 1ml of working solution (containing Borate-KCN buffer solution 1000 µl/test and 20 µl/test dithiothreitol) and vortexed. The tubes were then loosely capped and placed in a covered boiling water bath (100 °C) for 20 minutes.
2. Tubes were removed from the boiling water bath, and allowed to cool at ambient water bath for 5 minutes.

3. 350 µL of the treated sample was pipetted to an IMMULITE® sample cup and put into an IMMULITE® 1 autoanalyzer and vitamin B₁₂ concentration was obtained.

3.7.2 Reaction principle

Vitamin B₁₂ in the sample is released from carrier proteins by incubation at 100 °C in the presence of dithiothreitol and potassium cyanide to inactivate vitamin B₁₂-binding proteins as well as antibodies to intrinsic factor. After the heat denaturation step, the sample and hog intrinsic factor were simultaneously introduced into an IMMULITE® test unit containing a polystyrene bead coated with a B₁₂ analogue and incubated for 30 minutes at 37 °C with intermittent agitation. During this incubation, vitamin B₁₂ in the treated sample competes with the B₁₂ analogue on the solid phase for a limited number of vitamin B₁₂ binding sites on the purified intrinsic factor. Alkaline phosphatase labelled anti-hog intrinsic factor is introduced, and the test unit was incubated for another 30 minute cycle. Unbound enzyme conjugate was removed by a centrifugal wash.

3.7.3 Calibration

The IMMULITE vitamin B₁₂ assay uses low and high vitamin B₁₂ calibrators to generate a calibration curve (calibration range of 100-1200 pg/ml) for calculation of vitamin B₁₂ levels in the samples by a fully automated procedure using the vitamin B₁₂ adjusters (IMMULITE cat. No. LVBL and LVBH).
3.7.4 Quality control

Vitamin B₁₂ control with a concentration of 332 pg/ml was tested before assay of each batch of samples to ensure that the assay control values were within ± 5% of the specified concentration.

Within run coefficient of variation (% CV) of vitamin B₁₂ assay ranged from 5.3- 11.4 whilst the between run % CV ranged from 6.1-7.4. The analytical sensitivity of vitamin B₁₂ assay was 50 pg/ml.

3.8 Quantitative analysis of folic acid

3.8.1 Folic acid assay procedure

A competitive, liquid-phase, ligand-labelled, protein binding chemiluminescent analysis principle was used to assay the concentration of folic acid using an IMMULITE® auto analyzer.

The folic acid assay was carried out as follows

1. 200 μl of serum was added to tubes containing 1ml of working solution (containing Borate-KCN buffer solution 1000 μl/test, ligand labelled folate 20 μl/test and 20 μl/test dithiothreitol) and vortexed. The tubes were then loosely capped and placed in a covered boiling water bath (100 °C) for 20 minutes.

2. Tubes were removed from the boiling water bath, and allowed to cool at ambient water bath for 5 minutes.

3. 350 μl of the treated sample was pipetted to an IMMULITE® sample cup and put into an IMMULITE® autoanalyzer and the concentration of the samples obtained.
3.8.2 Reaction principle

IMMULITE® Folic Acid assay is a boil, competitive, liquid-phase, ligand-labelled, protein binding chemiluminescent assay with in situ immobilization, and with an anti-ligand detection system. The solid phase, a polystyrene bead enclosed within an IMMULITE® Test Unit, is coated with a murine monoclonal antibody specific for folic acid binding protein.

After the sample preparation procedure, the patient sample, ligand-labeled folic acid analog and folic acid binding protein are simultaneously introduced into the Test Unit, and incubated for approximately 30 minutes at 37 °C with intermittent agitation. During this time, folic acid in the sample competes with the ligand-labeled folic acid analog for a limited amount of folic acid binding protein, and the folic acid binding protein is captured by the antibody on the bead. (Unbound analog is then removed by a centrifugal wash.)

3.8.3 Calibration

The IMMULITE® folic acid assay uses low and high folic acid calibrators (IMMULITE® cat. No: LFOL, LFOH) to generate a calibration curve using folic acid concentrations in the range of 0.5 - 2.4 ng/ml.

3.8.4 Quality control

Folic acid control with a concentration of 8.4 ng/ml was tested before assay of each batch of samples to ensure that the assay control values were within ± 5% of the specified concentration.
The coefficient of variation (% CV) of folic acid assay shows a within run % CV of 9.0, 4.2 and 5.2 for low, medium and high concentrations respectively whilst the between run % CV were 8.9, 4.0 and 7.4 for low, medium and high respectively. The analytical sensitivity of the folic acid assay was 0.3 ng/ml.

3.9 Quantitative analysis of homocysteine in study 2:

3.9.1 Homocysteine assay procedure

Homocysteine assay was carried out by Fluorescence Polarization Immunoassay (FPIA) technology using an AxSYM® auto analyzer as follows:

1. Specimens were mixed thoroughly after thawing by low speed vortexing.

2. 200 μl of specimen was pipetted and transferred into a sample cup and concentration measured on the Abbott AxSYM® auto analyzer.

The homocysteine reagents and sample are pipetted in the following sequence inside the auto analyzer.

1. Sample and homocysteine reagents required for one test are pipetted by the sampling probe into various wells of a Reaction Vessel (RV).

2. 200 μl of sample, pretreatment solution, line diluent and SAH Hydrolase are pipetted into one well of the RV to make up the predilution mixture.

3. The RV is immediately transferred into the Processing Center.

4. Further pipetting is done in the Processing Center by the Processing Probe.

5. An aliquot of the predilution mixture, Antibody, and Solution line diluent are transferred to the cuvette of the RV.
6. Fluorescein tracer and a second aliquot of the predilution mixture are transferred to the cuvette.

7. SAH and labeled Fluorescein Tracer compete for the sites on the monoclonal antibody molecule.

8. The intensity of polarized fluorescent light is measured by the FPIA optical assembly.

3.9.2 Biological principle of the procedure

Bound HCY (oxidized form) is reduced to free HCY that is enzymatically converted to S-adenosyl-L-homocysteine (SAH) as follows.

Reduction: Homocystine, mixed disulfide, and protein-bound forms of HCY in the sample are reduced to form free HCY by the use of dithiothreitol (DTT).

\[
\begin{align*}
\text{HCY-SS-HCY (Homocystine)} & \quad \text{DTT} \\
\text{R1-SS-HCY (R1 = thiol residue)} & \quad \text{Free Homocysteine} \\
\text{Protein-SS-HCY} & \\
\end{align*}
\]

Enzymatic Conversion: Free HCY is converted to S-adenosyl-L-homocysteine (SAH) by the use of SAH hydrolase and excess adenosine. Under physiological conditions, SAH hydrolase converts SAH to homocysteine. Excess adenosine in the Pretreatment Solution drives the conversion of HCY to SAH by the bovine SAH Hydrolase.

\[
\begin{align*}
\text{HCY + Adenosine} & \quad \text{SAH hydrolase} \\
& \quad \text{S-adenosyl-L-homocysteine} \\
\end{align*}
\]
3.9.3 Calibration

Homocysteine assay was calibrated using the homocysteine calibrators (Abbott AxSYM® cat. No. 9F84-01) with a calibration range of 0-50 μmol/L by a four parameter logistic curve fit method which generated a standard calibration curve for the calculation of homocysteine levels in the samples.

3.9.4 Quality control

The three homocysteine controls having homocysteine concentrations of 7, 12.5 and 25 μmol/L (Abbott AxSYM® cat. No. 9F84-10) were tested before assay of each batch of samples to ensure that the assay control values were within ± 5% of the specified concentration.

Homocysteine assay shows a within run % coefficient of variations (CVs) of 2.4- 4.5 for low, 1.8- 2.6 for medium and 1.4-2.1 for high concentrations of homocysteine whilst the between run % CVs were 3.1-5.1 for low, 2.6- 3.6 for medium and 2.0- 2.8 for high concentrations of homocysteine. The sensitivity of the homocysteine assay is 0.8 μmol/L.
3.10 Quantitative analysis of apolipoprotein A-I

3.10.1 Apolipoprotein A-I assay procedure

An immunoprecipitation method was used to assay the concentration of apolipoprotein A-I using a Konelab 20® auto analyzer.

The apolipoprotein A-I assay was carried out as follows

1. Serum samples were mixed thoroughly after thawing by low speed vortexing.
2. 200 μl of each serum sample was pipetted to sample cups and concentrations were determined on a Konelab® auto analyzer.

3.10.2 Reaction principle

The apolipoprotein A-I assay method is based on measurement of immunoprecipitation enhanced by polyethylene glycol at 340 nm. Specific antiserum is added in excess to buffered samples. The increase in absorbance caused by immunoprecipitation is recorded when the reaction has reached its endpoint. The change in absorbance is proportionate to the amount of apolipoprotein A-I (antigen) in solution.

3.10.3 Calibration

Calibration of apolipoprotein A-I was carried out using apolipoprotein calibrators with a calibration range of 0.15 - 12.5 g/l. The Konelab 20® auto analyzer generates a calibration curve from the measured calibrators using the spline fit method for the calculation of apolipoprotein A-I levels in the samples.
3.10.4 Quality control

Apolipoprotein A-I control sample with a concentration of 1.06 g/l provided by the manufacturer (Lipo Trol. Code 981653) was tested before assay of each batch of samples to ensure that the assay control values were within ± 5% of the specified concentration.

Apolipoprotein A-I shows a within run % coefficient of variations (CVs) of 1.6 and 1.7 for low and high concentrations of apolipoprotein A-I respectively whilst the between run % CVs were 3.4 and 2.8 for low and high concentrations of apolipoprotein A-I respectively with a determination limit of 0.05 g/l apolipoprotein A-I.

3.11 Quantitative analysis of apolipoprotein B

3.11.1 Apolipoprotein B assay procedure

Apolipoprotein B levels of samples were analyzed by an immunoprecipitation method using a KONELAB 20 auto analyzer.

Apolipoprotein B assay was carried out as follows

1. Serum samples were mixed thoroughly after thawing by low speed vortexing.
2. 200 μl of each serum sample was pipetted to sample cups and concentrations were determined on a Konelab auto analyzer.

3.11.2 Reaction principle

The apolipoprotein B assay method is based on measurement of immunoprecipitation enhanced by polyethylene glycol at 340 nm. Specific antiserum is added in excess to buffered samples. The increase in absorbance caused by
immunoprecipitation is recorded when the reaction has reached its endpoint. The change in absorbance is proportionate to the amount of apolipoprotein B (antigen) in solution.

3.11.3 Calibration

Calibration of apolipoprotein B was carried out using apolipoprotein calibrators with a calibration range of 0.05-13.1 g/l. The Konelab 20® auto analyzer generates a calibration curve from the measured calibrators using the spline fit method for the calculation of apolipoprotein B levels in the samples.

3.11.4 Quality control

Apolipoprotein B control sample with a concentration of 0.75 g/l provided by the manufacturer (Lipo Trol. Code 981653) were used before assay of each batch of samples to ensure that the assay control values were within ± 5% of the specified concentration.

Apolipoprotein B assay shows a within run % coefficient of variations (CVs) of 1.6 and 1.7 for low and high concentrations of apolipoprotein B respectively whilst the between run % CVs were 3.4 and 2.8 for low and high concentrations of apolipoprotein B respectively with a detection limit of 0.05 g/l apolipoprotein B.
3.12 Quantitative analysis of glutathione peroxidase activity

3.12.1 Glutathione peroxidase assay procedure

Glutathione peroxidase assay was carried out with whole blood samples using Randox® reagents (U.K) as follows

1. Heparinized whole blood 0.05 ml was added to a 1.5 ml eppendorf tube containing 1 ml diluting agent, mixed well and incubated at room temperature for 5 minutes.

2. To inactivate the other peroxidases in human blood which may give falsely elevated results, Drabkin’s solution, containing cyanide which serves to inhibit this positive interference, was added and the contents mixed well.

3. 500 μl of pretreated samples were analyzed within 20 minutes of preparation in a Konelab 20® auto analyzer. The analyzer was programmed to compare the decrease in sample absorbance with a reagent blank prepared automatically inside the analyzer and give the glutathione concentration in Units/L of haemolysate after correcting for the dilution factor.

4. The value obtained was converted to units GPx per gram haemoglobin by using the following equation

\[
\text{Sample value in Units GPx /gm Haemoglobin} = \frac{\text{Sample value in Units GPX/ 100 ml}}{\text{Haemoglobin concentration (g/dl)}}
\]

(The haemoglobin concentration/full blood count was assayed by a Kleheuwer® haematological analyzer using EDTA blood)
3.12.2 Reaction principle

Glutathione Peroxidase (GPx) catalyses the oxidation of Glutathione (GSH) by Cumene Hydroperoxide. In the presence of Glutathione Reductase (GR) and NADPH the oxidized Glutathione (GSSG) is immediately converted to the reduced form with a concomitant oxidation of NADPH to NADP⁺. The decrease in absorbance at 340 nm is measured.

\[
\text{Glutathione peroxidase (GPx)} \\
2\text{GSH} + \text{ROOH} \xrightarrow{\text{GPx}} \text{ROH} + \text{GSSG} + \text{H}_2\text{O}
\]

\[
\text{Glutathione reductase (GR)} \\
\text{GSSG} + \text{NADPH} + \text{H}^+ \xrightarrow{\text{GR}} \text{NADP}^+ + 2\text{GSH}
\]

3.12.3 Calibration

Calibration of GPx was carried out using RANSEL® GPx controls (Cat. No. SC 692) with a calibration range of 83-34400 Units/L haemolysate. The Konelab 20® auto analyzer generates a calibration curve from the measured calibrators for the calculation of GPx levels in the samples.

3.12.4 Quality control

Glutathione peroxidase control sample provided by the manufacturer (RANSEL® control cat. No. SC 692) were tested before assay of each batch of samples to ensure that the assay control values were within ± 5% of the specified concentration.
3.13 Genotyping to detect MTHFR C677T and A1298C polymorphisms

3.13.1 Polymerase chain reaction / restriction fragment length polymorphism

Restriction fragment length polymorphism (RFLP) was used to genotype the samples for MTHFR C677T and A1298C polymorphisms.

Polymorphisms in genes sometimes either create or abolish recognition sites for restriction endonucleases, commonly referred to as restriction enzymes. The presence or absence of such recognition sites can be made use for genotyping by the method referred to as restriction fragment length polymorphism based on the following principles:

1. The genomic DNA flanking the polymorphic restriction site which contains the segment of DNA of interest is amplified by the polymerase chain reaction (PCR) after extraction of genomic DNA from the samples.

2. The PCR product containing the amplified DNA segment of interest is digested with the appropriate restriction endonuclease.

3. The digested fragments are visualized by resolving the restriction endonuclease digested PCR products on an agarose gel and inspecting the patterns created depending on the presence or absence of the polymorphism of interest.

3.13.2 DNA extraction

Venous blood collected into K$_3$ EDTA containing tubes and stored at −20 °C prior to DNA extraction were utilized for DNA extraction after thawing. DNA extraction was performed using Promega Wizard® DNA purification kits (Promega Cooperation., U.S.A. cat. No: A1120) according to the manufacturers protocol. This
protocol involves lysis of leukocytes, lysis of nuclei, inactivation of ribonuclease, removal of proteins and other possible contaminants, and isolation of DNA as a pellet.

The Promega Wizard® DNA purification procedure was carried out as follows:

1. To a 1.5 ml microcentrifuge tube containing 900 µl of cell lysis solution, 150 µl of K₃ EDTA whole blood was added and mixed by inverting the tube 5-6 times.

2. The mixture was incubated for 10 minutes at room temperature (26-28 °C). The sample tube was inverted 2-3 times once during the incubation.

3. Incubated sample was centrifuged at 13,000 rpm for 20 seconds, supernatant removed and washed with cell lysis solution until the pellet became white.

4. The pellet was resuspended in 20 µl of lysis solution by vortexing vigorously for 10-15 seconds and then to the solution 150 µl of nuclei lysis solution was added and incubated for 5 minutes at 60°C for 5 minutes in a dry bath.

5. Following incubation the tube was centrifuged briefly to remove drops from the inside of the lid.

6. 0.75 µl of RNAse solution was added to the nuclear lysate and sample mixed by inverting the tube 2-5 times. Sample tube was then incubated for 15 minutes at 37 °C and following incubation allowed to come to room temperature.

7. Following incubation, the tube was centrifuged briefly to remove drops from the inside of the lid and then 50 µl of protein precipitation solution was added and mixed by vortexing for 30 seconds.

8. Sample mix was then centrifuged at 13,000rpm for 3 minutes and supernatant was transferred to a labelled new 1.5ml microcentrifuge tube containing 300 µl of isopropanol and mixed by inverting 2-3 times.
9. Sample mixture was centrifuged again at 13,000 rpm for 60 seconds and supernatant decanted without disturbing the pellet.

10. To the tube containing the DNA pellet 150 µl of 70% ethanol was added and the tube inverted gently several times to wash the DNA pellet.

11. Ethanol was pipetted out without disturbing the pellet and DNA sample air dried by inverting the tube on clean absorbent paper and keeping it for 10-15 minutes.

12. 50 µl of DNA hydration solution was added to the DNA and mixed well and the prepared DNA samples were labeled and stored at 2-8 °C until needed.

3.13.3 Amplification of DNA segments of interest to detect MTHFR C677T and A1298C polymorphisms

Amplification of DNA segments of interest was done using the polymerase chain reaction (PCR).

Please note that all the PCR temperatures given below are after optimization.

3.13.3.1 Principle of the PCR procedure.

Amplification by PCR involves simultaneous primer extension on the selected segment of complementary strands of DNA with two oligonucleotide primers, specific to each strand that flank the genomic region to be amplified. This amplification is brought about by the use of thermostable Taq DNA polymerase enzyme in the presence of deoxynucleotides and a reaction buffer containing Mg²⁺ as described by Saiki et al in 1988 (305).

PCRs were performed on Robocycler® (Stratagene Corporation, U.S.A.) programmable thermal cycler.
3.13.3.2 PCR amplification of DNA for MTHFR C677T polymorphism analysis

The MTHFR C677T polymorphism creates a restriction site which can be cleaved by the restriction endonuclease *Hinfl*. Thus the MTHFR C677T polymorphism was genotyped using a method described by Frosst et al. in 1995. This method described by Frosst et al. does not contain an internal control to distinguish the homozygous wild type from undigested DNA fragments due to restriction endonuclease inhibitors. Thus initially digest control amplification with DMD 48 was multiplexed into the C677T amplification to control for the presence of restriction endonuclease inhibitors. The digest of this multiplex gave fragment sizes of 198 base pairs (bp) due to uncut DNA when the mutation was not found as in the case of homozygous wild type, and 189 due to the DMD 48 control digest being cut by *Hinfl*. These two bands were found to be difficult to differentiate in the agarose gel, both occurring very much nearer in various concentrations of agarose gel, thus necessitating the development of a new control digest to run with the amplified DNA fragment as described later in the section.

All PCR buffers and enzymes used in these investigations were from Promega Corporation U.S.A. The MT 1, MT 2, DMD forward and reverse primers and bacteriophage λ 154bp forward and reverse primers were from Sigma-Aldrich Corporation U.S.A.

i. PCR amplification of genomic DNA segment to analyze MTHFR C677T polymorphism using DMD 48 as internal control

PCR was conducted in a reaction mix containing 2.0 µl of sample template genomic DNA (containing aprox. 18ng DNA), 2.5 µl of 2 mM deoxynucleotides (dNTPs: dATP, dCTP, dGTP and dTTP), 2.5 µl of 10X PCR reaction buffer (containing
MgCl$_2$), 1.0 µl of 5 µM MT 1 MT 2 primer mix, 2 µl pf 5 µM DMD 48 primer mix (containing the forward and the reverse primers; Table 3.2), 0.25 µl of Taq DNA polymerase and 14.75 µl of distilled water to make a total reaction volume of 25 µl.

The PCR amplification involved an initial heat denaturing step at 94 °C for 5 minutes followed by 35 repeated cycles of heat denaturation of the genomic DNA at 94 °C for 30 seconds, annealing of the primers to their complementary sequences at an annealing temperature of 65 °C for 30 seconds and extension of the annealed primers at 72 °C for 30 seconds. The temperature cycles were concluded with 4 minutes at 72 °C. The amplified DNA segments were cooled at 4 °C and started on restriction digestion immediately or kept at 2-8 °C until restriction digestion. To determine the presence of PCR amplified products, 5 µl of amplified DNA was electrophoresed on a 2% agarose gel as described in section 3.13.5.
<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5' – 3')</th>
<th>Orientation</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MT-1</td>
<td>TGA AGG AGA AGG TGT CTG CGG GA</td>
<td>Sense</td>
<td>198</td>
</tr>
<tr>
<td>MT-2</td>
<td>AGG ACG GTG CGG TGA GAG TG</td>
<td>Antisense</td>
<td></td>
</tr>
<tr>
<td>DMD 48F</td>
<td>TTG AAT ACA TTG GTT AAA TCC CAA CAT</td>
<td>Sense</td>
<td>574</td>
</tr>
<tr>
<td>DMD 48R</td>
<td>CTT GAA TAA AGT CTT CCT CAC AC</td>
<td>Antisense</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.2. PCR primers used for the PCR/RFLP genotyping assays to genotype the MTHFR C677T polymorphism and the initial internal control DMD 48.
ii. PCR amplification of genomic DNA segment to analyze MTHFR C677T polymorphism and amplification of bacteriophage λ DNA segment to use as internal control.

a). PCR amplification of genomic DNA segment to analyze MTHFR C677T:

PCR was conducted in a reaction mix containing 2.0 µl of sample template genomic DNA (containing approx. 18 ng DNA), 2.5 µl of 2 mM deoxynucleotides (dNTPs: dATP, dCTP, dGTP and dTTP), 2.5 µl of 10X PCR reaction buffer (containing MgCl₂), 1.0 µl of 5 µM MT 1 MT 2 primer mix, 0.25 µl of Taq DNA polymerase and 16.75 µl of distilled water was added to make a total reaction volume of 25 µl.

The PCR amplification involved an initial heat denaturing step at 94 °C for 5 minutes followed by 35 repeated cycles of heat denaturation of the genomic DNA at 94 °C for 30 seconds, annealing of the primers to their complementary sequences at an annealing temperature of 65 °C for 30 seconds and extension of the annealed primers at 72 °C for 30 seconds. The temperature cycles were concluded with 4 minutes at 72 °C. The amplified DNA segments were cooled at 4 °C and started on restriction digestion immediately or kept at 2-8 °C until restriction digestion. To determine the presence of PCR amplified products 5 µL of amplified DNA was electrophoresed on a 2% agarose gel as described in section 3.13.5.

b). Preparation of primers to generate 154 bp bacteriophage λ fragment:

GenBank database at the National Centre for Biotechnology Information (NCBI), U.S.A, a free database accessed online at URL: http://www.ncbi.nlm.nih.gov was searched to obtain DNA sequence of bacteriophage λ and gene mapping information. Primer3 software, a free software program that can be accessed online at
URL: http://www.broad.mit.edu/genome_software/other/primer3.html was used to design oligonucleotide primers for amplification of bacteriophage λ 154 bp fragment.

NEB cutter version 2.0, a free program that can be accessed online at URL: http://tools.neb.com/NEBcutter2 was used to analyze restriction enzyme recognition sites on bacteriophage λ DNA sequence.

Using the bacteriophage λ DNA sequence and NEB cutter, a bacteriophage λ segment of 154 bp was selected which had a Hinf I restriction site giving fragments of 18 bp and 132 bp in length. Suitable forward and reverse primers, (Lambda. F and Lambda. 154.R; Table 3.3) to amplify the selected bacteriophage λ segment were generated using Primer3 software program.
<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5' – 3')</th>
<th>Orientation</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lambda. F</td>
<td>TGC AAT GCC ACA AAG AAG AG</td>
<td>Sense</td>
<td>154</td>
</tr>
<tr>
<td>Lambda. 154. R</td>
<td>GCG AAT TAA CCC ATC GTT GA</td>
<td>Antisense</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.3. PCR primers developed for amplification of selected bacteriophage λ 154 bp segment.
c). PCR amplification of bacteriophage λ DNA segment to use as an internal control

PCR was conducted in a reaction mix containing 2.0 μl of sample bacteriophage λ template DNA (containing approx. 8ng DNA), 2.5 μl of 2mM deoxynucleotides (dNTPs: dATP, dCTP, dGTP and dTTP), 2.5 μl of 10X PCR reaction buffer (containing MgCl₂), 2.5 μl of 5 μM forward primer, 2.5 μl of 5 μM reverse primer, 0.25 μl of Taq DNA polymerase and 12.75 μl of distilled water to make a total reaction volume of 25 μl.

The PCR amplification was done using the same PCR conditions as for amplification of genomic DNA segment for MTHFR C677T polymorphism and involved an initial heat denaturing step at 94 °C for 5 minutes followed by 35 repeated cycles of heat denaturation of the genomic DNA at 94 °C for 30 seconds, annealing of the primers to their complementary sequences at an annealing temperature of 65 °C for 30 seconds and extension of the annealed primers at 72 °C for 30 seconds. The temperature cycles were concluded with 4 minutes at 72 °C. The amplified DNA segments were cooled at 4 °C and started on restriction digestion immediately or kept at 2-8 °C until restriction digestion. To determine the presence of PCR amplified products 5 μL of amplified DNA was electrophoresed on a 2% agarose gel as described in section 3.13.5.

3.13.3.3 PCR amplification of DNA for MTHFR A1298C polymorphism analysis

The MTHFR A1298C polymorphism abolishes a restriction site which can be cleaved by the restriction endonuclease Mbo II. Thus the MTHFR A1298C polymorphism was genotyped based on a method described initially by van der Put et al. in 1998 (109).
Compared to MTHFR C677T polymorphism analysis, this does not require a separate internal control to verify activity of the restriction endonuclease \textit{Mbo} II as the amplified segment of genomic DNA has other restriction sites for the \textit{Mbo} II restriction enzyme, which will not be digested if the endonuclease is inhibited. Thus in case of the DNA segment not being digested it would give rise to a single band in agarose gel electrophoresis compared to more than one band that could be visualized when it is digested.

All PCR buffers and enzymes used in these investigations were from Promega Corporation U.S.A. The MT 3 and MT 4 primers were from Sigma-Aldrich Corporation U.S.A.

PCR was conducted in a reaction mix containing 2.0 \( \mu \text{l} \) of sample template genomic DNA (containing aprox. 18ng DNA), 2.5 \( \mu \text{l} \) of 2 mM deoxynucleotides (dNTPs: dATP, dCTP, dGTP and dTTP), 2.5 \( \mu \text{l} \) of 10X PCR reaction buffer (containing \textit{MgCl}_2), 1.0 \( \mu \text{l} \) of 5 \( \mu \text{M} \) MT 3 MT 4 primer mix (Table 3.4), 0.25 \( \mu \text{l} \) of \textit{Taq} DNA polymerase and 16.75 \( \mu \text{l} \) of distilled water to make a total reaction volume of 25 \( \mu \text{l} \).
<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5' - 3')</th>
<th>Orientation</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MT-3</td>
<td>CTT TGG GGA GCT GAA GGA CTA CTA C</td>
<td>Sense</td>
<td>163</td>
</tr>
<tr>
<td>MT-4</td>
<td>CAC TTT GTG ACC ATT CCG GTT TG</td>
<td>Antisense</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.4. PCR primers used for the PCR/RFLP genotyping assays to genotype the MTHFR A1298C polymorphism.
The PCR amplification involved an initial heat denaturing step at 94 °C for 5 minutes followed by 35 repeated cycles of heat denaturation of the genomic DNA at 94 °C for 30 seconds, annealing of the primers to their complementary sequences at an annealing temperature of 65 °C for 30 seconds and extension of the annealed primers at 72 °C for 30 seconds. The temperature cycles were concluded with 4 minutes at 72 °C. The amplified DNA segments were cooled at 4 °C and started on restriction digestion immediately or kept at 2-8 °C until restriction digestion. To determine the presence of PCR amplified products, 5 µl of amplified DNA was electrophoresed on a 2% agarose gel as described in section 3.13.5.

3.13.4 Restriction endonuclease digestion of amplified DNA segments to detect MTHFR C677T and A1298C polymorphisms

All restriction endonuclease buffers and enzymes used in these investigations were from Promega Corporation U.S.A.

3.13.4.1 Restriction endonuclease digestion of amplified DNA segments with Hinf I to detect MTHFR C677T polymorphism

The restriction endonuclease Hinf I, (from Haemophils influenzae) was used to digest the PCR amplified DNA segment to detect MTHFR C677T polymorphism. Hinf I restriction endonuclease recognize the sequence depicted below and at the position indicated by ▼, it cuts the double stranded DNA where ever this sequence is found (N denotes any nucleotide base).

\[ 5' \cdots G\text{▼}\text{ANT} \text{C} \cdots 3' \]

\[ 3' \cdots \text{C TNA▼G} \cdots 5' \]
i. Restriction endonuclease digestion of amplified DNA segments with *Hinf* I to detect MTHFR C677T polymorphism using DMD as internal control.

Restriction endonuclease digestion with *Hinf* I of the amplified segment of DNA to detect MTHFR C677T polymorphism with DMD 48 as the internal control was conducted in a reaction mix containing 10 μl of PCR product (containing aprox. 10μg DNA), 1.5 μl of 10X restriction enzyme buffer B (containing 60 mM Tris HCl pH 7.5, 500 mM NaCl, 60 mM MgCl₂ and 10 mM Dithiothreitol at 37 °C), 0.15 μl of 0.1 mg/ml bovine serum albumin (BSA) as an enhancer of enzyme activity, 0.5 μl of 10 units/μl *Hinf* I restriction endonuclease and 2.85 μl of distilled water to make a reaction volume of 15 μl. After mixing the contents well, the samples were incubated at 37 °C overnight in a dry bath. The digested PCR fragments were visualized by agarose gel electrophoresis as described in section 3.13.5.

ii. Restriction endonuclease digestion of amplified DNA segments with *Hinf* I to detect MTHFR C677T polymorphism using bacteriophage λ 154 bp PCR product as internal control.

Restriction endonuclease digestion with *Hinf* I of the amplified segment of DNA to detect MTHFR C677T polymorphism with bacteriophage λ 154 bp PCR product as the internal control was conducted in a reaction mix containing 10 μl of sample PCR product(containing aprox. 10μg DNA), 5.0 μl of 154 bp bacteriophage λ product (containing aprox. 8μg DNA), as internal control, 2.0 μl of 10X restriction enzyme buffer B (containing 60 mM Tris HCl pH 7.5, 500 mM NaCl, 60 mM MgCl₂ and 10 mM Dithiothreitol at 37°C), 0.15μl of 0.1 mg/ml bovine serum albumin (BSA) as an
enhancer of enzyme activity, 0.5 µl of 10 units/µl *Hinf* I restriction endonuclease and 2.35 µl of distilled water to make a reaction volume of 20 µl. After mixing the contents well, the samples were incubated at 37 °C overnight in a dry bath. The digested PCR fragments were visualized by agarose gel electrophoresis as described in section 3.13.5.

3.13.4.2 Restriction endonuclease digestion of amplified DNA segments with *Mbo II* to detect MTHFR A1298C polymorphism

The restriction endonuclease *Mbo II*, (from *Moraxella bovis*) was used to digest the PCR amplified DNA segment to detect MTHFR A1298C polymorphism. *Mbo II* restriction endonuclease recognize the sequence depicted below and at the position indicated by , it cuts the double stranded DNA whereever this sequence is found (N denotes any nucleotide base).

\[
\begin{align*}
5' & \ldots GAAGA(N)\Upsilon \ldots 3' \\
3' & \ldots CTTCT(N)\Upsilon \ldots 5'
\end{align*}
\]

Restriction endonuclease digestion with *Mbo II* of the amplified segment of DNA to detect MTHFR A1298C polymorphism was conducted in a reaction mix containing 10 µl of PCR product (containing approx. 10 µg DNA), 1.5 µl of 10X restriction enzyme buffer B (containing 60 mM Tris HCl pH 7.5, 500 mM NaCl, 60 mM MgCl₂ and 10 mM Dithiothreitol at 37 °C), 0.15 µl of 0.1 mg/ml bovine serum albumin (BSA) as an enhancer of enzyme activity, 0.02 µl of 10 units/µl *Mbo II* restriction endonuclease and 3.33 µl of distilled water to make a reaction volume of 15 µl. After mixing the contents well, the samples were incubated at 37 °C overnight in a dry bath.

The digested PCR fragments were visualized by agarose gel electrophoresis as described in section 3.13.5.
3.13.5 Visualization of DNA fragments with agarose gel electrophoresis

The standard method used to separate and identify different sized DNA fragments is agarose gel electrophoresis. During this electrophoretic process negatively charged DNA molecules migrate towards the anode depending mainly on the size with smaller fragments having greater mobility in the agarose gel. As the DNA fragments are not visible to the naked eye, the locations of the different fragments of DNA have to be visualized by staining. This can be done directly by staining the DNA fragments with low concentrations of ethidium bromide which intercalates with DNA as it migrates in the agarose gel and fluoresces when examined under ultra violet (UV) light in a UV transilluminator.

3.13.5.1 Preparation of the agarose gel

2% agarose gels were prepared as follows:

1. A gel mould was prepared by sealing the edges of a clean dry Perspex plate with tape to make a tray. A comb was positioned 0.5-1 mm above the plate so that a well will be formed when the agarose is added and the plate kept in a horizontal position on the bench.

2. 2% Agarose gels were prepared by weighing in 1 g of agarose (Sigma-Aldrich Corporation U.S.A.) in a 200 ml conical flask and adding 1X TAE buffer (containing 40 mM Tris-acetate and 1 mM EDTA) to make 50 ml. The flask was loosely capped and the slurry heated in a microwave until the agarose dissolved.

3. The solution was cooled to 60 °C.
4. The solution was poured into the mould and the mould inspected to make sure that air bubbles were not formed under or between the teeth of the comb.

5. The gel was left to set for 30-45 minutes at room temperature.

6. After the gel was completely set, the comb and the tape were carefully removed and the gel transferred to the electrophoresis tank. 1X TAE electrophoresis buffer was added to cover the gel to a depth of ~1 mm and to the buffer 2 μl of ethidium bromide was added.

3.13.5.2 Electrophoresis of prepared DNA samples using the prepared agarose gels.

Prepared DNA samples were electrophoresed as follows:

1. To 15 μl of sample 1.5 μl of 10X gel loading buffer (Sigma-Aldrich Corporation U.S.A.) was added and mixed well.

2. Each sample of DNA to be resolved mixed with gel loading buffer were placed on separate wells of the submerged gel using a micropipette. A 50 bp DNA ladder was also loaded for each gel.

3. A 50 volt current was applied to the electrophoretic tank after closing the lid of the electrophoretic tank so that the DNA moved towards the anode. The gel was run until the blue lines migrated an appropriate distance through the gel.

4. When the DNA samples migrated a sufficient distance through the gel as indicated by the dye, the electrical current was turned off, the electrical leads and the lid of the electrophoretic chamber were removed, gel examined under UV light in a UV transilluminator and photographed.
3.13.6 Quality control

In PCR, before RFLP assays, DNA free controls were used and if any of these negative controls were found to have PCR amplified DNA products, the entire PCR batch was analyzed again. In restriction digestion step in the RFLP, constant restriction sites were used for internal control of each restriction digestion reaction and if the internal control was not digested, the samples were reassayed. Two independent observers read all gels and autoradiographs and when the observers did not agree, those samples were regenotyped.
3.14 Calculation of severity of ischaemia in study 2

Coronary angiogram reports of subjects were analysed and were given a score according to three techniques to assess the severity of coronary stenosis based on the method described by Sullivan et al. in 1991 (297) using

1. Vessel score
2. Stenosis score
3. Extent score

The angiogram findings were interpreted by a cardiothoracic anaesthetist in consultation with cardiothoracic surgeons and findings noted. The interpreters of the angiograms were kept blind to the investigation results.

3.14.1 Calculation of vessel score

This was calculated as the number of vessels with a significant stenosis (70% or greater reduction in lumen diameter). Depending on the number of vessels involved (left anterior descending artery, the left circumflex artery and the right coronary artery) vessel score ranged from 0 to 3. Left main artery stenosis was scored as single vessel disease (306).

3.14.2 Calculation of stenosis score

Stenosis score was calculated by a modified Gensini score as described by Reardon et al in 1985 (299) and Hamsten et al in 1986 (307) which places emphasis on the severity of stenosis while including some measure of the extent of coronary artery disease. Briefly, the most severe stenosis in each of eight segments (left main coronary artery, left anterior descending artery, main diagonal branch, first septal perforator, left...
circumflex artery, obtuse marginal and postero-lateral vessels, right coronary artery and
the main posterior descending branch) was graded according to severity, that is; a grade
of 1 for 1-49% reduction in lumen diameter, 2 for 50-74%, 3 for 75-99% and 4 for total
occlusion. The scores in each of the eight segments were added together to give a total
score out of a theoretical maximum of 32.

3.14.3 Calculation of extent score

Extent score was calculated according to a method described by Sullivan et al. in
1990 (297) which indicates the proportion of the coronary arterial tree involved by
angiographically detectable atheroma. The proportion of each vessel involved by
atheroma, identified as luminal irregularity was multiplied by a factor for each vessel:
left main artery, 5; left anterior descending artery, 20; main diagonal branch, 10; first
septal perforator, 5; left circumflex artery, 20; obtuse marginal and postero-lateral
vessels, 10; right coronary artery, 20; and main posterior descending branch, 10. When
the major lateral wall branch was a large obtuse marginal on intermediate vessel, this
was given a factor of 20 and the left circumflex artery a factor of 10. The scores for
each vessel or branch were added to give a total score out of 100, being the percentage
of the coronary intimal surface area involved by atheroma. Data regarding the extent
score was determined by a cardiothoracic anaesthetist in consultation with a
cardiothoracic surgeon.
3.15 Data processing and analysis

Data were double entered to Microsoft® Excel (Redmond, Washington) and analysed using SPSS for Windows version 11.5 (Chicago, Illinois) and STATA version 5 (College Station, Texas). Bivariate analysis between variables was tested using t-test, chi square tests and Fisher’s exact tests. Multivariate analysis was tested using a conditional logistic regression model to control for other confounding factors in the analysis of association between hyperhomocysteinaemia and CAD and ANOVA for testing of association between MTHFR gene polymorphisms with homocysteine, vitamin B12 and folate. P-values of less than 0.05 were considered to be significant.

3.16 Ethical considerations

Ethical clearance to conduct the studies was obtained from the Ethical Review Committee of the Faculty of Medical Sciences, University of Sri Jayewardenepura (Appendix 4, 5 and 6). The study objectives were explained in Sinhala (or English, where appropriate) to all participants by way of direct communication and prepared leaflets in study 1 (Appendix 7 and 8). Informed written consent was obtained from all participants by way of an approved consent form (Appendix 9 and 10). Participants in the study were not identified by name and address and data collected from them and the investigation findings were only accessible to the investigators of the study.
4. RESULTS

Results are described under two sections. Section I describes the findings of the study on the association between coronary artery disease and hyperhomocysteinaemia. Section II describes the findings of the association between vitamin B₁₂, folate, and MTHFR polymorphisms (A1298C and C677T) with homocysteine levels and the association between vitamin B₁₂, folate, homocysteine, apolipoprotein A-I, apolipoprotein B and glutathione peroxidase to the severity of coronary artery disease.

4.1 Study I – Study on the association between coronary artery disease and hyperhomocysteinaemia

4.1.1 Characteristics of study subjects

The study population consisted of 163 males and 58 females each in the control group made up of apparently healthy subjects and the test subjects made up of patients diagnosed as having an acute coronary syndrome (ACS). No significant differences were observed between the patients and controls in the prevalences of diabetes, family history of CAD, smoking, involvement in regular physical activity, regular vitamin supplementation, being vegans and alcohol ingestion and in height, weight and BMI (Table 4.1 and Table 4.2).

Results revealed that the patients with ACS had a significantly higher prevalence of hypertension (p < 0.001) and hypercholesterolaemia (p < 0.001) when compared with control subjects. (Table 4.1).
Plasma homocysteine concentration ranged from 3.42 – 27.61 μmol/L in the patients (n = 221) with a mean of 13.95 μmol/L whilst in the controls (n = 221) plasma homocysteine concentration ranged from 2.21 – 32.84 μmol/L with a mean of 12.65 μmol/L (Table 4.3). Due to the non-availability of a reference range for homocysteine concentrations in Sri Lankan and in Asians, the 90th percentile of the controls in this study (18.38 μmol/L) was taken as the upper limit of normal for plasma homocysteine.
Table 4.1 Prevalence characteristics of the subjects in study 1.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Patients</th>
<th>Controls</th>
<th>McNemar’s Odds Ratio* (p-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No:</td>
<td>%</td>
<td>No:</td>
</tr>
<tr>
<td><strong>Hypertension</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>90</td>
<td>40.72</td>
<td>34</td>
</tr>
<tr>
<td>No</td>
<td>131</td>
<td>59.28</td>
<td>187</td>
</tr>
<tr>
<td><strong>Hypercholesterolaemia</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>71</td>
<td>32.13</td>
<td>10</td>
</tr>
<tr>
<td>No</td>
<td>148</td>
<td>66.97</td>
<td>211</td>
</tr>
<tr>
<td><strong>Diabetes mellitus</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>57</td>
<td>25.79</td>
<td>42</td>
</tr>
<tr>
<td>No</td>
<td>164</td>
<td>74.21</td>
<td>179</td>
</tr>
<tr>
<td><strong>Smoking</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>130</td>
<td>58.82</td>
<td>127</td>
</tr>
<tr>
<td>No</td>
<td>91</td>
<td>41.18</td>
<td>94</td>
</tr>
<tr>
<td><strong>Alcohol intake</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>112</td>
<td>50.68</td>
<td>107</td>
</tr>
<tr>
<td>No</td>
<td>109</td>
<td>49.32</td>
<td>114</td>
</tr>
<tr>
<td><strong>Family History of CAD</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>23</td>
<td>10.40</td>
<td>15</td>
</tr>
<tr>
<td>No</td>
<td>198</td>
<td>89.60</td>
<td>206</td>
</tr>
<tr>
<td><strong>Regular physical exercise</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>34</td>
<td>15.38</td>
<td>30</td>
</tr>
<tr>
<td>No</td>
<td>187</td>
<td>84.62</td>
<td>191</td>
</tr>
<tr>
<td><strong>Regular vitamin supplementation</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>36</td>
<td>16.29</td>
<td>26</td>
</tr>
<tr>
<td>No</td>
<td>185</td>
<td>83.71</td>
<td>195</td>
</tr>
<tr>
<td><strong>Vegan</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>21</td>
<td>09.50</td>
<td>15</td>
</tr>
<tr>
<td>No</td>
<td>200</td>
<td>90.50</td>
<td>206</td>
</tr>
</tbody>
</table>

*Chi square test comparing patients and controls
Table 4.2 Weight, height and BMI of patients and controls in study 1.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Patients (n=221)</th>
<th>Controls (n=221)</th>
<th>p-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
<td></td>
</tr>
<tr>
<td>Weight in kg</td>
<td>57.38 ± 9.93</td>
<td>55.89 ± 6.95</td>
<td>0.79</td>
</tr>
<tr>
<td>Height in meters</td>
<td>1.59 ± 0.09</td>
<td>1.59 ± 0.08</td>
<td>0.68</td>
</tr>
<tr>
<td>BMI</td>
<td>22.66 ± 3.58</td>
<td>22.12 ± 2.40</td>
<td>0.63</td>
</tr>
</tbody>
</table>

* Student t-test comparing patients and controls.

Table 4.3 Homocysteine concentrations of the subjects in the study

<table>
<thead>
<tr>
<th>(µmol/L)</th>
<th>Patients (µmol/L)</th>
<th>Controls (µmol/L)</th>
<th>p-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>13.95</td>
<td>12.65</td>
<td>0.005**</td>
</tr>
<tr>
<td>SD</td>
<td>5.30</td>
<td>4.58</td>
<td></td>
</tr>
<tr>
<td>Minimum</td>
<td>3.42</td>
<td>2.12</td>
<td></td>
</tr>
<tr>
<td>Maximum</td>
<td>27.61</td>
<td>32.84</td>
<td></td>
</tr>
</tbody>
</table>

*McNemars test comparing patients and controls (18.38µmol/L was taken as the upper limit of normal for plasma homocysteine).

** Significant association
Age of the control subjects ranged from 25-76 years with a mean of 55.4 years (± 9.55SD). Age of patients ranged from 26-76 years with a mean of 55.6 years (± 9.33SD). There was no significant correlation between age and the homocysteine concentrations in controls subjects as well as in patients with an ACS alone or when both control subjects and patients with an ACS were combined. (Figures 4.1 & 4.2 and Table 4.4).
Figure 4.1. Distribution of serum homocysteine with age in normal subjects of study 1.

Figure 4.2. Distribution of serum homocysteine with age in patients with an ACS of study 1.
Table 4.4. Correlation between plasma homocysteine concentration and age in the study population

<table>
<thead>
<tr>
<th></th>
<th>Correlation coefficient</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control subjects</td>
<td>0.052</td>
<td>0.440</td>
</tr>
<tr>
<td>Patients with an ACS</td>
<td>-0.015</td>
<td>0.830</td>
</tr>
<tr>
<td>Controls &amp; patients with an ACS</td>
<td>0.020</td>
<td>0.682</td>
</tr>
</tbody>
</table>

*Spearman correlation*
4.1.2 Association between Hyperhomocysteinaemia and CAD.

When conditional logistical regression analysis was performed, hyperhomocysteinaemia was found to be a significant predictor of CAD after controlling for hypertension and hypercholesterolemia (adjusted odds ratio 2.38) (Table 4.6). Subjects with hypercholesterolemia were 7 times more likely to have CAD whilst the subjects with hypertension were more than 2 times likely to have CAD. As patients and controls were matched on age, age was considered a continuous variable and was not a significant predictor of CAD after controlling the other variables.
Table 4.5  Conditional logistic regression analysis of confounding variables

<table>
<thead>
<tr>
<th>Variable</th>
<th>Regression coefficient</th>
<th>p-value</th>
<th>Odds ratio</th>
<th>95% confidence interval of odds ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>0.255</td>
<td>0.277</td>
<td>1.290</td>
<td>0.815-2.041</td>
</tr>
<tr>
<td>Hypertension</td>
<td>0.867</td>
<td>0.001</td>
<td>2.379</td>
<td>1.417-3.995</td>
</tr>
<tr>
<td>Hypercholesterolaemia</td>
<td>1.950</td>
<td>&lt;0.001</td>
<td>7.027</td>
<td>3.381-14.604</td>
</tr>
<tr>
<td>Hyperhomocysteinaemia</td>
<td>0.870</td>
<td>0.004</td>
<td>2.387</td>
<td>1.325-4.299</td>
</tr>
</tbody>
</table>

1 Age was considered a continuous variable.

2 Hypertension was recorded if the blood pressure (BP) was documented to be > 140 mm Hg systolic or > 90 mm Hg diastolic. Reference is persons without hypertension.

3 Hypercholesterolaemia refers to a total cholesterol concentration > 240 mg/dl. Reference persons without hypercholesterolaemia.

4 Hyperhomocysteinaemia refers to a total Homocysteine concentration > 18.38 μmol/L. Reference persons without hyperhomocysteinaemia.
4.1.3 Association between hyperhomocysteinaemia and CAD in the young and adults over 50 years of age:

Analysis of results revealed that hyperhomocysteinaemia was a significant predictor of CAD in the young but not in the elderly (Table 4.6). Among persons below 50 years, persons with hyperhomocysteinaemia were 4.5 times more likely to develop CAD as compared to those with normal concentrations of plasma homocysteine. When young and the elderly patients with an ACS were compared, hyperhomocysteinaemia was a significant predictor ($p=0.027$) of CAD in the young compared to persons over 50 years of age on chi square analysis.
Table 4.6. Association between hyperhomocysteinaemia and CAD by age group.

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Patients</th>
<th>Controls</th>
<th>McNemar's</th>
<th>p- value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Young</td>
<td></td>
<td></td>
<td>Odds ratio</td>
<td></td>
</tr>
<tr>
<td>Hyperhomocysteinemic</td>
<td>19</td>
<td>05</td>
<td>4.50</td>
<td>0.004</td>
</tr>
<tr>
<td>Normal homocysteine levels</td>
<td>44</td>
<td>58</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Elderly</td>
<td></td>
<td></td>
<td>Odds ratio</td>
<td></td>
</tr>
<tr>
<td>Hyperhomocysteinemic</td>
<td>26</td>
<td>17</td>
<td>0.61</td>
<td>0.188</td>
</tr>
<tr>
<td>Normal homocysteine levels</td>
<td>132</td>
<td>141</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
4.1.4 Association of risk of CAD in persons with two or less traditional risk factors for the development of CAD with patients with 3 or more traditional risk factors:

Analysis of results revealed that among patients with an ACS, 9% did not have any traditional risk factors for the development of CAD compared to 25.3% in the control group. Out of the total in each group 62.8% among the patients with an ACS and 85% amongst the control subjects had two or less traditional risk factors for development of CAD. Compared to this, 37.2% of the patients with an ACS and only 15% of the control subjects had three or more traditional risk factors for the development of CAD (Table 4.7).

Results from the study revealed that the presence of three or more traditional risk factors was significantly associated ($p<0.001$) with the risk of developing CAD when compared to subjects with two or less risk factors (Table 4.8).

When patients with two or less traditional risk factors for development of CAD were compared with patients with three or more traditional risk factors, there was no significant association ($p = 0.229$) between the number of risk factors and hyperhomocysteinaemia (Table 4.9).
Table 4.7. Frequency distribution of number of traditional risk factors* in patients with an ACS and controls

<table>
<thead>
<tr>
<th>Number of risk factors</th>
<th>ACS Patients</th>
<th>Normal Subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number</td>
<td>Percentage</td>
</tr>
<tr>
<td>None</td>
<td>20</td>
<td>9.0</td>
</tr>
<tr>
<td>1</td>
<td>46</td>
<td>20.8</td>
</tr>
<tr>
<td>2</td>
<td>73</td>
<td>33.0</td>
</tr>
<tr>
<td>3</td>
<td>49</td>
<td>22.2</td>
</tr>
<tr>
<td>4</td>
<td>26</td>
<td>11.8</td>
</tr>
<tr>
<td>5</td>
<td>6</td>
<td>2.7</td>
</tr>
<tr>
<td>6</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td>7</td>
<td>1</td>
<td>0.5</td>
</tr>
<tr>
<td>Total</td>
<td>221</td>
<td>100</td>
</tr>
</tbody>
</table>

* Presence of hypertension, hypercholesterolaemia, diabetes mellitus, current smoking, excess alcohol intake, overweight and obesity (BMI > 25), and a family history of CAD were taken as traditional (conventional) risk factors.
Table 4.8. Association between the number of traditional risk factors and development of CAD

<table>
<thead>
<tr>
<th>Number of traditional risk factors</th>
<th>Controls</th>
<th>Patients</th>
<th>p-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Two or less</td>
<td>150</td>
<td>87</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Three or more</td>
<td>71</td>
<td>134</td>
<td></td>
</tr>
</tbody>
</table>

* Chi square test comparing two or less traditional risk factors with three or more traditional risk factors in patients and controls.

Table 4.9. Association between homocysteine levels and number of traditional risk factors in patients with an ACS.

<table>
<thead>
<tr>
<th></th>
<th>2 or less risk</th>
<th>3 or more risk</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal homocysteine concentrations (n=)</td>
<td>107</td>
<td>69</td>
<td>0.229</td>
</tr>
<tr>
<td>Hyperhomocysteinaemia (n=)</td>
<td>32</td>
<td>13</td>
<td></td>
</tr>
</tbody>
</table>

* Chi square test comparing subjects with normal homocysteine concentrations and subjects with hyperhomocysteinaemia and number of traditional risk factors.
4.2 Study II: Study on the association between vitamin B\textsubscript{12}, folate, and MTHFR polymorphisms (A1298C and C677T) with homocysteine levels and the association between vitamin B\textsubscript{12}, Folate, Homocysteine, Apolipoprotein A-I, apolipoprotein B and Glutathione Peroxidase to the severity of coronary heart disease.

4.2.1 Characteristics of study subjects

4.2.1.1 General characteristics of the study subjects

Age of the study population, most of whom were males, ranged from 34-77 years with a mean of 57.8 years (± 9.6SD).

A majority of study subjects had hypercholesterolaemia whereas hypertension and diabetes mellitus was not seen in a majority of the subjects. The personal characteristics of the study subjects are summarized in table 4.10.

4.2.1.2 Biochemical and anthropometric characteristics of the study population.

In the study population, the BMI ranged from 20.21 to 45.42 with a mean of 25.98 indicating that most of the study population was either overweight or obese.

The mean vitamin B\textsubscript{12} level of the study sample was 577.51 pg/ml which is above the lower limit of normal of 200 pg/ml (308) with 8 CAD patients amounting to 10.13% of the study population having values below normal.

The mean folate level of the study sample was 4.91ng/ml which is above the lower limit of normal of 3 ng/ml (308) with 27 CDA patients amounting to 34.18% of the study population having values below normal.
Table 4.10. Characteristics of subjects in study 2.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Number</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sex</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>61</td>
<td>77.2</td>
</tr>
<tr>
<td>Female</td>
<td>18</td>
<td>22.8</td>
</tr>
<tr>
<td><strong>Hypertension</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>32</td>
<td>59.5</td>
</tr>
<tr>
<td>No</td>
<td>47</td>
<td>40.5</td>
</tr>
<tr>
<td><strong>Diabetes mellitus</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>35</td>
<td>44.3</td>
</tr>
<tr>
<td>No</td>
<td>44</td>
<td>55.7</td>
</tr>
<tr>
<td><strong>Hypercholesterolaemia</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>73</td>
<td>92.4</td>
</tr>
<tr>
<td>No</td>
<td>06</td>
<td>07.6</td>
</tr>
<tr>
<td><strong>Family history of myocardial infarction</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>15</td>
<td>19.0</td>
</tr>
<tr>
<td>No</td>
<td>64</td>
<td>81.0</td>
</tr>
<tr>
<td><strong>Smoking</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smoker</td>
<td>14</td>
<td>17.7</td>
</tr>
<tr>
<td>Former smoker</td>
<td>10</td>
<td>12.7</td>
</tr>
<tr>
<td>Non-smoker</td>
<td>55</td>
<td>69.6</td>
</tr>
</tbody>
</table>
Table 4.10. Characteristics of subjects in study 2 contd.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Number</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regular physical activity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>21</td>
<td>26.6</td>
</tr>
<tr>
<td>No</td>
<td>58</td>
<td>73.4</td>
</tr>
<tr>
<td>Vitamin supplementation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>00</td>
<td>00</td>
</tr>
<tr>
<td>No</td>
<td>79</td>
<td>100.0</td>
</tr>
<tr>
<td>Regular alcohol consumption</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>16</td>
<td>20.3</td>
</tr>
<tr>
<td>No</td>
<td>63</td>
<td>79.7</td>
</tr>
<tr>
<td>Vegan</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>03</td>
<td>03.8</td>
</tr>
<tr>
<td>No</td>
<td>76</td>
<td>96.2</td>
</tr>
</tbody>
</table>
The mean GPx concentration of the study sample was 120.44 units GPx/mg Hb which is well below the normal value of more than 275 units GPx/mg Hb. Only 2 subjects amounting to 2.53% of the study population had GPx levels above the normal lower limit.

The mean homocysteine concentration of the study population was 13.77 μmol/L, which is below the normal reference value of 18.35 μmol/L calculated from the 90th percentile value of the controls in study 1. Nine subjects in the study population had a homocysteine concentration above 18.35 μmol/L.

The mean apolipoprotein A-I concentration of the study population was 0.71 g/l which is below the recommended limit of above 0.89 g/l (32), thus indicating a higher risk for cardiovascular disease. Fifty eight subjects amounting to 73% of the study population had apolipoprotein A-I levels below 0.89 g/l.

The mean apolipoprotein B concentration of the study sample was 0.64 g/l which is below the high risk level of 1.35 g/l (32), thus indicating a higher risk for cardiovascular disease. None of the subjects in the study population had apolipoprotein B levels above 1.35 g/l.

The mean apolipoprotein B/A-I ratio of the study sample was 1.12 which is above the recommended limit of 0.90 (213). Thirty nine subjects amounting to almost half the study population had apolipoprotein B/A-I ratio above 0.90.

The biochemical parameters and the anthropometric measurements of subjects are summarized in table 4.11.

There was no significant correlation (Spearman correlation coefficient 0.102; p-value 0.371) between age and the homocysteine concentrations in subjects of study 2 (Figure 4.3).
Table 4.11. Biochemical and anthropometric characteristics of subjects in study 2.

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>SD</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (kg)</td>
<td>66.74</td>
<td>11.20</td>
<td>44-98</td>
</tr>
<tr>
<td>Height (m)</td>
<td>1.60</td>
<td>0.09</td>
<td>1.36-1.82</td>
</tr>
<tr>
<td>BMI</td>
<td>25.98</td>
<td>3.98</td>
<td>20.21-45.42</td>
</tr>
<tr>
<td>Vitamin B₁₂ (pg/ml)</td>
<td>577.51</td>
<td>299.88</td>
<td>100-1198</td>
</tr>
<tr>
<td>Folate (ng/ml)</td>
<td>4.91</td>
<td>2.91</td>
<td>0.46-10.62</td>
</tr>
<tr>
<td>Glutathione peroxidase</td>
<td>120.44</td>
<td>36.42</td>
<td>56.09-295.27</td>
</tr>
<tr>
<td>(units GPx/mg Hb)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Homocysteine (μmol/L)</td>
<td>13.77</td>
<td>5.21</td>
<td>3.56-42.14</td>
</tr>
<tr>
<td>Apolipoprotein A-I (g/l)</td>
<td>0.71</td>
<td>0.27</td>
<td>0.17-1.23</td>
</tr>
<tr>
<td>Apolipoprotein B (g/l)</td>
<td>0.64</td>
<td>0.23</td>
<td>0.20-1.18</td>
</tr>
<tr>
<td>Apolipoprotein B/A-I ratio</td>
<td>1.12</td>
<td>0.71</td>
<td>0.17-3.63</td>
</tr>
</tbody>
</table>
Figure 4.3. Distribution of serum homocysteine with age in CAD patients of study 2.
4.2.2 Association of vitamin B₁₂, and folate with Homocysteine concentrations in patients with coronary artery disease.

Both vitamin B₁₂ and folate levels showed a significantly (p < 0.01) negative correlation with the serum homocysteine concentrations of the study population (Table 4.12).

There was no significant association between normal homocysteine levels, and hyperhomocysteinaemia with normal and abnormal vitamin B₁₂ and folate levels when 18.35 µmol/L was used as the upper normal limit cut off value for plasma homocysteine, However when the cutoff value for the upper limit of normal of 15.0 µmol/L, as used in the Western world, was used there was a significant association between hyperhomocysteinaemia and low folate levels (Table 4.13).
Table 4.12. Association of vitamin B$_{12}$ and folate concentrations to Homocysteine concentrations

<table>
<thead>
<tr>
<th></th>
<th>Correlation coefficient$^#$</th>
<th>p- value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin B$_{12}$</td>
<td>-0.346</td>
<td>0.002*</td>
</tr>
<tr>
<td>Folate</td>
<td>-0.345</td>
<td>0.002*</td>
</tr>
</tbody>
</table>

$^#$ Spearman correlation

* Correlation is significant at the 0.01 level

Table 4.13. Significance (p-values$^#$) of the association between vitamin B$_{12}$ and folate with normal homocysteine levels and hyperhomocysteinaemia at different cut off values.

<table>
<thead>
<tr>
<th></th>
<th>Vitamin B$_{12}$</th>
<th>Folate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homocysteine cutoff of 18.35µmol/L</td>
<td>0.106</td>
<td>0.261</td>
</tr>
<tr>
<td>Homocysteine cutoff of 15.0µmol/L</td>
<td>0.520</td>
<td>0.015*</td>
</tr>
</tbody>
</table>

$^#$ Chi square tests between normal and abnormal

* Significant correlation
4.2.3 MTHFR C677T/A1298C polymorphism analysis:

4.2.3.1 Comparison of the internal controls and results of agarose gel electrophoresis in MTHFR C677T polymorphism analysis

MTHFR C677T genotype analysis indicated that when DMD 48 was used as the internal control for *Hinf* I digestion of the amplified DNA segment of interest in the analysis of MTHFR C677T polymorphism, the DMD gave DNA fragments of 236 bp, 189 bp and 81 bp in length. The digested DNA fragment of interest gave either a 198 bp fragment if uncut when the mutation is not found as in the case of homozygous wild type or a fragment/s of 175 bp and 23 bp in length depending on the presence of the mutation. Differentiation between the undigested fragments and the genotypes for the MTHFR C677T polymorphism became difficult due to the close proximity of the 198 bp band from the DNA fragment of interest and the 189 fragment from the DMD 48 internal control in the agarose gel (Plate 4.1).

When the 154 bp bacteriophage λ DNA fragment was used as the internal control in the *Hinf* I digestion to identify the MTHFR C677T genotypes, the internal control gave DNA fragments of 18 bp and 132 bp in length. These occurring far away from the 198 bp and 175 bp fragments from digestion of the DNA fragment of interest were easily distinguishable in the agarose gel. 18 bp and 23 bp fragments could not be differentiated on the 2% agarose gel. Visualization of the 132 bp fragment was sufficient to show that digestion by *Hinf* I had occurred and visualization of 198 bp and/or the 175 bp fragments was sufficient to assign a genotype for the MTHFR C677T polymorphism (Plate 4.2).
Plate 4.1. MTHFR 677C>T genotyping: The pattern created by electrophoresing the *Hinf*I digested PCR products of samples of the three genotypes and the DMD 48 internal control on a 2% agarose gel.
Plate 4.2. MTHFR 677C>T genotyping: The pattern created by electrophoresing the \textit{Hinf} I digested PCR products of samples of the three genotypes and the 154 bp bacteriophage \( \lambda \) DNA internal control on a 2\% agarose gel.
4.2.3.2 MTHFR A1298C polymorphism analysis using agarose gel electrophoresis

In the analysis of MTHFR A1298C polymorphism, Mbo II digestion of the PCR amplified DNA fragment of interest gave bands of 84bp, 56bp, 31bp, 30bp and 28bp in length on electrophoresing on 2% agarose gel. 28bp, 30bp and 31bp fragments could not be differentiated on the 2% agarose gel. Visualization of the 56bp and the 84bp fragments was sufficient to assign a genotype for the MTHFR A1298C polymorphism (Plate 4.3).

4.2.4 Association between Hyperhomocysteinaemia and MTHFR gene mutations (A1298C, C677T polymorphism):

Genotypes were obtained from all 79 CAD subjects. Genotype frequencies of all polymorphisms studied were in Hardy-Weinberg equilibrium. Amongst the study population the heterozygous mutant AC genotype was the commonest in the MTHFR A1298C polymorphism, whereas the wild type CC genotype was the commonest in the MTHFR C677T polymorphism. The homozygous mutant TT genotype showed the least occurrence amongst all polymorphisms studied with only 4 individuals showing the genotype. The frequencies of the different MTHFR A1298C and C677T genotypes are summarized in Table 4.14.

The frequencies for the variant alleles of the MTHFR A1298C and C677T polymorphisms were 43.04% and 17.72% for the C allele of A1298C polymorphism and the T allele of the C677T polymorphism respectively.
Plate 4.3. MTHFR A1298C genotyping: The pattern created by electrophoresing the Mbo II digested PCR products of samples of the three genotypes on a 2% agarose gel.

Table 4.14. Genotype frequencies for MTHFR A1298C and C677T polymorphisms of the study sample (n=79).

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>MTHFR A1298C</th>
<th>MTHFR C677T</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotype</td>
<td>AA</td>
<td>AC</td>
</tr>
<tr>
<td>Frequency</td>
<td>29</td>
<td>32</td>
</tr>
<tr>
<td>Percentage</td>
<td>36.7</td>
<td>40.5</td>
</tr>
</tbody>
</table>
Genotype frequencies showed that the associations of MTHFR polymorphisms and folate, vitamin B₁₂ and homocysteine concentrations were similar in males and females, thus both groups were combined for all subsequent analyses. According to the results of MTHFR C677T polymorphism analysis, the highest mean homocysteine concentration was observed in persons with the homozygous mutant TT genotype. Furthermore, the subjects with the homozygous mutant TT genotype of the MTHFR C667T polymorphism also had the lowest mean concentrations of both serum vitamin B₁₂ and serum folate concentrations (Table 4.15).

Results of the MTHFR A1298C polymorphism analysis indicated that the highest mean homocysteine concentration was observed in persons with the heterozygous mutant AC genotype whilst subjects with the homozygous wild type AA genotype of the MTHFR A1298C polymorphism had the lowest mean concentrations of both serum vitamin B₁₂ and serum folate concentrations (Table 4.16).

Homocysteine concentrations did not differ significantly with the three genotypes of the MTHFR C677T polymorphism as well as the MTHFR A1298C polymorphism. Vitamin B₁₂ and folate concentrations also did not show a significant difference between the MTHFR A1298C and C677T genotypes.

Highest mean homocysteine concentration of 19.75 µmol/L was seen in the MTHFR 1298AA/677TT combination, whereas the lowest was among the MTHFR 1298CC/677CT combination. When mean homocysteine concentrations between different combinations was compared with MTHFR double wild type homozygous genotype (A1298AA/677CC) no significant differences (p>0.05) were observed in any of the combinations (Table 4.17).
Table 4.15. Relationship between MTHFR C677T genotype and homocysteine, folate and vitamin B\textsubscript{12} concentrations.*

<table>
<thead>
<tr>
<th>MTHFR C677T genotype</th>
<th>P value#</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC (n=55)</td>
<td>CT (n= 20)</td>
</tr>
<tr>
<td>Serum homocysteine</td>
<td>13.72 (5.5)</td>
</tr>
<tr>
<td>Serum vitamin B\textsubscript{12} (pg/ml)</td>
<td>584.95 (310.4)</td>
</tr>
<tr>
<td>Serum folate (ng/ml)</td>
<td>4.38 (2.92)</td>
</tr>
</tbody>
</table>

*Homocysteine, folate and vitamin B\textsubscript{12} concentrations are expressed as the mean (SD).\#

Anova test between genotypes
Table 4.16. Relationship between A1298C genotype and homocysteine, folate and vitamin B$_{12}$ concentrations.*

<table>
<thead>
<tr>
<th>MTHFR A1298C genotype</th>
<th>P value$^#$</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA (n= 29)</td>
<td></td>
</tr>
<tr>
<td>AC (n= 32)</td>
<td></td>
</tr>
<tr>
<td>CC (n= 18)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Serum homocysteine (µmol/L)</th>
<th>Serum vitamin B$_{12}$ (pg/ml)</th>
<th>Serum folate (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA</td>
<td>13.37 (3.4)</td>
<td>569.03 (265.7)</td>
<td>4.66 (3.0)</td>
</tr>
<tr>
<td>AC</td>
<td>14.36 (7.0)</td>
<td>579.13 (352.9)</td>
<td>5.15 (3.10)</td>
</tr>
<tr>
<td>CC</td>
<td>13.35 (3.8)</td>
<td>588.28 (262.6)</td>
<td>4.88 (2.57)</td>
</tr>
</tbody>
</table>

*Homocysteine, folate and vitamin B$_{12}$ concentrations are expressed as the mean (±SD).$^\#$ Anova test between genotypes
Table 4.17. Relationship between MTHFR A1298C/C677T genotype combinations and homocysteine concentrations.

<table>
<thead>
<tr>
<th>MTHFR genotype (A1298C/C677T)</th>
<th>Number</th>
<th>Mean homocysteine Concentration (μmol/L)</th>
<th>Standard deviation (±)</th>
<th>p-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA/CC</td>
<td>17</td>
<td>12.75</td>
<td>2.81</td>
<td>-</td>
</tr>
<tr>
<td>AA/CT</td>
<td>10</td>
<td>13.16</td>
<td>2.85</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>AA/TT</td>
<td>2</td>
<td>19.75</td>
<td>5.83</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>AC/CC</td>
<td>23</td>
<td>14.47</td>
<td>7.65</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>AC/CT</td>
<td>7</td>
<td>13.57</td>
<td>5.27</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>AC/TT</td>
<td>2</td>
<td>16.03</td>
<td>8.00</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>CC/CC</td>
<td>15</td>
<td>13.68</td>
<td>3.78</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>CC/CT</td>
<td>3</td>
<td>11.72</td>
<td>4.43</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>CC/TT</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* Anova test; MTHFR 1298AA/677CC as reference
4.2.5 Severity of coronary artery disease in the study population

The severity of ischaemia as assessed by the vessel score, stenosis score and extent score by perusing the coronary angiogram reports showed a mean vessel score of 2.04 out of a possible maximum of 3 which indicates that most of the patients in the study population had involvement of at least two out of the three main coronary vessels. Four patients had a vessel score of zero in this study sample.

The mean stenosis score of the study population was 9.86 out of a theoretical maximum of 32 indicating that more than one fourth of the collective vessel diameter of the segments studied is having significant stenosis.

The mean extent score of the study was 46.42% out of a theoretical maximum of 100 according to the method described by Sullivan et al. in 1990 (297). (Table 4.18). Thus in the study sample, according to the extent score, almost 50% of the coronary musculature has been involved with stenosis in most of the CAD patients. Thus overall, the results indicate that the severity of ischaemia is high in the study population.

4.2.6 Association between vitamin B₁₂, folate, and homocysteine concentrations on the severity of coronary artery disease.

Serum homocysteine and serum folate concentrations were not significantly related to the severity of coronary artery disease. Serum vitamin B₁₂ concentrations showed a significant negative correlation with both the vessel score (p <0.05) and the extent score (p < 0.01) (Table 4.19).
Table 4.18. Severity of ischaemia in the study sample.

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>95% CI</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vessel score</td>
<td>2.06</td>
<td>1.83 - 2.25</td>
<td>0- 3</td>
</tr>
<tr>
<td>Stenosis score</td>
<td>9.86</td>
<td>9.14 – 10.38</td>
<td>4- 16</td>
</tr>
<tr>
<td>Extent score</td>
<td>46.42</td>
<td>43.19 – 48.71</td>
<td>23.8-73.0</td>
</tr>
</tbody>
</table>

Table 4.19. Association of vitamin B<sub>12</sub>, folate, and homocysteine concentrations to severity of CAD.

<table>
<thead>
<tr>
<th></th>
<th>Vessel Score</th>
<th>Stenosis Score</th>
<th>Extent Score</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Correlation</td>
<td>p-value</td>
<td>Correlation</td>
</tr>
<tr>
<td>coefficient#</td>
<td></td>
<td></td>
<td>coefficient#</td>
</tr>
<tr>
<td>Serum homocysteine</td>
<td>0.085</td>
<td>0.46</td>
<td>0.025</td>
</tr>
<tr>
<td>Serum vitamin B&lt;sub&gt;12&lt;/sub&gt;</td>
<td>-0.251</td>
<td>0.02*</td>
<td>-0.21</td>
</tr>
<tr>
<td>Serum folate</td>
<td>-0.127</td>
<td>0.27</td>
<td>-0.19</td>
</tr>
</tbody>
</table>

* Spearman correlation

* Significant correlation
4.2.7 Association of Apolipoprotein A-I, Apolipoprotein B and Glutathione peroxidase with severity of coronary artery disease.

Apolipoprotein A-I:

Apolipoprotein A-I concentrations showed a significant inverse correlation with the stenosis score (p<0.01) and the extent score (p<0.001). Apolipoprotein A-I did not show a significant correlation with the vessel score (Table 4.20).

Apolipoprotein B:

Apolipoprotein B concentrations showed a significant correlation with the vessel score (p<0.01). Apolipoprotein B did not show a significant correlation with both the stenosis and the extent scores (Table 4.20).

Apolipoprotein B/A-I ratio:

Apolipoprotein B/A-I ratio showed significant correlations with the stenosis (p<0.001) and extent scores (p<0.001). Apolipoprotein B/A-I ratio did not show a significant correlation with the vessel score (Table 4.20).

Glutathione peroxidase:

Glutathione peroxidase concentrations showed a significant inverse correlation (p<0.001), with the vessel, the stenosis and the extent scores (Table 4.20).
Table 4.20. Association of apolipoprotein A-I, apolipoprotein B and glutathione peroxidase to severity of CAD.

<table>
<thead>
<tr>
<th></th>
<th>Vessel Score</th>
<th>Stenosis Score</th>
<th>Extent Score</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Correlation</td>
<td>p-value</td>
<td>Correlation</td>
</tr>
<tr>
<td></td>
<td>coefficient#</td>
<td></td>
<td>coefficient#</td>
</tr>
<tr>
<td>Apolipoprotein A-I</td>
<td>-0.151</td>
<td>0.185</td>
<td>-0.316</td>
</tr>
<tr>
<td>Apolipoprotein B</td>
<td>0.244</td>
<td>0.030*</td>
<td>0.139</td>
</tr>
<tr>
<td>B/A-I ratio</td>
<td>0.219</td>
<td>0.053</td>
<td>0.350</td>
</tr>
<tr>
<td>Glutathione peroxidase</td>
<td>-0.311</td>
<td>0.005**</td>
<td>-0.428</td>
</tr>
</tbody>
</table>

# Spearman correlation

* Significant correlation
4.2.8 Association of age with Apolipoprotein A-I, Apolipoprotein B, glutathione peroxidase concentrations in coronary artery disease patients:

Results show that there was no significant association between apolipoprotein B, apolipoprotein B/A1 ratio and glutathione peroxidase with age of CAD patients. Age had a significant association ($p < 0.5$) with the apolipoprotein A-I concentrations in the study population and showed an increase with the age of CAD patients (Table 4.21).
Table 4.21. Influence of age on the association of apolipoprotein A-I, apolipoprotein B, glutathione peroxidase on coronary artery disease.

<table>
<thead>
<tr>
<th></th>
<th>Correlation coefficient</th>
<th>p- value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apolipoprotein A-I</td>
<td>0.230</td>
<td>0.041*</td>
</tr>
<tr>
<td>Apolipoprotein B</td>
<td>0.058</td>
<td>0.612</td>
</tr>
<tr>
<td>Apolipoprotein B/A-I ratio</td>
<td>0.233</td>
<td>0.136</td>
</tr>
<tr>
<td>Glutathione peroxidase</td>
<td>0.204</td>
<td>0.072</td>
</tr>
</tbody>
</table>

* Spearman correlation

* Significant correlation
5. DISCUSSION

5.1 Association between Hyperhomocysteinaemia and CAD

5.1.1 High performance liquid chromatography in the assay of Homocysteine

Chromatography is an analytical method with wide applications for the separation, identification and determination of chemical components in complex mixtures. This technique is based on the separation of components in a mixture (the solute) due to the difference in migration rates of the components through a stationary phase by a gaseous or liquid mobile phase. Those components held preferentially in the stationary phase are retained longer in the system than those that are distributed selectively in the mobile phase. As a consequence, solutes are eluted from the system as local concentrations in the mobile phase in the order of their increasing distribution coefficients with respect to the stationary phase. This concept is the basis of different types of chromatography including high-performance liquid chromatography (HPLC).

High performance liquid chromatography is basically a highly improved form of column chromatography where instead of a solvent being allowed to drip through a column under gravity, it is forced through under high pressures of up to 400 atmospheres, thus making the process much faster. This allows the use of a very much smaller particle size for the column packing material which gives a much greater surface area for interactions between the stationary phase and the molecules flowing past it. This leads to a much better separation of compounds in a mixture. The other major improvement over column chromatography concerns the detection methods which are highly automated and extremely sensitive. These include electrochemical and spectroscopic detection systems.
There are two forms of HPLC referred to as Normal Phase HPLC and Reversed Phase HPLC.

In normal phase HPLC the column is filled with polar silica particles, and the solvent is non-polar. Polar compounds in the mixture being passed through the column will stick longer to the polar silica than non-polar compounds, thus allowing the passage of non-polar components more quickly through the column than polar compounds.

In reversed phase HPLC (RP-HPLC), which is the most commonly used form of HPLC, the silica is modified to make it non-polar by attaching long hydrocarbon chains to its surface - typically with either 8 or 18 carbon atoms in them. A polar solvent is used - for example, a mixture of water and an alcohol such as methanol. Thus in RP-HPLC, there is a strong attraction between the polar solvent and the polar molecules in the mixture that passes through the column. Compared to this, the attraction between the hydrocarbon chains attached to the silica (the stationary phase) and the polar molecules in the solution is less. Polar molecules in the mixture will therefore spend most of their time moving with the solvent. The non-polar compounds in the mixture tend to form attractions with the hydrocarbon groups because of van der Waals dispersion forces. They will also be less soluble in the solvent because of the need to break hydrogen bonds as they squeeze in between the polar solvent (water or methanol molecules, for example). They therefore spend less time in solution in the solvent and this will slow them down on their way through the column. Thus in RP-HPLC, it is the polar molecules that will travel through the column faster than the non-polar compounds.

Several methods of measuring plasma total homocysteine have been described (309-313). One of the most widely used methods of total plasma homocysteine (and other aminothiol) measurement is reversed phase High Performance Liquid
Chromatography (HPLC) with fluorescence detection after derivatization of plasma amino thiols with ammonium 7-fluorobenzo-2-oxA-I,3 diazole sulfonate (SBD-F) (311, 312). Plasma amino thiols exist as free reduced and oxidized compounds or as protein bound ones and one of the most critical steps in the sample processing is the reduction of disulfide bonds before derivatization so that total homocysteine in plasma is measured. For this step, trialkylphosphines are used and tri-butyl phosphate (TBP) and \textit{tris} (2-carboxyethyl) phosphate (TCEP) are the most widely used. TBP has an irritating odour, is poorly water soluble and it has to be dissolved in toxic dimethylformamide, whereas TCEP is less toxic, soluble in aqueous solutions and stable (313). Thus recently, researchers have preferred TCEP over TBP as the reductant in the analysis of aminothiols in human plasma.

One of the methods using the above was described by Pfeieffer et. al. in 1999 and this method has several advantages such as being a rapid, user friendly assay which uses a stable and non-hazardous reducing agent in TCEP, and rapid isocratic separation of aminothiols of interest using a mobile phase of mild pH using only 50 μl of plasma (304).

Use of an internal standard is a standard requirement in HPLC and this holds true for the RP-HPLC method used in the analysis of homocysteine too to compensate for variations in thiol derivatization and sample injection procedures. But most of the earlier published methods use external calibration alone for quantitation of homocysteine by RP-HPLC because of the difficulty in selecting an internal standard (314). Recently different internal standards have been used in RP-HPLC analysis of homocysteine and these include N-(2-mercaptopropionyl) glycine, 2-mercaptoethylamine (ME), N-acetylcysteine and Cystamine dihydrochloride (315).
Thus in this study we initially used N-(2-mercaptopyrrolonyl) glycine as the internal standard and found that it lengthened the chromatogram up to 3-4 times compared to running without an internal standard. This fact has been observed in other studies using the above internal standards too (315). In comparison when cystamine dihydrochloride was used as the internal standard the retention time for the internal standard was shorter by more than 50% and an excellent chromatographic pattern was observed. Thus the results of the present study have revealed that cystamine dihydrochloride was a better internal standard when compared to N-(2-mercaptopyrrolonyl) glycine, as the retention time of cystamine dihydrochloride is less, thus saving on time and the amount of solvents when samples are run. Cystamine dihydrochloride has also been shown to have the advantage of overcoming the matrix effect of plasma when compared with calibrators prepared in PBS or water (304).

The present assay method was modified by having the column at room temperature (28-32 °C) thus negating the difficult task of maintaining a controlled column temperature when compared with the assay method described by Pfeiffer et al. (1999) whose assay method was carried out at a controlled column temperature. However it was found that baseline separation of the thiols and the internal standard with the present assay method was not optimal at 30 ml/L methanol in acetic acid–acetate buffer (pH 5.5). This improved as the methanol concentration was decreased gradually and thereby giving a chromatogram with good base line separation at a methanol concentration of 12 ml/L. These results suggest that methanol at 12 ml/L in acetic acid – acetate buffer is a better way of getting good chromatograms in conditions described above.
Calibration curves obtained using the above modification for homocysteine was linear up to a homocysteine concentration of 150 μmol/L. Hyperhomocysteinaemia is graded according to the fasting plasma levels as mild to moderate (15-30 μmol/L), intermediate (31-100 μmol/L), and severe (> 100 μmol/L) (74). Thus this method is able to differentiate between normal and hyperhomocysteinemic persons as well as grading them according to the severity of hyperhomocysteinaemia.

The assay performance characteristics (coefficient of variation) of homocysteine and internal standard gave consistent within and between run assay CVs indicating that the test method could be reproduced with ease over a longer period of time. Thus, the present assay method is an acceptable cost effective way of assaying total homocysteine and it could be used as a reliable and reproducible method for routine determination of plasma homocysteine under normal laboratory temperatures in our country.

5.1.2 Association between Hyperhomocysteinaemia and coronary artery disease

Homocysteine concentrations have been shown to vary among different ethnic groups or geographic areas thus necessitating the development of reference values for countries (40). Due to the non-availability of a reference range for homocysteine concentrations in Sri Lankan and in Asians, the 90th percentile of the controls in this study (18.38 μmol/L) was taken as the upper limit of normal for plasma homocysteine. Even this value was within the moderate hyperhomocysteinaemia concentration based on the gradings used by western countries. Hence, it is possible that even subjects with a plasma homocysteine concentration between 15-18.38 μmol/L may be at a higher risk of getting CAD and other diseases associated with hyperhomocysteinaemia.
Among Indians, it has been reported that plasma homocysteine concentration is high as compared to populations in the western world (316). This remains true for Indians living in or outside India (316-319). The mean (± SD) plasma homocysteine concentrations in normal subjects in three studies conducted in India by Lakshmi et al. (2001), Sastry et al. (2001), and Misra et al. (2002) were reported as 19.83 μmol/L (±1.25), 18.04 μmol/L (± 10.69) and 23.9 μmol/L (±5.9) respectively (316, 317, 319). A study carried out with Asian Indians living in the United states reported that the plasma homocysteine levels were significantly higher at 14 μmol/L (±6.5) in Indians compared to Caucasians (318). Similar evidence has been reported by Mendis et al. (1997, 2002) in Sri Lankan subjects in which the mean plasma homocysteine concentration of healthy subjects was found to be 23.8 μmol/L (±12.9) whilst in the other study the 90th percentile for healthy subjects was found to be 18.2 μmol/L (320, 321). Results of this study are comparable with the second of these studies with the 90th percentile value of the control subjects being 18.38 μmol/L.

These data indicates that populations in the Indian subcontinent may have higher fasting total homocysteine levels when compared to the values reported in the west. This is quite significant in that the levels observed in these studies all fall above the norms for other populations. The importance of this lies in the fact that these homocysteine concentrations fall within mild to moderate hyperhomocysteinemic levels in the western categorization which has been found to be a risk factor for coronary artery disease as well as other diseases. Further epidemiological and population studies have to be conducted in this region to find out the norms and to find out whether this observed increase in total homocysteine levels are true as well as to see whether these
hyperhomocysteinemic populations are at increased risk of developing disease conditions associated with hyperhomocysteinaemia.

One important finding of this study was that the expected rise in the homocysteine concentration with age was not observed in both study I and study II subjects.

Age and kidney function are reported to be the primary correlates of fasting homocysteine levels in normal subjects (322). Studies have found age to be a significant correlate with plasma homocysteine, with levels increasing with increasing age (5, 322-327). However one must note that most of these studies have been conducted with western populations.

Many studies have reported this association between plasma homocysteine concentrations with age and an increase in plasma homocysteine concentrations in males compared to post menopausal females (127, 324, 325, 328-330) which has led to establishment of reference intervals specific for age and for fasting plasma homocysteine (77). This increase in fasting plasma homocysteine concentration with age has been attributed to changes in renal function (85, 331)), impaired renal metabolism of homocysteine with age (136) or vitamin status (145, 332).

The present study findings are not in accordance with the established norm of plasma homocysteine levels increasing significantly with increasing age. The two populations studied showed no significant association between age and plasma homocysteine among both the control group in study I and the CAD patients in both studies. The control subjects in study I and the CAD patients in study 2 showed a trend towards an increase in homocysteine concentrations with age although this trend was not statistically significant (p >0.05). In comparison, patients with an ACS in study 1
showed an inverse trend i.e. a decrease in homocysteine concentration with increasing age, although again this association was not statistically significant (p>0.05).

According to literature, studies not showing a significant correlation between age and plasma homocysteine are rare. However, some studies conducted in the Western world have reported on the absence of correlation between age and homocysteine (71, 127, 333).

In a study conducted in Sri Lanka Mendis et al. (1997) reported that there is no significant correlation between age and homocysteine in their study sample (320). A study conducted in India by Misra et al. (2002) involving an apparently healthy study sample whose mean age was approximately 24 years, reported that the mean homocysteine concentration of 23.9 μmol/L was the highest recorded in India. Other studies conducted in India involving older subjects reported that the mean homocysteine concentrations were much lower than the values reported by Misra et al. (2002) (319). Thus the assumption that homocysteine concentration increases with age in all populations may not be applicable to persons living in the Indian subcontinent.

Majority of the studies on the association of homocysteine and diseases have been performed with age and sex matched controls, especially after the initial reporting on the association of age and sex with homocysteine. Thus direct correlation of age with plasma homocysteine levels have not been documented in these studies which have been performed mainly in the western world. Even research conducted in neighboring India fail to document a direct correlation between age and homocysteine concentrations. The reason for the absence of a significant correlation between increasing age and plasma homocysteine levels may be due to differences in dietary habits with age, which may be unique from that observed in the western world, which
would have led to a higher level of homocysteine in the young compared to older populations.

The association between hyperhomocysteinaemia and CAD reported in this study are similar to those reported in other studies done in Sri Lanka (320, 321). Mendis et al. (2002) reported that in a multivariate analysis using a logistic regression model hyperhomocysteinaemia was a significant predictor of CHD after controlling for age, sex and presence or absence of diabetes mellitus with an adjusted odds ratio of 2.881 (95% confidence interval 1.138-7.294) (321). In another study a crude odds ratio of 3.2 (95% confidence interval 1.0-11.3) was obtained for the risk of development of CAD due to hyperhomocysteinaemia (320). The results of the present study was similar, with an adjusted odds ratio of 2.387 (95% confidence interval 1.325-4.299) after controlling for hypercholesterolaemia and hypertension when a conditional logistic regression analysis was performed. According to the study findings of Mendis et al. (2002), the age and presence of diabetes mellitus have been identified as potential risk factors for coronary artery disease whilst the gender has not been identified as a risk factor. In this study, in bivariate analysis, hypertension and hypercholesterolaemia were found to be significant potential risk factors. However the presence of diabetes mellitus was not found to be a significant predictor in the present study. In this study, we were unable to assess the association of age and sex with CAD as the patients and controls were matched on age and sex.

Thus all three studies including the present study show a 3 fold increase in the risk of CAD among persons with hyperhomocysteinaemia as compared to subjects with normal homocysteine levels. These studies have been conducted over a period of time on different study populations, the 3 fold risk in development of CAD in persons with
hyperhomocysteinaemia compared to subjects with normal homocysteine levels. Hence these findings may be of value in using homocysteine as a tool in predicting the risk of CAD for Sri Lankans.

This study also revealed that subjects with hypercholesterolemia were 7 times more likely to have CAD whereas subjects with hypertension were 2 times more likely to have CAD. Thus the findings of the present study confirm that hypercholesterolaemia is an important risk factor for CAD in Sri Lankan subjects too. Furthermore, the findings of this study suggest that hyperhomocysteinaemia is a far more important risk factor for development of CAD when compared to hypertension.

The present study further suggested that the addition of other conventional risk factors did not increase the risk of CAD implying that hyperhomocysteinaemia by itself is one of the most important predictors of CAD.

Hyperhomocysteinaemia has emerged as one of the novel coronary risk factors during the last 30-40 years. A positive association between hyperhomocysteinaemia and CAD has been found in many studies mainly involving Caucasians. These include mainly retrospective studies and some prospective studies. However these studies have shown conflicting results. Majority of the retrospective studies have shown a significant association between hyperhomocysteinaemia and CAD whilst only some prospective studies have shown this association. The main argument against hyperhomocysteinaemia being a risk factor of CAD is the conflicting results that have come up from prospective studies with most not showing a significant association between hyperhomocysteinaemia and CAD.

According to Boushey et al. (1995), each 5 μmol/L increase in plasma homocysteine concentration is associated with a 1.6 and 1.8 fold increase in risk of
developing CHD for men and women respectively (158). A prospective study carried out by Arnesen et al. (1995) revealed that in the general population serum total homocysteine is an independent risk factor for CHD and that there was no threshold level above which serum homocysteine is associated with CHD events (73). A consistent graded association was also found in the British BUPA cohort (334). A graded relationship between all cause mortality and plasma homocysteine levels was observed in a study of Norwegian population with angiographically confirmed coronary artery disease (159). Furthermore, some studies have found that patients with acute myocardial infarction who have elevated plasma homocysteine levels are at a higher risk of recurrent coronary events and death independent of other risk factors and the extent of coronary artery disease (335).

However, some studies have suggested that there was no significant association between homocysteine and atherosclerotic disease including myocardial infarction (162, 336, 337). Furthermore, in a review of prospective and retrospective studies Christen et al. (2000) commented that in contrast to cross-sectional and case-control studies, results of prospective studies indicate less or no predictive ability for plasma homocysteine in cardiovascular disease. Furthermore, it was suggested that elevated homocysteine level may well be an acute-phase reactant that is predominantly a marker of atherogenesis, or a consequence of other factors more closely linked to risks of cardiovascular disease (338).

In summary, although subjects had the same age range and sex distributions, the research findings suggest that the normal homocysteine concentrations in the Western world are very much lower compared to the values of Asians, and particularly those living in the Indian subcontinent. Thus data pertaining to studies conducted in the region...
has to be evaluated to see whether findings in the West are comparable to what was observed in people living in the Indian subcontinent.

Furthermore it appears that findings of the studies on populations in the Indian subcontinent and populations from the subcontinent domiciled in western countries on hyperhomocysteinaemia as a risk factor for CAD were not convincing (339-341). Existing results from some Indian studies have also not found a positive association between elevated plasma homocysteine levels and coronary heart disease (316, 339, 342, 343) whilst some have reported a positive association (344).

However in Sri Lanka, only a few studies have reported on hyperhomocysteinaemia and CAD and it has been found that hyperhomocysteinaemia was a contributory factor associated with increased risk of CAD (320, 321) which is in agreement with the findings of this study.

5.2 Association between hyperhomocysteinaemia and CAD in the young (age ≤ 50 years) compared to the elderly (age > 50 years).

The present study revealed that in subjects below 50 years there was a strong association (p=0.004) between hyperhomocysteinaemia and CAD with a 4.5 fold increase in risk of developing CAD when compared to persons of similar age with normal concentrations of plasma homocysteine. In comparison, there was no significant association (p= 0.188) between hyperhomocysteinaemia and CAD among persons over 50 years of age.

This association between hyperhomocysteinaemia and CAD in the two age groups could not be elucidated further by adjusting for hypertension and hypercholesterolaemia, the other two risk factors which were found to be significantly
associated with CAD, in a conditional logistic regression analysis as age was a matching variable in this study.

To assess whether hyperhomocysteinaemia had more predictive power of CAD in patients of one age group over the other, a chi square analysis was performed and this analysis indicated that when young and the elderly patients with CAD were compared, hyperhomocysteinaemia was a significant predictor of CAD in young CAD patients when compared to CAD patients who were over 50 years of age ($p=0.027$). Therefore, the present study confirmed a strong CAD risk association in hyperhomocysteinaemic young population who are below 50 years of age when compared with hyperhomocysteinaemic elderly population in Sri Lanka. This is the first study to report such an association of hyperhomocysteinaemia and CAD in Sri Lankans.

Hyperhomocysteinaemia has been found to be associated with increased risk of cardiovascular disease in the elderly in most of the studies. These include studies such as the European Concerted Action Project (345), the Rotterdam study (346), and others (347) which reported that among elderly subjects an elevated homocysteine level is associated with an increased risk of cardiovascular disease.

A significant association between hyperhomocysteinaemia and CAD in persons below 55 years of age was reported by Marcucci et al. in 2005. They found that the odds ratio for CAD at young age significantly increased in the fourth quartile of the distribution of fasting homocysteine concentrations (OR 14.9; 95% CI 4.1-58; $P<0.0001$). However this study was not designed to identify whether this association was different in persons over 55 years of age (348). Two studies carried out with young CAD patients whose age was below 35 years and females below 45 years of age
reported that patients with premature MI had higher homocysteine levels when compared with controls in the same age groups (349, 350).

Literature further reveals that high homocysteine levels on admission strongly predict late cardiac events in young patients with acute coronary syndrome (351). However some studies have failed to find elevated homocysteine levels as a risk factor for CAD in the young. Valentine et al. (1996) reported that there was no significant association between homocysteine concentration and the risk of CAD in a cohort below 45 years of age (352).

Although most of the studies conducted with either young or elderly patients have failed to compare whether any difference between the two groups exist, they have also not been able to find out whether elevated homocysteine is more of a risk factor in the young or the elderly age groups. Studies involving a cross section of both the young as well as the elderly populations have also failed to compare the two groups to see whether any difference is present (73, 337, 353, 354). Hence, this study adds a new finding to the literature on the association of hyperhomocysteinaemia and CHD.

Whereas coronary heart disease rate has decreased by 50% in the West in the past 30 years, the rates have doubled in India without any signs of a downturn (355). In Sri Lanka, between 1970 and 2003 hospital deaths due to IHD has doubled from 9.3 to 18.8 deaths per 100,000 population (4, 356). It has also been observed that in India the average age of first myocardial infarction has decreased by 20 years during the past 30 years and 25% of acute MI occur under the age of 40 years and 50% under the age of 50 years (357, 358). Furthermore, unlike in Caucasians, CAD in young Asian Indians have been found to be severe, extensive and malignant and this has been attributed to an accelerated atherosclerotic process that begins early in life (359-361). It has been
reported that high rates of CAD in Asian Indians are accompanied by low rates of conventional risk factors except for diabetes (362). Thus these findings together indicate that CAD rates as well as its incidence in the young is steadily rising and that conventional risk factors are unable to fully explain the excess burden of CAD in them. Thus for the younger population, most research is limited to the established CAD risk factors and further investigations of recently identified CAD risk factors are needed (363).

There is a paucity of data regarding the incidence patterns of CAD in the younger population of Sri Lanka. But we could assume from the trends observed in the hospitals that the situation is similar in our country to what is observed in India.

In this context findings of this study are important not only as a new finding but also with respect to management of patients, especially the young, in that strategies may need to be different or they may have to be changed for different age groups. More emphasis may have to be placed on the young compared to the elderly, especially regarding strategies to reduce the incidence of novel risk factors like elevated plasma homocysteine.

The management of hyperhomocysteinaemia mainly involves therapy with vitamins B₁₂ and folate, and vitamin B₆ in persons having post methionine load hyperhomocysteinaemia. This is a cheap and cost effective way of reducing homocysteine concentrations and, thus, a good way of reducing the incidence of CAD in the population. Thus further studies are required in this respect, especially population based studies involving both the young and the elderly as cheap and effective interventions may be applied with ease if found to be necessary.
5.3 Association between Hyperhomocysteinaemia and the number of risk factors (having two or less traditional risk factors compared to 3 or more traditional risk factors) among CAD patients in the study sample

Results of this study show that there is no significant association between the number of risk factors and hyperhomocysteinaemia when patients with two or less traditional risk factors were compared with patients with 3 or more traditional risk factors.

Traditional or established risk factors like hypertension, hypercholesterolaemia, diabetes mellitus, physical inactivity, smoking etc. have been long recognized as the major risk factors for the development of CAD. Furthermore some researchers have reported that combinations of major coronary vascular disease risk factors place women and men at a higher risk of coronary heart disease and all cause mortality (364-366). Studies involving apparently healthy people have found that even in them a positive relationship exists between the number of cardiovascular risk factors and the risk of development of cardiovascular disease as assessed by assessment of aortic compliance by non-invasive methods such as Doppler ultrasound assessment (295).

This concept has been questioned recently due to findings of new epidemiological studies. Some of these studies have found that approximately half of all patients with CAD do not have any of the traditional risk factors (367-369). However this concept has been subject to argument on that the conventional risk factors are found in 80-90% of patients with CAD (370-372).

Studies reported in the literature have questioned the validity of homocysteine as a risk factor for CAD, arguing that hyperhomocysteinaemia arises as a consequence of
CAD due to the traditional risk factors and hyperhomocysteinaemia by itself is not a significant predictor of CAD (338, 373, 374).

In this study, at least one of the conventional risk factors [presence of hypertension, hypercholesterolaemia, diabetes mellitus, current smoking, excess alcohol intake, overweight and obesity (BMI > 25), and a family history of CAD was present in 91% of patients with an ACS and there was a significant association (P<0.001) between the number of risk factors (viz. two or less compared to three or more) and development of CAD, reemphasizing the importance of traditional risk factors on development of CAD. Along with this there was a significant association between hyperhomocysteinaemia and CAD in this study and this remained significant after adjusting for the conventional risk factors like hypercholesterolaemia and hypertension, thus highlighting the importance of hyperhomocysteinaemia by itself as a risk factor. Studies have also reported that there is an increase in the homocysteine concentrations as the number of conventional risk factors increase among patients with CAD (366, 375). Findings of this study suggest that the presence of two or less risk factors compared to three or more risk factors did not have an influence on the homocysteine levels.

Thus in this study, the addition of other conventional risk factors did not have an influence on the homocysteine levels implying that hyperhomocysteinaemia is one of the most important predictors of CAD.
5.4 Deficiency vitamin/s (vitamin B\textsubscript{12} and folate) giving rise to hyperhomocysteinaemia in patients with coronary artery disease.

This study is the first to report on the association between hyperhomocysteinaemia and vitamin B\textsubscript{12} and folate in CAD patients in Sri Lanka.

Results of this study show a significantly negative correlation (Spearman correlation $p < 0.01$) between both vitamin B\textsubscript{12} and folate with plasma homocysteine levels.

Vitamin B\textsubscript{12} and folate are the two major vitamins that are involved in the remethylation pathway of homocysteine metabolism and they determine the homocysteine levels in the fasting state whilst vitamin B\textsubscript{6} is the primary vitamin that is involved in the transsulfuration pathway of homocysteine metabolism and it determines the post methionine load homocysteine levels (84).

Studies conducted on healthy subjects have found that deficiency of vitamin B\textsubscript{12} and folate is the major cause of hyperhomocysteinaemia in the fasting state (71, 96-98, 113, 114). In patients with coronary artery disease too, vitamin B\textsubscript{12} and folate deficiency has been found to be the major cause for hyperhomocysteinaemia (119, 376). But some others have found MTHFR gene mutations to be a major cause of hyperhomocysteinaemia in healthy as well as CHD patients (377-381).

Further evidence for the association between vitamin B\textsubscript{12} and folate levels with homocysteine concentrations come from vitamin supplementation studies. In a population based study involving Norwegians, median plasma homocysteine concentrations were found to decrease with vitamin B\textsubscript{12} and folate supplementation over a period of 6 years.
The increase in each unit of plasma folate (nmol/L) and vitamin B₁₂ (pmol/L) was associated with reductions in plasma homocysteine concentrations of 0.2 and 0.1 μmol/L respectively. Among the study population, those who have taken folate and vitamin B₁₂ supplements had shown significant reductions in plasma homocysteine concentrations (141). In a different study, supplementation with high-dose multivitamin preparations did not show a significant reduction in plasma homocysteine levels when compared to a placebo treated group. Furthermore it was reported that even subjects with normal plasma homocysteine levels showed reduced homocysteine levels with multivitamin supplementation (70).

However it has also been reported that folate and B₁₂ are closely related to hyperhomocysteinaemia and CAD separately but not when hyperhomocysteinaemia and CAD are taken together. Siri et al. ((1998) reported that although low vitamin B₁₂ concentrations were associated with an increased risk of coronary atherosclerosis this association was partly independent of homocysteine levels. They also reported that although low folate status was a strong determinant of elevated homocysteine concentrations, it was not associated with increased risk of coronary atherosclerosis (376). Several studies have found that although folic acid supplementation does reduce plasma homocysteine levels, it does not reduce the risk of cardiovascular disease (382) nor the risk of recurrent cardiovascular disease after myocardial infarction (383).

According to literature, most of the studies related to vitamin B₁₂, folate and hyperhomocysteinaemia have been conducted mainly with western populations. Thus its applicability to the Asian region had to be questioned as the dietary habits of persons of Western origin and Asian origin as well as those living in the Western world and in the Indian subcontinent are different which could have a significant influence on vitamin
B₁₂ and folate levels. Hence it is more appropriate to relate findings of this study with Asian Indians living in the west and in the Indian subcontinent.

Carmel et al. (2002) reported that in apparently healthy Asian Indians living in the U.S., hyperhomocysteinaemia was common and that it was due to vitamin B₁₂ deficiency. In this study vitamin B₁₂ deficiency was reported to be affecting more than half of the Indian men studied and the authors postulated that this may largely be subclinical and probably largely dietary in origin. They further reported that folate deficiency was very rare among this normal healthy population (384). A similar study conducted with Asian Indians living in the United States showed that plasma homocysteine concentrations were higher among Asian Indian compared to Caucasians. The plasma folate levels of the Asian Indians were in the normal range and did not show a correlation with the homocysteine levels whereas vitamin B₁₂ showed a significant negative correlation with plasma homocysteine concentrations (318).

A study conducted in Maharashtra India has indicated that 47% of the study population had vitamin B₁₂ deficiency whilst 77% showed hyperhomocysteinaemia. The authors further suggested that approximately 75% of the study population exhibited metabolic signs compatible with vitamin B₁₂ deficiency which could be only partly explained by their dietary habits. Only 2.5% of the study population in this study had folate deficiency indicating that vitamin B₁₂ deficiency was the main reason for the observed increase in homocysteine levels (385). These reports highlight mainly the importance of vitamin B₁₂ compared to folate in the development of hyperhomocysteinaemia. In contrast, a study carried out with a Bangladeshi population living in the United Kingdom showed a significant inverse correlation between plasma homocysteine concentrations and fasting serum folate levels (386). These findings were
similar to work reported by Michie et al. which involved Pakistanis and Indians living in the United Kingdom (387).

Thus the possibility exists that deficiencies of both vitamin B₁₂ and folate are involved in the development of hyperhomocysteinaemia in different communities in the Asian region.

The results of this study show that both vitamins B₁₂ as well as folate are strongly correlated with the homocysteine levels in our Sri Lankan study population indicating that both may be important in giving rise to hyperhomocysteinaemia in Sri Lanka. In addition a significant association between hyperhomocysteinaemia and decreased folate levels were observed when the upper limit of normal for homocysteine was taken as 15 μmol/L, the cut off value being used in most countries. As discussed before, it was decided to take the 90th percentile value for the controls in study 1 as the upper limit of normal as there was no established reference range for homocysteine for Sri Lankan subjects and this value falls under the moderate hyperhomocysteinaemia category which has been found to be a risk factor for CAD. Thus there may be a possibility that hyperhomocysteinemic subjects could be categorized as normal. No significant association was observed between hyperhomocysteinaemia and vitamin B₁₂ at both homocysteine cut off values indicating that out of the two vitamins studied, the main reason for hyperhomocysteinaemia in the study population was folate deficiency.

Folate deficiency should be rare in our country because of the increased intake of green leafy vegetables. Vitamin B₁₂ deficiency could arise due to inadequate intake of meat products, which is common in our country due to religious reasons, as well as due to inadequate intake even among non-vegetarians. But data supporting this do not exist as nothing much has been done on this topic in Sri Lanka. The mean folate
concentration observed in this study (4.91) is higher than the reported 4.49 ng/ml and 3.66 ng/ml in two other studies on rural adolescent school girls and urban adolescent girls and women in Sri Lanka respectively (388, 389). The percentage of subjects with low folate status (34.18%) in this study was lower than that reported by Thoradeniya et al. (2006) while it was higher than the study report by Atukorala (1998). The low serum folate levels reported in the studies, including the present study could be either due to low folic acid content in their diet or destruction of folate due to the method of preparation of food (388).

The present study found that the serum vitamin $B_{12}$ values were within the normal range except for 10.13% of the study population who showed serum levels indicative of vitamin $B_{12}$ deficiency. These findings were in agreement with previous studies reported in Sri Lanka regarding prevalence of vitamin $B_{12}$ deficiency (388, 389).

Micronutrient deficiencies and nutritional anaemia are major problems in South East Asian countries (390). Deficiency of vitamin $B_{12}$ and folate are supposed to be two of the common vitamin deficiencies (along with vitamin A) in Sri Lanka and they have been implicated in nutritional anaemia in the country although the incidence of these two vitamin deficiencies is thought to be decreasing now (391). Thus there was the possibility of either one or both these vitamins may be involved in the development of hyperhomocysteinaemia in Sri Lanka especially in patients with CAD as depicted from the results reported in the present study.

Thus the findings of this study bridge the gap of existing problem associated with the cause of hyperhomocysteinaemia in relation to folate and vitamin $B_{12}$ deficiency in that folate deficiency is the main cause of hyperhomocysteinaemia, at least in CAD patients.
Therefore, advocacy of diets which contain higher folate levels as well as supplementation of folate in vulnerable groups, such as persons who have had a previous MI and also including the young would enable us to reduce the incidence of hyperhomocysteinaemia and its consequences.

As far as the general population in Sri Lanka is concerned, introducing foods rich in folate or supplementation may be an efficient cost effective long term method of reducing the morbidity and mortality associated with CAD and hyperhomocysteinaemia.

5.5 Association between Hyperhomocysteinaemia and MTHFR gene mutations (A1298C, C677T polymorphisms) using restriction fragment length polymorphism analysis

5.5.1 Use of bacteriophage λ DNA as an internal control in restriction enzyme digestion in the MTHFR C677T gene mutation analysis by RFLP agarose gel electrophoresis

Restriction enzymes protect bacteria from invasion from DNA by recognizing and cleaving specific sequences in double stranded DNA (392). DNA of the bacteria containing these restriction enzymes is protected from digestion by these restriction endonucleases through methylation or modification of restriction sites, thus enabling them to survive without their DNA being digested by their own restriction enzymes. These restriction enzymes found in certain bacteria are now being used for different molecular biology procedures.

Restriction endonucleases are useful in the analysis and characterization of PCR products. Digestion of PCR products of interest with restriction endonucleases is used to confirm amplification of desired sequences when the size of the digested fragments
could be predicted from known restriction sites. In addition it can be used to identify sequence variants and provide linkage analysis and in diagnosis of disease (393, 394).

There are numerous restriction sites throughout any region of a given DNA. Some of these sites are polymorphic in that on a given allele the site may be present or absent and this determines whether the restriction endonuclease is able to cleave the DNA fragment or not (395). When a gene with a mutation which can lead to a disease or change in a metabolic process is identified and characterized, restriction fragment length polymorphism analysis may be used to screen samples for the same mutations if they create or abolish a restriction site. For this purpose, the sequence flanking the mutation is amplified and digested with the appropriate restriction enzyme and the generated fragments resolved by gel electrophoresis. Depending on the restriction enzyme and the DNA segment, DNA fragments of different lengths could be visualized to get the correct genotype. Unfortunately, failure of the restriction endonuclease to digest a PCR product can lead to misleading results in RFLP analysis. Thus control samples, homozygous and heterozygous for both alleles, have to be amplified at the same time as the unknown to detect undigested samples (396). Additional endogenous restriction sites within the PCR product have been used as an internal control for complete digestion. When such a constitutive site is not found for an amplicon being investigated, coamplification of a sequence in an irrelevant gene has been used as a restriction digest control (397). Therefore, the DNA sequence of the relevant DNA segments must be known as primers are prepared depending on the DNA sequence of interest. Thus in some studies to overcome this problem an addition of engineered restriction site/s into DNA segments of interest have been used (398) as restriction digestion controls.
In this study, when the \textit{Hinf I} digestion of the amplified C677T mutation site was performed, the undigested as well as the homozygous wild type gave a 198 bp fragment, thus necessitating the use of an internal restriction digestion control to distinguish the undigested from the homozygous wild type. Thus, the DMD 48 gene, which is found in an irrelevant gene, was used as a co amplicon to be used as the internal control for \textit{Hinf I} restriction enzyme digestion in the MTHFR C677T gene mutation analysis by RFLP - agarose gel electrophoresis. The \textit{Hinf I} digested fragments arising from digestion of the DNA segment of interest in the MTHFR C677T analysis and the fragments generated by \textit{Hinf I} digestion of the DMD 48 internal control gave bands which were very close together in agarose gel electrophoresis (198 bp and 189 bp) and were difficult to distinguish apart.

When bacteriophage \(\lambda\) DNA fragment with the \textit{Hinf I} digestion site was used as the restriction digestion control we were able to overcome this problem as the digested bacteriophage \(\lambda\) restriction digestion control gave a 132 bp band on gel electrophoresis. Thus visualization of the 132 bp fragment was sufficient to show that digestion by \textit{Hinf I} had occurred and visualization of 198 bp and/or the 175 bp fragments was sufficient to assign a genotype for the MTHFR C677T polymorphism.

When comparing the two restriction digestion controls, use of DMD 48 was easier as in the same PCR this fragment with the \textit{Hinf I} restriction site could be multiplexed by using suitable primers along with primer for MTHFR C677T polymorphic site. When using the bacteriophage \(\lambda\) DNA fragment as the restriction digestion control, the fragment had to be amplified separately and added to the sample mix for the restriction digestion with \textit{Hinf I}, thus necessitating two PCR’s. But we were able to overcome this by using the same PCR conditions as for the C677T polymorphic
site amplification to amplify the bacteriophage λ DNA fragment, thus enabling us to amplify the samples and the necessary restriction digestion control in the same PCR run.

Use of another restriction site which is far apart from the segment of interest has been one of the ways to overcome the problem of identifying whether digestion of a sample has taken place (399). Unfortunately this can be achieved only if the DNA sequence on either side of the segment of interest is known and only if that site contains a restriction site for a given restriction enzyme. Thus if the sequence on either side is unknown, use of an internal control to distinguish uncut or partially cut segments becomes a problem. In MTHFR C677T polymorphism analysis too, researchers have used Hinf I digestion sites further apart from the C677T polymorphic site as the restriction digestion control (399). Therefore, we could have followed the same procedure to identify undigested fragments from the wild type homozygous state. But one of the aims of this study was to find out a restriction digestion control which has the potential to be used with samples of DNA from organisms whose DNA sequences were not known and whose RFLP patterns would help in the identification of species or diseases, especially as DNA sequencing is not freely available in our country.

The advantage of using bacteriophage λ as a restriction digestion control was that it could undergo PCR at the same conditions as that of the MTHFR C677T containing DNA segment. Furthermore bacteriophage λ DNA has multiple restriction sites for different restriction enzymes (400). Thus it may be used as an internal control in other RFLP assays too. This is especially true as bacteriophage λ PCR amplification is relatively easy under different PCR conditions as observed by many researchers who have performed these analyses.
Thus this new method developed by us utilizing bacteriophage \( \lambda \) as a restriction digestion control in the MTHFR C677T gene mutation analysis by RFLP agarose gel electrophoresis has the potential to be used as an internal control in MTHFR gene analysis as well as in RFLP analysis of DNA segments where the sequence is not known. Further studies using bacteriophage \( \lambda \) as a restriction digestion control would give a definite insight to its potential use in RFLP.

5.5.2 Prevalence of MTHFR C677T polymorphism in the study sample

The prevalence of T allele frequency of MTHFR C677T gene in this study is 17.72% which was higher than that reported for a Sri Lankan populations previously (399, 401, 402). Thus the allele frequency in this study population was not in agreement with previous studies. Two of the previous studies failed to find MTHFR 677TT homozygotes in Sri Lankans (401, 402) while in the other only two 677TT homozygotes were found (399). In the present study, four 677TT homozygotes were identified comprising three males and one female.

The MTHFR 677T allele frequencies in different communities vary according to research reports from around the world. For example, the allele frequency in Europeans is 24-40% (403), 26-37% in Japanese populations (404, 405) and 11% in African Americans. In neighbouring India, the allele frequency for 677T has been reported to be between 10-11% (406, 407). Thus findings of this study give a higher 677T allele frequency than that observed previously in Sri Lanka and India. The reason for the higher frequency observed merits further investigation as 677T allele is the allele that is associated most with hyperhomocysteinaemia.
5.5.3 Prevalence of MTHFR A1298C polymorphism in the study sample

This study is the first to report on the MTHFR A1298C polymorphism in Sri Lanka. The prevalence of the C allele in this study was 43%. Prevalence of MTHFR 1298C allele outside the Indian subcontinent varies among different populations with a prevalence of 35% in neonates in Canada (408), 33% among healthy individuals in the Netherlands (109), 33% among healthy individuals in Italy (409), 28% among an elderly population in the United Kingdom and 31.7% among individuals with vascular disease from the United States (410). All these studies indicated that the average prevalence of 1298C allele of MTHFR gene was approximately 30-35%. The highest prevalence of the MTHFR 1298C allele outside the Indian subcontinent was reported as 49% among healthy Lebanese subjects (411) whilst the lowest of 16% was reported in Japan (411).

In India, the allelic frequencies for the MTHFR 1298C allele has varied from a high prevalence rate of 43% in New Delhi (412) to a lowest prevalence rate of 10% in Chandigarh (413). A study in Tamil Nadu, India reported that the 1298C allele frequency among patients with MI and healthy controls were 40.4% and 35% (104). Thus the prevalence of the MTHFR 1298C allele frequency in this study appears to be very much similar to the prevalence rates reported in India.

Unfortunately, there are no data available in Sri Lanka regarding the prevalence of the MTHFR 1298C allele and its association with increased levels of homocysteine compared to the 1298A allele. Further population studies need to be carried out to investigate its prevalence in Sri Lanka which will enable us to come to a conclusion regarding its possible association with homocysteine in Sri Lankan subjects.
Double homozygosity for the mutant MTHFR 677 and 1298 polymorphism was not observed among the study population of this study. This was in accordance with the findings of the rest of the world (111, 414) as the double-homozygosity for these mutations is very rare or non existent as we were not able to peruse any reports of occurrence of this mutant double homozygosity for the MTHFR 1298 and 677 polymorphisms.

5.5.4 Association of MTHFR C677T gene mutations (A1298C, C677T polymorphisms) with homocysteine concentrations in CAD patients in the study sample.

This study is the first to report on the association of MTHFR C677T gene mutation (A1298C, C677T polymorphisms) with hyperhomocysteinaemia in CAD patients of Sri Lanka.

In this study the highest mean homocysteine concentration was observed among subjects with the MTHFR 677TT genotype although this value was not statistically significant when compared with other groups. Similarly subjects with the MTHFR 677TT genotype also had the lowest folate and vitamin B₁₂ concentrations amongst the study population.

The missense mutation at position 677 of the MTHFR cDNA has been found to produce a thermolabile form of the enzyme resulting in increased plasma homocysteine (415). Folate is an important cofactor in the conversion of homocysteine to methionine. It is known that 677TT homozygosity results in the need for a higher folate intake to obtain normal homocysteine levels. It has also been reported that homozygosity for 677T when associated with a decreased intake of or an increased requirement for folate can lead to vascular disorders especially as the individual ages (402, 416).
The effect of 677T allele on homocysteine concentration has been found to be more pronounced in homozygous TT subjects with low folate concentrations (378), and folic acid supplementation has been reported to cause a marked decrease in plasma homocysteine in TT subjects who initially had the same homocysteine concentrations as subjects with the CC genotype (417, 418).

Thus the findings in this study that subjects with the homozygous mutant allele of the MTHFR 677 polymorphism (subjects with MTHFR 677TT) had the lowest concentration of the two vitamins and the highest concentration of homocysteine suggest that these subjects, who actually need more folate to overcome the effects of the mutant thermolabile form of the MTHFR gene, are having below normal folate levels. This in turn can lead to a much higher level of homocysteine in their plasma with more pronounced vascular disease. A lower concentration of vitamin B₁₂ was observed in these subjects compared to the other two genotypes, although these levels are above the lower limit of normal of 200 pg/ml (309). This may also be of importance as decreased vitamin B₁₂ concentrations can lead to the folate trap, thus aggravating the already depleted folate deficiency leading to a much higher plasma homocysteine level.

In this study the highest mean concentrations of homocysteine and folate was observed among subjects with the 1298AC genotype although this value was not statistically significant when compared with other groups. Furthermore, highest vitamin B₁₂ levels were observed among subjects with MTHFR 1298CC genotype.

A significant decrease in the MTHFR enzyme activity has been observed in the homozygous 1298CC as well as in the heterozygous 1298AC state of the MTHFR gene (109), with the highest reduction of enzyme activity being observed in subjects with 1298CC genotype. The result of this decrease in MTHFR enzyme activity should be an
increase in plasma homocysteine concentrations in subjects having these two genotypes. But many population based studies have given conflicting results regarding the association between these two genotypes and plasma homocysteine levels. Different studies have reported highest homocysteine concentrations with different 1298 genotypes. Studies have reported high homocysteine concentrations with 1298CC genotype (74, 109, 419-421), 1298AC genotype (410) and 1298AA wild type (422). Thus there is controversy regarding the association of plasma homocysteine levels with the different genotypes of the MTHFR A1298C polymorphism. The finding of the present study was also unable to explain the above controversy regarding the association of homocysteine levels and the A1298C polymorphism.

Results of this study also do not make this clearer other than to adding more fuel to the fire of this ongoing controversy.

Subjects with the combined 1298AA/67TT are reported to have significantly elevated homocysteine levels (109, 421). This study also showed that subjects with 1298AA/67TT have the highest plasma concentrations although this was not statistically significant (p>0.05) when compared to 1298AA/677CT combination. According to previous studies it has been reported that there is an increase in homocysteine levels in subjects who are heterozygous for both MTHFR mutations, the 1298AC/677CT genotype, when compared with subjects having the 1298AA/677CC genotype (109, 410, 422). The homocysteine concentrations of these subjects with 1298AC/677CT genotype have been reported to be lower only to those having the 1298AA/677TT genotype and the 1298AC/677TT genotype. This increase in homocysteine concentration with the 1298AC/677CT genotype was not observed in this study population with even subjects who were having the 1298AC/677CC and
1298CC/677CC genotypes showing higher mean plasma homocysteine levels. Although findings of this study show that there is no significant association between homocysteine levels and the combined MTHFR 1298 and 677 genotypes, it is insufficient to draw a definite conclusion regarding its validity to be applied to the general population due to the smaller sample size in each of the combinations.

Hence the results of this study suggest that MTHFR gene polymorphisms are not associated with hyperhomocysteinaemia.

5.6 Association between, vitamin B₁₂, Folate, and Homocysteine levels with the severity of coronary artery disease.

The severity of myocardial ischaemia in this study was calculated by using the vessel, the stenosis and the extent scores as described previously. The vessel score indicates the number of vessels involved with significant stenosis (70% or greater reduction in lumen diameter). Stenosis score places emphasis on the severity of stenosis while including some measure of the extent of coronary artery disease whilst the extent indicates the proportion of the coronary arterial tree involved by angiographically detectable atheroma.

The mean vessel score obtained in this study population is 2.04 which indicated that most of the study population had involvement of the three main vessels. Thus the conclusion we could arrive from the vessel score obtained is that most of the study population were having severe CAD and this was evident as all were undergoing coronary artery bypass grafting. But dependence on the vessel score alone in the assessment of the severity of CAD would lead to problems as four subjects in this study who were awaiting coronary artery bypass grafting had a vessel score of zero. The
vessel score places emphasis only on the number of vessels with significant stenosis (stenosis of 70% or greater in the left anterior descending, left circumflex, and right coronary arteries). The four patients who had a vessel score of zero had stenosis scores in the range of 7-11 out of a theoretical maximum of 32, thus highlighting the inadequacy of the vessel score alone as a tool to assess the extent of coronary stenosis.

Mean value obtained for the stenosis score was 9.86 out of a theoretical maximum of 32 in this study indicating that the severity of ischaemia is relatively high in the study population as according to the stenosis score close to one third of the musculature of the heart is affected by stenosis. Stenosis score obtained from different studies involving subjects with MI have varied and on average severity of ischaemia assessed by the stenosis score in this study is similar to that reported by others (423-425).

Extent score as described by Sullivan et al (1990) gives an indication of the extent of coronary muscular affected by ischaemia and in this study the mean score was 46.42 indicating that almost 50% the coronary musculature has been involved with stenosis in most of the CAD patients. The extent scores in MI have varied and on average severity of ischaemia assessed by the extent score in this study is similar to that reported by others.

The vessel, stenosis and extent scores have been validated and used extensively in previous studies as a tool in the assessment of myocardial ischaemia (423-427). Although these scores give different values depending on the severity of ischaemia in the population studied, the values obtained in this study as described above are in accordance with values reported in other studies. Out of these the stenosis score and the extent score have more power as tools in assessing the severity and extent of coronary
ischaemia than the vessel score (297). Thus, depending on the above, the use of these three scores as a tool in assessing severity of myocardial ischaemia in this study could be validated.

Serum homocysteine and serum folate concentrations were not significantly related to the severity of coronary artery disease as assessed by the vessel, stenosis and extent scores in this study. Although hyperhomocysteinaemia is now increasingly recognized as an independent risk factor for coronary artery disease, evidence to its association with the severity of ischaemia has not been proven conclusively. Several studies have found a significant association between severity and extent of coronary ischaemia as assessed by the above three scores and homocysteine concentrations. Chao et al. (1999) showed a significant correlation between plasma homocysteine concentrations and the severity and extent of coronary artery disease (423). Some other studies also have shown a significant association between homocysteine concentrations and severity and extent of coronary heart disease (428-430). Depending on these reports, homocysteine has been proposed as a marker of severity and extent of coronary artery disease without symptoms of CAD (429). These reports are contradicted by findings in some other studies where they have not found an association between homocysteine concentrations and severity of ischaemia (431, 432).

Findings from this study also indicates that there is no correlation between homocysteine and severity and extent of coronary heart disease. Thus its possible use as a predictor of severity of CAD cannot be proposed depending on the results of this study population.

Serum folate has been implicated in the development of CAD by increasing homocysteine levels as well as by independent means. Voutilainen et al. (2004) have
reported that in a Finnish population, CAD risk was not associated with homocysteine levels and in the same subjects moderate-to-high serum folate concentrations were associated with a greatly reduced incidence of acute coronary events (433). Furthermore, they have also reported that low serum folate concentrations are associated with an excess incidence of acute coronary events (434). These findings were further confirmed by Ford. et al. (1998) who have also highlighted that low levels of serum folate may be associated with mortality from all causes and cardiovascular disease (435).

According to the literature, no work has been reported on the association of folic acid with severity of ischaemia. However findings of this study suggested that the folate concentrations did not show any significant correlation with the parameter used in this study to assess the severity of ischaemia such as the vessel, stenosis and extent scores.

In this study serum vitamin B₁₂ concentrations showed a significant negative correlation with both the vessel score (p <0.05) and the extent score (p < 0.01). This was in conjunction with the fact that homocysteine did not show a significant correlation with these two scores of severity of ischaemia in this study. No previous studies have reported whether there would be a direct association of vitamin B₁₂ with CAD. Most of the records of vitamin B₁₂'s association with CAD have been through its association with hyperhomocysteinaemia, where vitamin B₁₂ deficiency is one cause which can lead to hyperhomocysteinaemia. Thus findings of this study are quite surprising as other related work on the topic had not found an association of vitamin B₁₂ with severity of ischaemia independent of homocysteine. Thus further research on this association and a
possible molecular mechanism by which such an association could lead to CAD need to be investigated.

5.7 Association of Apolipoprotein A-I, Apolipoprotein B and Glutathione peroxidase with severity of coronary artery disease.

Present findings show that Apolipoprotein A-I (inversely) and the Apolipoprotein B/A-I ratio correlate significantly with the stenosis and extent scores but not with the vessel score. Apolipoprotein B correlated significantly only with the vessel score.

The vessel score as described before is a primitive way of assessing the severity and extent of ischaemia compared to the stenosis and extent scores. Thus its association with a biochemical marker may not be that significant clinically in predicting the severity of ischaemia. Although apolipoprotein B levels in this study correlated significantly with the vessel score it did not show a significant correlation with the other two major scoring systems which gives a more meaningful indication about the severity and extent of coronary ischaemia. However, both apolipoprotein A-I and the Apolipoprotein B/A-I ratio showed significant correlations with the stenosis and extent scores.

Studies performed recently have shown that measurement of different forms of apolipoprotein may improve cardiovascular risk prediction. Among these novel risk factors apolipoprotein B, apolipoprotein A-I, and the apolipoprotein B/A-I ratio have been reported to be better predictors of cardiovascular events than non HDL-cholesterol and HDL-cholesterol, even in subjects taking lipid-modifying therapy (252). Apolipoproteins regulate the synthesis and metabolism of lipoprotein particles in the
body and also stabilizes the lipoprotein structure. As discussed previously, the total value of apolipoprotein B indicates the number of potentially atherogenic lipoproteins. Apolipoprotein A-I is important in removing excess cholesterol from tissues and incorporating it into HDL for reverse transport to the liver (436). Thus the apolipoprotein B/A-I ratio reflects the balance of cholesterol transport. Therefore higher the apolipoprotein B/A-I value, higher the chances of cholesterol deposition, and consequently higher the risk for atherogenesis (252).

Out of these novel risk predictors, apolipoprotein B/A-I ratio has grown worldwide acceptance as one of the most important risk predictors. Recent reports from prospective risk studies, such as AMORIS (437), INTERHEART (17), EPIC-Norfolk study (438), ULSAM (439), and the MONICA/KORA (440) studies have indicated that the apolipoprotein B/A-I ratio is a useful predictor of risk of both non-fatal and fatal myocardial infarction. A recent meta-analysis on the apolipoprotein B/A-I ratio also supports the use of apolipoprotein B/A-I ratio as a future risk marker of cardiovascular disease (441).

Apolipoprotein A-I is now been recognized as an independent risk factor for CAD even in patients who are considered to be at low risk (215) and studies indicate that high apolipoprotein A-I concentrations are associated with lower mortality and myocardial infarction after coronary artery bypass grafting (214).

Apolipoprotein B is also recognized as a risk predictor for myocardial infarction (248, 440, 442). But in this study we could not find a significant correlation with the severity of myocardial ischaemia.
The available knowledge has already led to an update of guidelines in certain countries such as Canada (443). Reference ranges and target values have already been proposed for the different lipoprotein parameters (234).

Findings from this study also support the notion that apolipoprotein A-I and apolipoprotein B/A-I ratio are important as risk predictors of myocardial ischaemia in our country. This is especially true as at the time of blood collection all subjects have been on lipid lowering therapy for different periods of time, and even in them, the association between the apolipoprotein A-I and the apolipoprotein B/A-I ratio with the severity of ischaemia was significant.

This study expands this observation further in that the apolipoprotein concentrations have the possibility to predict severity of myocardial ischaemia. Studies reported on apolipoprotein concentrations as predictors of severity of ischaemia are rare and the limited number of such studies are in agreement that lipid parameters could be used in the prediction of the severity of myocardial ischaemia (299, 444, 445). This association has to be analyzed fully to see whether this could apply to the general population as there is always the risk that extensive lipid lowering therapy could get the apolipoprotein levels to well below normal levels. Thus in persons on lipid lowering therapy, the value of using apolipoprotein parameters may be of limited use as it will not correlate with the ischaemic burden which has already set in. Thus according to the above findings, the use of apolipoproteins in the prediction of severity of myocardial ischaemia may have to be restricted to persons who are not on lipid lowering therapy. But findings of this study indicate that apolipoprotein levels correlate significantly with severity of CAD even when they are on lipid lowering therapy. The available knowledge regarding this at the moment is limited and further studies on the topic may
have to be done before we could apply apolipoproteins as predictors of severity of coronary ischaemia.

But based upon the current data available it is now time to update guidelines to include apolipoprotein A-I, Apolipoprotein B/A-I ratio, and apolipoprotein B, although findings of this study do not support its use, as primary risk variables for development of CAD at least along with the currently used LDL-Cholesterol and HDL-Cholesterol.

Thus we propose that apolipoprotein A-I, apolipoprotein B values indicating level of risk should be further investigated. Considering the existing conventional risk factors and the prevalence and incidence of CAD in Sri Lankans, the apolipoprotein B/A-I ratio could be a simple, robust, more sensitive risk indicator of great value in health screening even in persons on lipid-lowering therapy.

The present study further indicated a significant inverse correlation (p<0.001) between glutathione peroxidase and the vessel, the stenosis and the extent scores.

Reactive oxygen species (ROS), including peroxidized lipids capable of initiating cellular injury or cellular dysfunction, may be generated within endothelial cells, be present in plasma components or be derived from polymorphonuclear granulocytes or other blood borne cells. These are involved in the oxidative modification of lipids, an important element in the process of atherogenesis. This is achieved by these peroxidized lipids directly damaging endothelial cells, by enhancing the adhesion and activation of polymorphonuclear granulocytes and by enhancing the susceptibility of platelets to aggregate. Endothelial cell injury by these lipid hydroperoxides also increases the uptake of LDL into the vessel wall. These factors alone or in combination aid in sustaining and development of atherosclerosis. This process is kept in check by the primary ROS scavengers in the body, the antioxidant
enzymes in the plasma and the red blood cells (76). These include antioxidant enzymes such as superoxide dismutase, glutathione peroxidase, glutathione reductase and catalase. Two of these antioxidant enzymes found in the cells, namely catalase and superoxide dismutase do not play an important role in human vascular cells. It has been reported that catalase activity is lacking in human vascular cells (281) and superoxide dismutase has been found to be poorly effective in safeguarding the cells in the human vasculature from damage caused by oxidants (255, 282). Thus one of the main antioxidant enzymes that is responsible for protecting the vasculature from ROS by its scavenging activity is glutathione peroxidase. Thus a decrease GPx levels may lead to an increase in the atherosclerotic burden and consequent increase in cardiovascular events.

Recently, baseline levels of the erythrocyte GPx-1 activity have been shown to be inversely related to future cardiovascular risk in patients with coronary artery disease (446-448). Studies have also found that GPx-1 is associated with increase in the extent of atherosclerosis (446) as well as the severity of the disease (448). In these studies where the association between severity and GPx was analysed, the severity has been assessed primarily by looking at the number of vessels involved. Thus this study is the first to assess the severity of CAD in detail with regards to its association with GPx. According to findings of this study GPx has the ability to predict the severity of ischaemia along the full spectrum of parameters used in this study as the vessel, the stenosis and the extent scores all showed very significant correlations (p<0.001) with the GPx concentrations. Thus GPx has the potential to be a very sensitive cardiac risk predictor, especially regarding its severity. Only a few studies have been conducted in
GPx as a predictor of myocardial ischaemia. Thus more studies are required to draw a conclusion regarding its use in clinical practice.

5.8 Influence of age on the association of apolipoprotein A-I, apolipoprotein B and glutathione peroxidase concentrations in coronary artery disease patients.

Apolipoprotein A-I concentration in this study showed a significant positive correlation with age. This study did not find a significant correlation between age with apolipoprotein B, apolipoprotein B/A-I ratio.

Previous studies have shown a significant effect of age on total and LDL cholesterol levels with levels increasing with increasing age. This has been observed in both cross sectional (449-451) as well as in prospective studies (452-455). However most of these studies have focused mainly on subjects below 70 years of age. In subjects over the age of 70 years, studies have reported that total and LDL cholesterol levels decrease with age. Cross sectional studies have reported that total and LDL cholesterol levels decrease with age (456-461) whilst the HDL cholesterol concentrations have not been found to vary with age (456-460). However majority of prospective studies have reported that HDL cholesterol concentrations decrease with increasing age (452, 454, 455, 460).

Studies on age related variations in serum apolipoprotein A-I and apolipoprotein B levels are, few and are only confined to European, North American, and few Australian and African populations (241, 462). These studies have also found that apolipoprotein B concentrations increase with age throughout life (451, 463-466). Thus the use of apolipoprotein B instead of LDL cholesterol, which has been found to increase up to the age of 79 years and then decline, has been proposed as a better risk
marker for CAD than LDL cholesterol. However, findings of this study were not in agreement with these reports as the present study did not find a significant association between increasing age and apolipoprotein B levels.

It has been reported that Apolipoprotein A-I concentrations increase with age in some studies (241) while some studies have failed to find such an association (464, 467). Findings of this study are in agreement with previous studies showing an increase in apolipoprotein levels with increasing age. Unfortunately studies reporting on age related variations in serum apolipoprotein A-I levels are few and these studies have also shown different results. Therefore no conclusions have been made regarding the association of apolipoprotein A-I with age. Hence the findings of the present study i.e. apolipoprotein A-I concentrations increase with increasing age may be of importance and also adds a new finding to the existing literature.

Studies conducted on mice have found an increase in red cell GPx levels with increasing age (468-471). Studies regarding GPx levels and its association with age in humans are few and these studies have reported an increase in the GPx concentrations with increasing age (269). Glutathione peroxidase concentrations in this study sample did not show a significant positive correlation with age. Thus findings of this study are not in agreement with the few studies reported before.

It is hypothesized that there is an increase in exposure to oxidants with age and this acts as a signal to increase the activity and expression of antioxidant enzymes as this adaptation will help to protect tissues from oxidative stress. If this hypothesis is true, there should be a decrease in oxidation of lipids which is one of the primary causes behind formation of atheromatous plaques in the vasculature.

187
But studies have found that GPx activity is often absent in atherosclerotic plaques (273-276) probably due to inactivation by oxidant species (277, 278) especially by oxidants like hypochlorous acid modified proteins and myeloperoxidase found in human atherosclerotic lesions (279). Another mechanism for the decrease in GPx activity in vascular disease has been attributed to products of lipid peroxidation in vascular wall inactivating GPx (266-268, 277, 278, 280). It has been reported that specific antioxidant enzyme inactivation, especially involving GPx, occurs in atherosclerotic lesions which have to be considered as a pro-oxidant environment with lipoperoxide burden in vascular tissue. Thus the increase in GPx levels in red cells with age may not be sufficient to counteract the oxidant environment in vascular tissue.

Further studies may be of value in evaluating the possible advantage of increasing selenium intake as a way of further increasing the GPx levels in the body to counteract the actions of pro-oxidants in the vasculature.
Findings of this study indicates that,

1. Hyperhomocysteinaemia is an independent risk factor for Coronary artery disease (CAD) and subjects with hyperhomocysteinaemia have two to three fold increase in risk of developing CAD than those with normal homocysteine concentrations.

2. Hyperhomocysteinaemia is a significant predictor of CAD in the young but not in the elderly and among persons below 50 years, persons with hyperhomocysteinaemia were 4.5 times more likely to develop CAD as compared to those with normal concentrations of plasma homocysteine. (This is a new contribution to the literature from this study)

3. Homocysteine concentrations did not increase with age in the study sample in comparison to the studies previously reported in the literature.

4. An increase in the number of conventional risk factors does not have an influence on the homocysteine concentrations.

5. A decrease in either vitamin B_{12} or folate concentrations in serum is associated with higher homocysteine concentrations.

6. Methylenetetrahydrofolate reductase (MTHFR) A1298C and C677T gene mutations do not have an effect on the homocysteine concentrations among patients with CAD.

7. Serum homocysteine and folate concentrations are not associated with the severity of coronary artery disease as assessed by the vessel, stenosis and extent scores.
Additional contribution to knowledge

1. A modified HPLC method for routine laboratory measurement of total homocysteine was developed for future use.

2. Use of bacteriophage λ DNA as an internal control in human genetic analysis by Restriction Fragment Length Polymorphism (RFLP) which has the potential to be used in other RFLP analyses.

Limitations/shortcomings of the study

1. It would have been more informative if we could have compared Apolipoprotein A-I, Apolipoprotein B and the Apolipoprotein B/A-I ratio with Lipoprotein(a) and the conventional lipid parameters like HDL-cholesterol, LDL-cholesterol and the total cholesterol/HDL ratio in order to analyze whether the conventional or the novel lipid parameters are better at predicting the severity of CAD.

2. In the analysis of gene polymorphisms and its association with homocysteine levels, comparison with a control group would have been more meaningful although this was not included in the objectives and initial study design due to cost and time factor.

3. The findings regarding the frequency of MTHFR polymorphisms, especially the frequency of A1298C polymorphism which has not been studied/reported in Sri Lanka before, cannot be compared.

4. Inclusion of superoxide dismutase (SOD) and catalase levels with GPx would have provided more information on the total anti-oxidant capacity in the vasculature in these CAD patients although the SOD and catalase have been reported to show less anti-oxidant defense capacity in the vasculature.
Proposals for future studies

2. Studies to assess the influence of age on homocysteine levels in healthy individuals of Sri Lanka.
3. Population studies to assess the prevalence of MTHFR gene polymorphisms and its effects on homocysteine levels to confirm/refute its association with homocysteine in Sri Lankans.
4. Studies to confirm, and to postulate mechanisms of the influence of vitamin B\textsubscript{12} on the severity of CAD.
5. Population studies to investigate the prevalence of folate (± vitamin B\textsubscript{12}) deficiency in Sri Lankans.
6. Use of bacteriophage \lambda fragments with appropriate restriction sites as an internal control in different RFLP analyses, especially when dealing with unknown DNA sequences.
7. REFERENCES


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227


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APPENDIX 1

List of Publications and Communications from thesis

The following publications and scientific communications arose from this doctoral thesis.


**APPENDIX 2**

Questionnaire used in study 1

**Study on Hyperhomocysteinaemia and CAD**

<table>
<thead>
<tr>
<th>Name of patient</th>
<th>BHT No:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age in years:</td>
<td></td>
</tr>
<tr>
<td>Date of admission:</td>
<td></td>
</tr>
<tr>
<td>Time of Admission</td>
<td></td>
</tr>
<tr>
<td>Profession:</td>
<td></td>
</tr>
</tbody>
</table>

**Smoking**

<table>
<thead>
<tr>
<th>Current smoker*</th>
<th>Yes **</th>
</tr>
</thead>
<tbody>
<tr>
<td>Former/non-smoker#</td>
<td>No</td>
</tr>
</tbody>
</table>

* Ones who have smoked any tobacco in the previous 12 months including ones who have quit within past year.
# Individuals who had not smoked any tobacco at any stage and those who had quit more than a year earlier.
** Consumption of alcohol three or more times per week.

**Past History of:**

<table>
<thead>
<tr>
<th>Hypertension#</th>
<th>Yes</th>
<th>No</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diabetes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>High Cholesterol*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chronic Renal Failure</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cerebro Vasclar Disease</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Deep Vein Thrombosis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peripheral Vascular Disease</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Parental History of MI before 45 years</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Self reported High-Cholesterol, Cholesterol level $\geq 240$ mg/dl, or taking Lipid-lowering medications.
# Self reported systolic blood $\geq 40$ mmHg, diastolic blood pressure $\geq 90$ mmHg, or taking antihypertensive medication.
Regular physical exercise:  
Yes | No
---|---

Vitamin Supplementation:  
Yes | No
---|---

1 Regularly involved in walking, cycling, gardening, jogging, football, vigorous swimming or exercising for 4 hours or more a week.

2 On multi vitamins or B-complex for more than 3 days per week.

Vegetarian | Non-vegetarian
---|---

Presenting Complain
Height (Meters) | Weight (Kg)
---|---

Blood Pressure (in ward)

Investigation Results
ECG
CPK
Troponin

Diagnosis

Comments: ........................................................................................................
........................................................................................................

Sample collected on  
(Date) at  am/pm

Signature

262
APPENDIX 3
Questionnaire/Data sheet used in study 2

Novel Coronary Risk Factors and CAD

<table>
<thead>
<tr>
<th></th>
<th>Yes</th>
<th>No</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypertension #</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>High Cholesterol*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>History of MI (Parents/Siblings)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

# Self reported systolic blood pressure ≥ 140 mmHg, diastolic blood pressure ≥ 90 mmHg, or taking antihypertensive medication.

* Self reported High-Cholesterol, Cholesterol level ≥ 240 mg/dl, or taking Lipid-lowering medications.

Patient Characteristics:

<table>
<thead>
<tr>
<th>Smoking</th>
<th>Current smoker *</th>
<th>Former/Non smoker #</th>
</tr>
</thead>
</table>

* Ones who have smoked any tobacco in the previous 12 months including ones who have quit within past year.

# Individuals who had not smoked any tobacco at any stage and those who had quit more than a year earlier.
### Coronary Angiography Findings:

<table>
<thead>
<tr>
<th>Vessel</th>
<th>% Narrowing</th>
<th>Proportion involved with atheroma (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Main Left Coronary Artery (MLCA)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Left Anterior Descending Artery</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Main Diagonal Branch</td>
<td></td>
<td></td>
</tr>
<tr>
<td>First Septal Perforator</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Left Circumflex Artery</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Obtuse marginal &amp; Posterolateral Vessels</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Right Coronary Artery</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Main Posterior Descending Branch</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Is the main lateral wall branch a large obtuse marginal or intermediate vessel?

- [ ] Yes
- [ ] No

### Laboratory Values

- Serum Creatinine: 
- Blood Urea: 

### Patient Information

- Height (Meters): 
- Weight (Kg): 

### Notes

1. Regularly involved in walking, cycling, gardening, jogging, football, vigorous swimming or exercising for 4 hours or more a week.
2. On multi vitamins or B-complex for more than 3 days per week.
3. Consumption of alcohol three or more times per week.
APPENDIX 4
Ethical clearance for study 1

FACULTY OF MEDICAL SCIENCES
UNIVERSITY OF SRI JAYEWARDENEPURA, SRI LA

My Hers
Yours Tres

14th January, 2003

Dr. R P Rasika Perera
Department of Bio Chemistry
Faculty of Medical Sciences

Dear Dr. Perera

Application No: A 142

Hyperhomocysteinaemia & Ischaemic Heart Disease (IHD) in young in young Sri Lankan IHD patients

I am pleased to inform you that ethical clearance was granted for your proposal at the ethical review committee meeting held on 11th December 2002.

Dr. S.D Jayaratne
Chairman: Ethical Committee

Dr. Renu Wickrematunge
Secretary: Ethical Committee
APPENDIX 5

Ethical clearance for study 2 (excluding genetic analysis)

Ethical Review Committee
Faculty of Medical Sciences,
University of Sri Jayewardenpura,
Gangodawila, Nugegoda, Sri Lanka
14th September 2005

Dr. Rasika Perera
Lecturer
Department of Biochemistry
Faculty of Medical Sciences

Dear Dr. Perera

Application/Approval No A 258/05

Novel coronary risk factors/outcome predictors of ischemic heart disease in a Sri Lankan population

We are pleased to inform you that ethical clearance was granted for your proposal at the ethical review committee meeting held on September 14, 2005.

Dr. S.D. Jayaratne
Chairman/Ethical Review Committee

Dr. Renu Wickremasinghe
Secretary/Ethical Review Committee
APPENDIX 6

Ethical clearance for the genetic analysis in study 2

Ethics Committee
Faculty of Medical Sciences
University of Sri Jayewardenepura
Gangodawila, Nugegoda,
Sri Lanka

Chairman
Dr. S D Jayaratne

Secretary
Dr. B C V Senaratna

Committee Members
A De Alwis
Prof. S Deraniyagala
Dr. S S N Fernando
Mr. M A Gunawardene
Dr. S D Kamaladana
Prof. S T Kathiriarchchi
Prof. H Peiris
Prof. M S A Perera
Dr. C A Wansatunge
Dr. D R Wickremasinghe
Dr. I Wijesiriwardene
S G Yasowardene

21st August 2007

Dr. PPR Perera
Department of Biochemistry,
University of Sri Jayewardenepura.

Dear Dr. Perera,

Application / Approval No: 357 / 7

METHYLENETETRAHYDROFOLATE REDUCTASE (MTHFR) GENE C677T AND A12986 POLYMORPHISM AND ISCHEMIC HEART DISEASE

I am pleased to inform you that provisional ethical clearance was granted for your proposal at the Ethical Review Committee meeting held on 15th August 2007. Definitive clearance will be given once the study is completed and a report submitted to the Ethical Review Committee.

Dr. S D Jayaratne
Chairman

Dr. B C V Senaratna
Secretary

267
Research on Homocysteine and Ischaemic Heart Disease

- Homocysteine is chemically an amino acid.
- Studies conducted in other countries have found that there is an association between homocysteine and “Heart attacks” (Acute coronary syndromes - Myocardial infarction and Unstable angina)
- When Blood Homocysteine levels go above normal there is an increased risk of getting an Acute Coronary Syndrome (i.e. above)
- Blood Homocysteine levels can go up due to
  1. A genetic disease - e.g. Homocysteinuria (rare)
  2. Vitamin deficiency – especially Vitamin B₆, B₁₂ and Folic acid.
- Of the above, increased blood Homocysteine levels due to B vitamin deficiencies is much more common.

In Sri Lanka, vitamin deficiencies are common. Hence can increased blood Homocysteine levels be a cause of Acute Coronary Syndromes in Sri Lanka?

- The objective of the study is to find out the answer to the above.
- **Importance**: If increased blood Homocysteine levels is a cause of Acute Coronary syndromes in our country, we will be able to decrease the incidence of us getting “Heart Attacks” to some extent by eating foods that contain more B vitamins / vitamin B supplementation.
- This is inexpensive when compared to the money that we have to spend on long term preventive measures / in treating patients with “Heart Attacks”
- More importantly, by above measures, we ourselves can do something to decrease the risk of us getting “Heart Attacks”.

268
APPENDIX 8

Information leaflet used in study 1 (Sinhala)

ඩංගුන් තනතුරු මිළිං දේශපාලනය

❖ මම්මස්ථානය යට ඇතෙකන් බිඳීමට නියෝගය තිබේ.
❖ ඉහළ කෙටි ස්ථාන බහුලව විශේෂව සිට අනුමානය කාටලක් ලබා ගැනීමට නියෝගය තිබේ. ගුහු සිටිය කාටලක් ලබා ගැනීමට නියෝගය තිබේ.
❖ මම්මස්ථානය යට ඇතෙකන් යන අන්තර්ශ්‍ර පිටත් විය හැකිය.

1 වරට කාටලක් සිටියේ අර්ධවාහනය යනු
- කාටලක් (Homocysteinuria) කාටලක් - ඉහළ
  අර්ධවාහනය අදහා අරි බුදු කරන්න.

2 ආකාරයේ අර්ධවාහනය - ආකාරයන්නයට අර්ධවාහනය අංක අර්ධවාහනය (Folic Acid), මැටිව හෝ මොල් කරන්න
❖ ආකාරයේ අර්ධවාහනය අදහා ආකාරයන්නයට බිඳීමට සිටිය යන අදහාවාහනය නාලිකාවන්තර?

- ඉහළ අර්ධවාහනය අදහා ආකාරයන්නයට පිළිබඳ විද්වාත්වීමකට සිටියේ.
- ආකාරයන්නය - ඉහළ අර්ධවාහනය අදහා ආකාරයන්නයට පිළිබඳ විද්වාත්වීමකට සිටියේ ආකාරයන්නයට පිළිබඳ විද්වාත්වීමකට සිටියේ. ආකාරයන්නයට අදහා ආකාරයන්නයට පිළිබඳ විද්වාත්වීමකට සිටියේ.
- ආකාරයන්නය අදහා ආකාරයන්නයට පිළිබඳ විද්වාත්වීමකට සිටියේ ආකාරයන්නයට පිළිබඳ විද්වාත්වීමකට සිටියේ. ආකාරයන්නයට පිළිබඳ විද්වාත්වීමකට සිටියේ.

269
APPENDIX 9
Research consent form used in study 1- (English)

Research Consent Form

Project: Hyperhomocysteinaemia and Ischaemic Heart Disease in young Sri Lankan IHD patients.

Name of Investigators: Prof. H. Peiris, Dr. J. Indrakumar, Dr. P. P. Rasika Perera

I……………………………………………………………………………………… volunteer to donate 2 ml of venous blood to be used for research purposes.

The blood will be used to study Plasma Homocysteine.

The potentials risks of donating blood, none of which are likely to occur, include pain, bruising, fainting or an infection at the puncture site. There will be no direct benefit to me. I understand that my participation in this study will be confidential. It is also my understanding that I can withdraw from the study whenever I wish without giving any reason and that refusal to participate will not affect my treatment / care in any way.

........................................................ ........................................
Signature of subject Date

Full Address: .................................................................
.................................................................
.................................................................

I have been present while the above has gone through the information and I have witnessed his/her consent to take part.

........................................................ ........................................
Witness Signature Date

Full Name: .................................................................

Full address: .................................................................
APPENDIX 10

Research consent form used in study 1- (Sinhala)

Homocysteine and Ischaemic Heart Disease in Sri lankan IHD patients.

[Consent form text in Sinhala]

....................

[Signature]

[Date]

[Name]

[Position]
Research Consent Form

Project: Novel Risk factors of Ischaemic Heart Disease in a Sri Lankan population.

Name of Investigators: Prof. Lal Chandrasena, Prof. Hemantha Peiris, Dr. H.D. Waikar,
Dr. J. Indrakumar Dr. P. P. Rasika Perera

I volunteer to donate 6 ml (maximum) of venous blood to be used for research purposes.

The blood will be used to study Homocysteine, Folate Acid, Vitamin B₁₂, Apolipoprotein B, Cystatin C and Glutathione Peroxidase.

The potentials risks of donating blood, none of which are likely to occur, include pain, bruising, fainting or an infection at the puncture site. There will be no direct benefit to me. I understand that my participation in this study will be confidential. It is also my understanding that I can withdraw from the study whenever I wish without giving any reason and that refusal to participate will not affect my treatment / care in any way.

Signature of subject .......................................................... Date ____________________________

Full Address: ........................................................................................................................................

I have been present while the above has gone through the information and I have witnessed his/her consent to take part.

Witness’ Signature .......................................................... Date ____________________________

Full Name: ........................................................................................................................................

Full address: ........................................................................................................................................