ANTECEDENTS, CHARACTERISATION AND VALIDITY OF CARDIOVASCULAR DISEASE BIOMARKERS AMONGST SOUTH ASIANS IN THE UK

BY

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ABSTRACT

The increased risk of cardiovascular disease (CVD) amongst South Asians (SAs) is unclear. This thesis examined potential biomarkers to address this. Cross-sectional data on SAs from community (n=1304) and hospital (n=148) populations was collected. Biomarkers were analysed by genotyping, mass spectrometry, automated-immuno-colourimetric-assays, ELISAs, and a new in-house assay for a novel marker, ferritin bound to apolipoprotein B. Diagnostic performance was assessed using receiver operating curves, logistic and linear regression models. C-reactive protein (CRP) was a comprehensive marker of CVD risk, where a range of 1.43-2.30 mg/L maximised sensitivity and specificity. CRP SNP (single nucleotide polymorphism) -390C>T/A contributed minimally to variation in CRP levels. Non-fasting triglycerides discriminated SAs at increased CVD risk, where APOA5 SNP -1131T>*C* was an independent predictor of triglycerides but APOC3 SNP -455T>C and -482C>T were not associated with triglycerides. The performance of IL-6, vWF, D-dimer and P-selectin were poor in comparison to CRP and triglycerides. BNP discriminated SAs with systolic heart failure with a cut off value of 36.4 pmol/l. Of the newly investigated biomarkers, a link between haemoglobin abnormalities and CVD was observed potentially through a mechanism involving iron transportation on lipoproteins. CRP and triglycerides should be considered in the routine CVD risk assessment of SAs.

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Definitions used in the thesis

Risk factor	Defining level	Definition
Central obesity measured as waist circumference Men Women	 > 90 cm > 80 cm	The International Diabetes Federation (IDF)
Body mass index	≥ 27 kg/m^2	WHO (World Health Organisation) criteria
Elevated triglycerides	≥ 1.7 mmol/l	The National Cholesterol Education Program ATP III
Low HDL cholesterol Men Women	 <0.9 mmol/l <1.0 mmol/l	WHO (World Health Organisation) criteria
Hypertension	≥130/≥ 85 mmHg and/or anti-hypertensive therapy	The National Cholesterol Education Program ATP III
Diabetes mellitus	HbA1c ≥ 6.5% and/or anti-diabetic therapy	American Diabetes Association
Total cholesterol	>5.18 mmol/l	The National Cholesterol Education Program ATP III
LDL cholesterol	>4.14 mmol/l	The National Cholesterol Education Program ATP III
Metabolic syndrome	At least three of the following; Central obesity: Waist circumference ≥ 102 cm (men) or ≥ 88 cm (women) Dyslipidaemia*:* Triglycerides ≥ 1.7 mmol/l & HDL-c < 1.0 mmol/L (men) & < 1.2 mmol/L (women) Blood pressure ≥ 130/85 mmHg Fasting plasma glucose ≥ 6.1 mmol/L	The National Cholesterol Education Program ATP III
10-year coronary heart disease risk score	Existing atherosclerotic disease (yes/no), gender, age, systolic&diastolic blood pressure, smoker (yes/no), total cholesterol, HDL cholesterol, diabetic (yes/no)	Derived from Framingham Heart Study

List of abbreviations

ABCA1 - ATP binding cassette protein A1

ACS - Acute coronary syndrome

AHA - American Heart Association

AHA – American Heart Association

ALT - Alanine aminotransferase

ApoAI -Apolipoprotein A1

ApoB – Apolipoprotein B

ApoCIII - Apolipoprotein CIII

ApoE - Apolipoprotein E (apoE)

ARIC - Atherosclerosis Risk in Communities

AST- Aspartate aminotransferase

ATP-III - Adult Treatment Panel III

AUC – area under the curve

BMI – Body mass index

BNP - Brain natriuretic peptide

CAPS - Carotid Atherosclerosis Progression Study

CARDIA - The Coronary Artery Risk Development in Young Adults

CCGC - C reactive Protein Coronary Heart Disease Genetics Collaboration

CDC - Centers for Disease Control

CETP - Cholesteryl transfer protein

CHASE - Child Heart and Health Study in England

CHD – Coronary Heart Disease

CRP – C-reactive protein

CVD - Cardiovascular disease

ECAT - European Concerted Action of Thrombosis and Disabilities

ELISA – Enzyme linked immunoassay

EPIC – European Prospective Investigation into Cancer and Nutrition

ESI-MS - Electrospray ionization mass spectrometry

GGT - γ-glutamyltransferase

GWAS - Genome-wide association scans

HbA1c – Glycated haemoglobin

HDL-c – High density lipoprotein cholesterol

HF – Heart failure

HIV - Human immunodeficiency virus

HL - Hepatic lipase

CRP – High sensitivity C-reactive protein

IHD - Ischemic heart disease

IL-6 – Interleukin-6

IRAS - Insulin Resistance Atherosclerosis Study

KS - Kaposi sarcoma

LCAT - Lecithin cholesterol acyl transferase

LDL-c- Low-density lipoprotein cholesterol

LPL - Lipoprotein lipase

LVEF – Left ventricular ejection fraction

LVSD - Left ventricular systolic dysfunction

MI - Myocardial infarction

MTP - Microsomal triglyceride transfer protein

NIASTAR - Niaspan: Indo-Asian Stroke patient Atherosclerotic Regression

NPHSII - Second Northwick Park Heart Study

OR – Odds ratio

PAD - Peripheral artery disease

ROC - Receiver operating characteristics

RR - Relative risk

sICAM-1 - Soluble intercellular adhesion molecule 1

SLE - Systemic lupus erythematosus

SMR - Standardised mortality ratios

SNP - Single nucleotide polymorphisms

T1DM – Type 1 diabetes mellitus

T2DM - Type 2 diabetes mellitus

vWF –von willbrand factor

CHAPTER 1 INTRODUCTION

1.1 Antecedents of cardiovascular disease biomarkers

Cardiovascular disease (CVD) is a complex disease caused by many lifestyle, environmental and genetic risk factors (Lusis et al., 2004). Epidemiological studies have established that family history is an independent risk factor for CVD (Colditz et al., 1986; Parikh et al., 2007). Further genetically determined atherosclerotic risk factors have been identified (Table 1) (Lusis, 2004). Heritability is the proportion of phenotypic variance attributable to genetic variance. For example, a heritability estimate of 0.25 for a specific risk factor indicates that levels of this factor within a population will differ, and that a quarter of this variability could be attributed to genetic individual differences.

Table 1 Genetically determined risk factors for CVD

Risk factors	Heritability estimates
Risk factors with a significant genetic component	
LDL and VLDL cholesterol	40-60%
HDL-cholesterol	45-75%
Triglycerides	40-80%
Body mass index	25-60%
Systolic blood pressure	50-70%
Diastolic blood pressure	50-65%
Lipoprotein(a)	90%
Homocysteine	45%
Diabetes mellitus	40-80%
Fibrinogen	20-50%
C-reactive protein & Interleukin-6	35-40%

Gene polymorphism can alter proteins affecting their function, activity or expression. The impact of a polymorphism in a disease such as CVD may not manifest until it coexists with other genetic abnormalities or with specific environmental exposures (Hingorani et al., 2000). Dissecting the genetic basis for the intermediate phenotypes such as inflammatory and metabolic markers

involved in the development of atherosclerosis could reveal the underlying pathophysiology for increased CVD risk observed amongst the South Asian population. This section gives a brief overview of the candidate gene approach and the single nucleotide polymorphisms that contribute to the variation in inflammatory and metabolic phenotypes involved in the development of CVD.

1.1.1 Candidate gene approach

The completion of the human genome project in 2003 and the International HapMap project in 2005 have allowed scientists to explore novel biomarkers and pathways associated with disease conditions. The two main approaches in genetic studies are the hypothesis driven candidate gene association studies, whereby the selection of the genetic marker is based on the understanding of the biological processes involved in the development of the disease and the unbiased approach which includes genome-wide association scans (GWAS). In GWAS, single nucleotide polymorphisms (SNPs) which may not necessarily be involved in the expression of the disease are used as markers to identify individual or clusters of disease-associated alleles (Vasan, 2006, Iles, 2008).

In this thesis, candidate gene approach will be employed to establish genotype-phenotype relationship. Candidate gene association studies allow a selection of SNPs with known biological function including rare variants to be explored. Even SNPs that do not reach significance ($P \leq 10^{-7}$) in genome wide association studies can still reach significance levels for a candidate gene study ($p \leq 10^{-3}$-10^{-4}). Candidate gene approach is required in the post-GWAS stage mainly to identify the genuine causative variants and to determine the effect of genetic variants in a particular population. Moreover, in the absence of a detailed GWAS data in a given disease it is cost-effective to conduct a candidate gene study (Wilkening et al., 2008).

1.1.2 Candidate genes in inflammation

Inflammation plays a prominent role in the development of atherosclerosis (Ross, 1999) and so genes related to the inflammatory pathway have been the main focus for genomic studies in CVD. The variations in the inflammatory phenotypes and genotypes could contribute to the increased susceptibility for CVD observed amongst subjects of South Asian origin. This section will discuss genetic markers that contributed to intraindividual variations in C-reactive protein (CRP) and interleukin-6 (IL-6) levels in non-Asian population. Both the inflammatory markers have been associated with CVD. CRP is the widest in use inflammatory biomarker and elevated levels have been associated with increased risk of type 2 diabetes mellitus (T2DM), hypertension (Pradhan et al., 2001), and CVD (Ridker et al., 1997).

1.1.2.1 C-reactive protein

There are many confounding factors that contribute to the interindividual variation in CRP levels, but the use of genetic variants that regulate the levels of CRP is a form of natural randomised trial or otherwise termed as Mendelian Randomization. This is achieved by random assortment of alleles from parents to offspring during gamete formation and conception. Thus minimizing the effect of non-genetic factors and providing an unbiased approach to assess the role of CRP in CVD (Hingorani et al., 2006; Thanassoulis and O'Donnell, 2009).

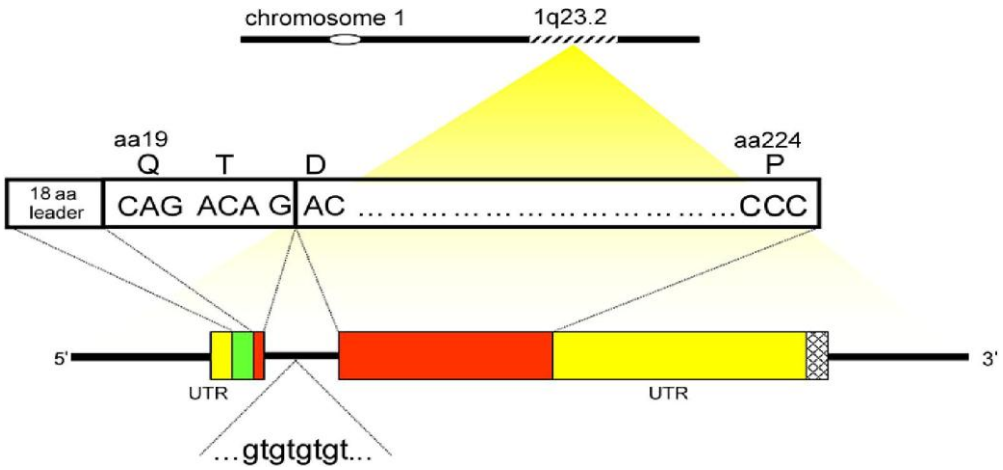
The mendelian randomization approach was used in the Rotterdam study (C reactive Protein Coronary Heart Disease Genetics Collaboration (CCGC), 2011), where established CVD risk factors increased significantly across increasing quartiles of CRP levels in European subjects but none differed across the four common gene variants of the CRP gene. The study showed a significant genotype-phenotype association and highlighted the unbiased approach of studying

the effect of CRP on disease risk though its gene. The notion behind this approach is if high levels of CRP contribute to disease risk, then subjects with common genetic variants of the CRP gene that raise CRP levels should have an increased risk of CVD (Kardys et al., 2006; Hingorani, 2006).

1.1.2.1.1 Structure of C-reactive gene

The human *CRP gene* is located on chromosome 1 and the promoter region of the gene is considered to be highly polymorphic that affect blood CRP levels (Lei et al., 1985 and Woo et al., 1985) and subsequently CVD risk. Family and twin studies suggest that about 35%-40% variation in the plasma CRP level is heritable (Pankow et al., 2001; Retterstol et al., 2003; MacGregor et al., 2004)

figure 1 *Structure of C-reactive gene*



Both Lei, 1985 and Woo, 1985 reported that CRP protein contains an 18 amino acid signal sequence and a mature protein of 206 amino acids. The CRP gene is composed of 1 intron which includes a GT repeat sequence that separates 2 exons (red boxes). The first exon encodes an 18 amino acid signal sequence (green box) and the first 2 amino acids of the mature protein whilst the second exon encodes the remaining 204 amino acids.
Reproduced from Hage et al., 2007, p.1116

1.1.2.1.1.1 Common single nucleotide polymorphisms of the C-reactive protein gene and phenotypic variation

Carlson et al., (2005) conducted a unique study with three experimental stages; initially the pattern of genetic variation across the *CRP* gene locus on chromosome 1q21-q23 was identified by resequencing the region in a multi-ethnic variation discovery panel. A set of 8 common haplotypes from seven TagSNPs were then defined in a population of European descent and African descent and studied in a much larger population (The Coronary Artery Risk Development in Young Adults-CARDIA study). The *CRP* gene haplotypes were significantly associated with variation in CRP levels ($p< 10^{-6}$), whereby haplotypes associated with low levels of CRP reduced the plasma levels of CRP by 1.5 mg/L per copy and vice versa. Similar results were reported in European descent in the Rotterdam study (Kardys, 2006), and in the third National Health and Nutrition Examination Survey (Crawford et al, 2006), where *CRP* gene haplotypes were associated with CRP levels.

Several genetic association studies of CRP have assessed the link between genotype and plasma CRP levels (Zee and Ridker, 2002: Brull et al., 2003; D'Aiuto et al., 2005). The majority of these association studies have investigated a small number of genetic polymorphisms, found in the promoter region (Kovacs et al., 2005) and 3'-untranslated region (UTR) (Brull, 2003) of the *CRP* locus. Hage (2007) identified two SNPs at the promoter region of the *CRP* gene which are functional, -409G>A (490 indicate the position of the SNP on the gene, here G is the wild type/major allele and A is the minor allele, the same format will be used throughout this thesis) (dbSNP ID-rs3093062) and -390C>T>A (rs3091244). These polymorphisms directly contributed to the interindividual variation of blood CRP levels. Higher CRP levels were observed in carriers of -409GG and -390TT. The two SNPs were reported to reside within the E box elements E-box

1 and E-box -2, which are functional since transcription factors bind to them and is altered in the presence of -409GG and -390TT SNPs.

Carlson (2005) and Szalai et al., (2005) confirmed that the rs3091244 triallelic SNP in the promoter region alters the affinity for transcription factors and affects the transcription of the gene which results in variation of blood CRP levels. In vitro study using CRP promoter-firefly luciferase reporters showed that individuals with -409G/-390T haplotype had a highest promoter activity, with high transcription factor binding version of E-box 1 and E-box 2 and high levels of CRP when compared to -409A/-390T individuals who showed weak promoter activity and hence lowest CRP levels (Szalai, 2005).

In the Women's Health Initiative Observational study (Lee et al., 2009); the genetic variants in the *CRP* gene were significantly associated with plasma CRP concentrations in a large multi-ethnic case control study of 3782 postmenopausal women. The minor allele frequencies for 13 SNPs in the CRP gene varied across the 4 ethnic groups (Whites, Blacks, Hispanics, Asians/Pacific Islanders, $P < 0.0001$). The minor alleles of SNPs rs3093068, rs1130864, and rs1417938 were associated with higher levels of CRP (geometric-mean increase per minor-allele change, 1.20 -1.25 mg/L) whilst the minor alleles of rs1205 and rs1800947 were associated with lower levels of CRP (decrease of 1.28 – 1.48 mg/L) in Whites. In Asians/Pacific Islanders the minor alleles at rs3093068 and rs1205 had a stronger association with higher levels of CRP than that seen in Whites (geometric- mean increase, 1.65 mg/L vs. 1.25 mg/L).

1.1.2.1.2 Genetic variants of C-reactive protein and disease associations

Prospective studies have shown that plasma levels of CRP predict incident CVD (Ridker, 1997; Ridker et al., 2002; Danesh et al., 1998) and recurrent CVD events (Haverkate et al., 1997; Speidl et al., 2002) and CVD mortality (Harris et al., 1999; Lindahl et al., 2000). Genetic variation of the *CRP gene* contributes to the CRP level in plasma. Hence, subjects with common genetic variants of the *CRP* gene that raise CRP levels may have an increased risk of CVD. This section summarizes the genetic disease association studies conducted on the inflammatory marker CRP.

1.1.2.1.2.1 Cardiovascular disease

In the Framingham heart study (Kathiresan et al., 2006), out of 13 common CRP SNPs the functional triallelic - rs3091244 SNP remained associated with plasma CRP levels even after adjustment for clinical covariates and accounting for correlation among SNPs in 1640 participants (stepwise $P<0.0001$). The triallelic SNP explained 1.4% of the variation in CRP levels. Subjects homozygous for CC genotype had the lowest level of CRP compared to heterozygotes (CT or CA genotypes) who had intermediate level of CRP and minor allele homozygotes (TT or AA) showed the highest level of CRP. The triallelic SNP rs3091244 was not associated with prevalent CVD (n=210, $P =0.62$ in multivariable adjusted model). The odds ratio for the group that included major allele heterozygotes (CT and CA) was 0.96 (95% CI 0.67 to 1.37), and for the group with both minor alleles (TA, TT, and AA), was 0.79 (95% CI 0.48 to 1.29) when compared with the major allele homozygotes (CC) as reference genotype. The SNP rs3091244 was also not associated with incident CVD (n=52, $P=0.18$ in multivariable adjusted model).

Suk Danik et al., (2006) assessed the relationship between seven common genetic variants within the *CRP* gene and the rise in plasma CRP levels after an acute coronary syndrome (ACS) in European American patients. The triallelic rs3091244 SNP was associated with the highest level of CRP. Kovacs (2005) associated the triallelic SNP with baseline CRP levels in patients with CHD. However this finding was not replicated in AIRGENE study (Kolz et al., 2008), which assessed the influence of genetic variants involved in the inflammatory pathway on the variability of CRP in patients with prior myocardial infarction (MI). Two minor alleles of the SNPs, rs1800947 and rs1205 within the *CRP* gene were associated with low CRP levels and demonstrated a dose dependent effect.

In a large study to establish the relationship between genetically elevated CRP levels and the risk of ischemic heart disease (IHD), the four CRP polymorphisms (rs3091244, rs1130864, rs1205, rs3093077) accounted for 64% increase in CRP levels, despite equal distribution of CVD risk factors across the genetic variants. However, the genotypes were not associated with increased risk of IHD (Zacho et al., 2008). Similarly, studies by Zee and Ridker (2002) and Kardy (2006) also failed to associate CRP genotypes with the risk of arterial thrombosis and the risk of coronary heart disease (CHD) respectively, despite showing a substantial genetic influence on CRP levels.

In the third National Health and Nutrition Examination Survey, the functional rs3091244 SNP was associated with CRP levels and with prevalent CHD in the non-Hispanic population (Lee, 2009). Since the functional significance of this triallelic SNP in the promoter region of CRP gene has been demonstrated in vitro, this association could be a true causal relationship (Crawford, 2006).

1.1.2.2 Interleukin 6

1.1.2.2.1 Structure of interleukin 6 gene

Bowcock et al., (1988) localized the *IL-6* gene to chromosome 7p21 using linkage studies. *IL-6* gene consists of four introns and five exons and encodes a 212 amino acid protein.

1.1.2.2.1.1 Common single nucleotide polymorphisms of interleukin 6 gene and plasma levels of interleukin -6

Fishman et al., (1998) identified a G>C polymorphism at position -174 within the promoter region of the *IL-6* gene on chromosome 7. This site involves a DNA binding site for the NF-IL-6 transcription factor, which is responsible for gene expression. In 102 healthy subjects, the G allele was associated with higher plasma levels of IL-6 compared to subjects with the C allele (GG: 274 pg/ml (95% CI, 2.43 -3.10); GC: 2.64 (2.35-2.97); CC: 1.63 (1.44-1.86), P=0.02). The study demonstrated that the 174G>C promoter polymorphism is functional, in that genetic variation influences the protein levels of IL-6, i.e. phenotypic expression. The functional activity of the 174G>C promoter polymorphism was detected by luciferase assay and demonstrated that G allele gene contruct showed higher expression of IL-6 compared to the C allele construct. The minor allele C had a reduced promoter activity which led to reduced expression of IL-6.

Fishman (1998) demonstrated the interethnic variation in the prevalence of this polymorphism in 383 Caucasians, 115 Gujarati Indians and 101 Afro Caribbean's. The frequency of the C allele was particularly rarer in Gujarati Indians and Afro Caribbean's 0.150 (95% CI, 0.094-0.184) and 0.05 (0.016-0.072) compared to Caucasians 0.403 (0.37-0.44).

Several studies have investigated the relationship between this promoter polymorphisms of *IL-6* and plasma levels of IL-6 but the results are conflicting. (Burzotta et al., 2001; Jerrard-Dunne et

al., 2003). Rauramaa et al., (2000) studied the effect of the 174G>C *IL-6* polymorphism in 87 asymptomatic Finnish men (aged 50 -60 years). Levels of IL-6 in those with GG genotype were comparable to those with the C allele.

1.1.2.2.1.2 Single nucleotide polymorphisms of interleukin-6 gene and plasma levels of C-reactive protein

Since IL-6 is a major inducer of CRP gene expression, and the -174G>C IL-6 promoter polymorphism is directly responsible for the phenotypic expression of IL-6. Studies have investigated the relationship between this polymorphism and plasma levels of CRP. Vicker et al., (2002) conducted a study of 588 members from 98 nuclear families. The strength of such 'family' studies is in the genetic homogeneity of the study subjects. The baseline CRP levels were highly heritable (h^2 =0.39, P<0.0000001), the results were similar to those reported by Pankow (2001). The -174 C allele was associated with high baseline CRP levels (P=0.01).

Humphries et al., (2001) showed in 494 middle-aged healthy UK men that carriers of the -174C allele had higher levels of CRP compared to non-carriers but the results were not significant (P=0.13).

1.1.2.2.2 Genetic variants of interleukin- 6 and disease associations

Prospective studies have shown that plasma levels of IL-6 predict incident CVD (Ridker et al., 2000a; Ridker et al., 2000b) and recurrent CVD events (Biasucci et al., 1996; Biasucci et al., 1999b) as well as mortality (Harris et al., 1999). The genetic variation in the IL-6 gene contribute to the variability of IL-6 in plasma, so ideally subjects with common genetic variants of the IL-6 gene that raise IL-6 levels should have an increased risk of CVD. This section summarizes the genetic disease association studies conducted on the inflammatory marker IL-6.

1.1.2.2.2.1 Cardiovascular disease

Humphries (2001) showed that the 174G>C IL-6 promoter polymorphism is associated with the increased risk of CHD in 2751 middle-aged healthy UK men, followed up for 6 years. The -174 C allele was significantly associated with higher systolic blood pressure (mean mmHg (95% CI); CC = 138.0 (136.3 -139.8); GC = 137.9 (136.9 – 138.9) compared to the G allele; GG = 135.5 (134.3 – 136.7), *P* =0.007) irrespective of their smoking status. This relationship between genotype and systolic blood pressure was greater in men with body mass index (BMI) >24.86 kg/m^2 (CC had 2.9 mmHg and GC 2.6 mmHg higher than GG, *P*=0.05) compared to men in the lowest tertile of BMI (CC + GC, 1.4 mmHg higher than GG, *P*=0.29). Carriers of the -174C allele had a relative risk (RR) of CHD of 1.54 (95% CI 1.0-2.23, P=0.048) compared to those with the G allele, this relationship was greatest in smokers (compared to GG non-smokers, RR 2.66, CI 1.64-4.32) and remained statistical significant after adjustment for CVD risk factors (*P*=0.04).

In 466 patients of the U.K. Small Aneurysm Trial (Jones et al., 2001), the -174C allele was associated with higher plasma levels of IL-6 (CC (median) 15.6 pg/ml; GC 4.8 pg/ml) compared to patients with GG genotype (1.9 pg/ml, *P*=0.047). Patients with small aneurysms who were carriers of the C allele had a higher risk of future CVD events and all cause mortalities compared to patients with the GG genotype: hazard ratios 0.51 (95% CI, 0.25-1.00), *P*=0.05 and 0.32 (95% CI, 0.12 -0.93), P=0.036 respectively.

The relationship between the -174G>C promoter polymorphism of *IL-6* gene, plasma levels of IL-6 and the length of hospital stay after coronary revascularization was demonstrated by Burzotta (2001) in 111 patients with multivessel CHD. The plasma levels of IL-6 were measured before surgery, after surgery and at discharge and showed a mean 17-fold increase with peak

values at 24 hours (P<0.0001). The levels of IL-6 were significantly different across the genotypic groups irrespective of comparable clinical and surgical characteristics. Sixty two patients homozygous for GG genotype had higher concentrations of IL-6 (mean ± SD, 69 ± 83 pg/ml at 48 hour after surgery) compared to thirty nine patients heterozygous for CG (49 ± 26 pg/ml) and ten homozygous for CC (33 ± 17 pg/ml), (P = 0.015). Patients who had GG homozygous stayed longer in the intensive care unit (2.5 ± 3.4 vs. 1.4 ± 0.9 days, P=0.02) and in the hospital (6.7 ± 4.0 vs. 5.3 ± 1.4 days, P=0.02) compared to patients who were carriers for C allele.

There are studies that have reported conflicting results on the -174G>C promoter polymorphism of *IL-6* gene, whereby high levels of IL-6 has been associated with the CC genotype. In the carotid atherosclerosis progression study (CAPS) (Jerrard-Dunne, 2003), the CC genotype was associated significantly with higher levels of IL-6 compared to GG/GC genotypes, despite comparable distribution of CVD risk factors across the genotype groups. The median (IQR) were GG, 1.51 (1.46) pg/ml; GC, 1.56 (1.30) pg/ml; and CC, 1.87 (1.86) pg/ml. Amongst those with IL-6 -174 CC genotype, the odds ratio (OR) for elevated IL-6 was 1.68 (95% CI, 1.00 -2.82, p=0.049) after age and gender adjustment and 2.07 (1.16-3.72, p=0.014) after adjustment for confounding factors. Moreover, the CC genotype was associated with elevated carotid artery intima-media thickness (P=0.001) and increased risk of carotid plaque (OR, 3.64 (95% CI, 1.15-11.54, P=0.028) in patients who were heavy alcohol drinkers.

1.1.2.2.2.2 Diabetes mellitus, insulin resistance and obesity

Type 1 diabetes mellitus (T1DM) is an autoimmune disease characterized by the destruction of the insulin producing islet beta cells. The genetic variants within the cytokine genes mainly *IL-6*

have been implicated in the genetic susceptibility to T1DM. Jahromi et al., (2000) demonstrated in a case control study that the IL-6-174G>C promoter polymorphism was associated with susceptibility to T1DM. The prevalence of the homozygous GG genotype was greater in patients compared to control subjects (50.6% vs. 33.3%, $p<0.002$). The CC genotype was less prevalent in the patient group compared to healthy subjects (12.5% vs. 24.2%, $p<0.004$).

The 174G>C promoter polymorphism of IL-6 has also been implicated in the pathogenesis of T2DM. In the European Prospective Investigation into Cancer and Nutrition (EPIC) Potsdam cohort, Mohlig et al., (2004) studied 188 T2DM cases and 186 controls. Cases were disease free at baseline and have developed the disease during the follow up of 2.3 years. The distribution of baseline characteristics including IL-6 concentrations was comparable in all genotypes except for age. The main finding was that the 174G>C IL-6 promoter polymorphism modified the association between BMI and the risk of T2DM. Amongst subjects with the CC genotype, the correlation between BMI and IL-6 levels was higher (0.52; 95 CI, 0.366–0.650; n = 103) compared to subjects with the GG genotype (0.2; 95% CI, 0.036–0.344; n = 150) and GC genotype (0.31; 95% CI, 0.209–0.410; n = 311). Amongst those with BMI greater than or equal to 28 kg/m^2, the risk of T2DM was estimated to be 17.68 (95% CI, 3.57–87.66) for the CC genotype, 3.44 (95% CI, 1.34–8.24) for the GG genotype and 2.94 (95% CI, 1.56–5.56) for the GC genotype.

Increased levels of IL-6 observed in T2DM and obesity have been associated with indices of adiposity and insulin resistance. Berthier et al., (2003) studied the association between the -174G>C promoter polymorphism of IL-6 and indices of adiposity in 270 healthy French-Canadian men. The G allele was more prevalent amongst lean subjects (BMI <25 kg/m^2, P=-0.007 and waist <100 cm, P=0.01). The G allele was also common amongst subjects with low

concentrations of insulin (<160pmol/l) and glucose (<4.6 mmol/l), *P*=0.03 and *P*=0.01 respectively. Across all genotypes, men who had CC and GC genotype showed a larger waist girth (102.76 ± 11.19 cm and 101.85 ± 10.62 cm) compared to those who were GG homozygous (97.98 ± 11.36 cm, *P*=0.02).

1.1.2.2.2.3 Lipid abnormalities

Fernandez-Real et al., (2000) studied the influence of the -174G>C IL6 promoter polymorphism on plasma lipids in 32 Caucasian healthy subjects (17 women). Age, sex, BMI, waist to hip ratio was comparable across all genotypes (CC vs. GC and GG). Healthy subjects with the G allele showed higher levels of plasma triglycerides (1.5 ± 0.9 vs. 0.90 ± 0.37 mmol/L; p=0.01), VLDL triglycerides (0.97 ± 0.69 vs. 0.42 ± 0.2 mmol/L; p=0.002), fasting (881vs 458 µmol/L, p=0.01) and post glucose load free fatty acids (229 vs. 90.5 µmoll/L, p=0.03), lower levels of HDL cholesterol (0.25 ± 0.14 vs. 0.39 ± 0.26 moll/L, p=0.058) but similar cholesterol and LDL cholesterol levels compared to those with the C allele. In univariate analysis, serum levels of IL-6 correlated positively with fasting triglycerides, VLDL triglycerides and post load free fatty acids (correlation coefficient, r=0.61, 0.65 and 0.60 respectively, p<0.001) and negatively with HDL cholesterol (r=-0.42, p<0.05). Subjects with the G allele showed the expected trend of higher serum IL-6 levels (9.9 ± 6.9 vs. 6.85 ± 1.7 pg/ml. p=0.09).

1.1.3 Candidate genes in lipid metabolism

Genetic studies so far have identified several loci that influence lipid metabolism and thereby increase the risk of CVD. Some of the major proteins that play a role in the generation of an atherogenic lipid profile are LDL receptor, apolipoprotein B-100, apolipoprotein E, lipoprotein (a), lipoprotein lipase, apolipoprotein CIII, cholesteryl ester transfer protein, apolipoprotein AI,

lecithin cholesterol acyl transferase and microsomal triglyceride transfer protein (Milewicz et al., 2000). Genetic variations within the genes involved in lipid metabolism have been proposed to explain the interindividual differences in plasma levels of total cholesterol, triglycerides and HDL-c and associated risk of CVD. Table 2 summarises the genes that are involved in the lipid metabolism that affect the overall lipoprotein integrity in the circulation.

Table 2 Genes involved in the lipid metabolism that affect the overall lipoprotein integrity in the circulation

Genes	Role in lipid metabolism
LDL receptor	Uptake of cholesterol rich LDL
Microsomal triglyceride transfer protein (MTP)	Assembly of plasma VLDL, precursor for ApoB containing lipoproteins in plasma
ATP binding cassette protein A1 (ABCA1)	Cholesterol efflux pump in reverse cholesterol transport
Apolipoprotein A1(apoA-1)	Protein component of HDL
Lecithin cholesterol acyl transferase (LCAT)	Etherification of HDL-C and maturation of HDL particle
Cholesteryl ester transfer protein (CETP)	Transfer of cholesteryl esters from HDL to ApoB containing lipoprotein in exchange for triglycerides
Apolipoprotein E (apoE)	LDR/ApoE receptor mediated uptake of atherogenic lipoproteins
Hepatic lipase (HL)	HDL metabolism, clearance of ApoB containing lipoproteins
Apolipoprotein CIII (ApoCIII)	Mediate clearance of triglyceride rich lipoproteins via ApoE mediated receptor
Apolipoprotein AV (ApoAV)	Regulator of triglycerides
Lipoprotein *lipase (LPL)*	Catabolism of triglycerides

Information taken from Lusis, 2004

It is beyond the scope of this thesis to mention all the candidate genes that contribute to interindividual differences in lipid levels in circulation and risk of CVD. The excess burden of CHD in South Asians is partly attributed to dyslipidaemia defined as having high levels of triglyceride, low levels of HDL-c and high levels of Lp (a) (Bhatnagar et al., 1995; Patel et al.,

2006). This section mainly summarizes the candidate genes that contribute to low HDL-c and high triglyceride levels and those that have been associated with an increased risk of CVD.

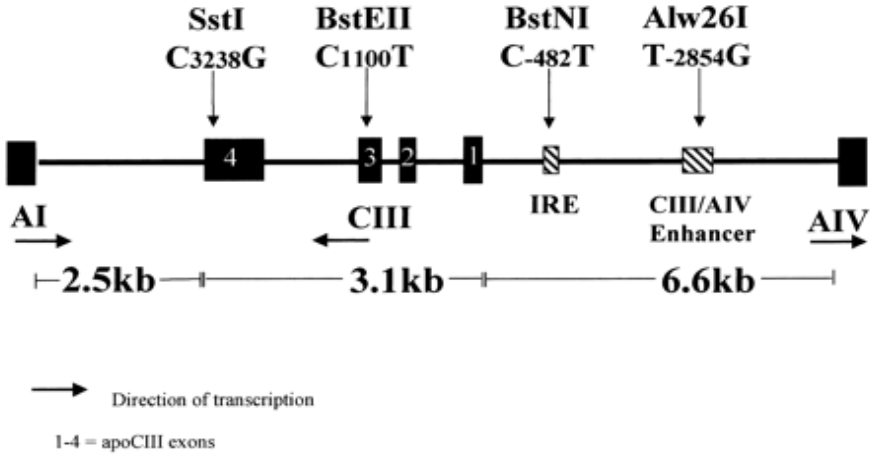
1.1.3.1 Candidate genes that contribute to low HDL and high triglyceride levels

1.1.3.1.1 Apolipoprotein C3 gene and Apolipoprotein A5 gene

1.1.3.1.1.1 Structure and function of Apolipoprotein C3

ApoCIII is a 79-residue glycoprotein and is synthesised in the liver and intestine as the protein component of VLDL and HDL particles (Ooi et al., 2008). Over expression of ApoCIII delays the catabolism of triglycerides by down regulating LPL and impairs the apoE mediated clearance of triglyceride-rich lipoprotein by the liver, resulting in hypertriglyceridemia (Li et al., 1995). The protein is encoded by the *APOC3* gene and is located within *A1/CIII/AIV/AV* gene cluster on chromosome 11q23-q24 (Protter et al., 1984). Figure 2 shows the *AI-CIII-AIV* gene region.

figure 2 *A1-CIII-AIV gene region*



APOC3 gene consists of 4 exons which encodes a 79 amino acid protein. Common APOC3 gene polymorphisms are C3238G (Sstl) in the 3' UTR (untranslated region), C1100T in exon 3, -482C>T & -455T>C (not shown here) in the promoter insulin-response element (IRE)
Reproduced from Waterworth et al., 1999, p.1873

Two common *APOC3* promoter polymorphisms –482C>T, -455T>C within the insulin response element are associated with hypertriglyceridemia (Dammerman et al., 1993) and susceptibility to T1DM. The expression of ApoCIII is down regulated by insulin (Waterworth et al., 2003); the mutant allele of the APOC3 promoter polymorphisms inhibits the ability of insulin to regulate ApoCIII expression. Increased plasma levels of ApoCIII promote β-cell destruction; reduce insulin secretion henceforth triggers the pathophysiological pathway to the onset of T1DM (Hokanson et al., 2006).

1.1.3.1.1.2 Single nucleotide polymorphisms of Apolipoprotein CIII gene and levels of triglycerides

In the multi-ethnic population based study, the Dallas Heart study and the Atherosclerosis Risk in Communities study, Kozlitina et al., 2011 demonstrated that the *APOC3* -482C>T and -455T>C polymorphisms were not related to triglyceride levels. Olivieri et al., (2003) demonstrated in subjects with metabolic syndrome that the C-allele of *APOC3* -455T>C polymorphism was associated with high triglycerides and increased ApoCIII activity. Waterworth et al., (1999) investigated the response of *APOC3* gene variants to oral glucose and fat tolerance tests in 405 young, healthy male offspring who had a family history of early MI and 415 age matched controls. The -482C>T polymorphism did not regulate postprandial changes in triglycerides but regulated response to oral glucose tolerance test whereby subjects with the rare allele had elevated levels of glucose (28.7% area under the curve (AUC), P=0.013) and insulin (20.5% AUC, $p<0.01$).

In the Second Northwick Park Heart Study (NPHSII), Waterworth et al., (2000) showed in a cohort of 2745 healthy men that −482T allele was associated with elevated triglycerides but was

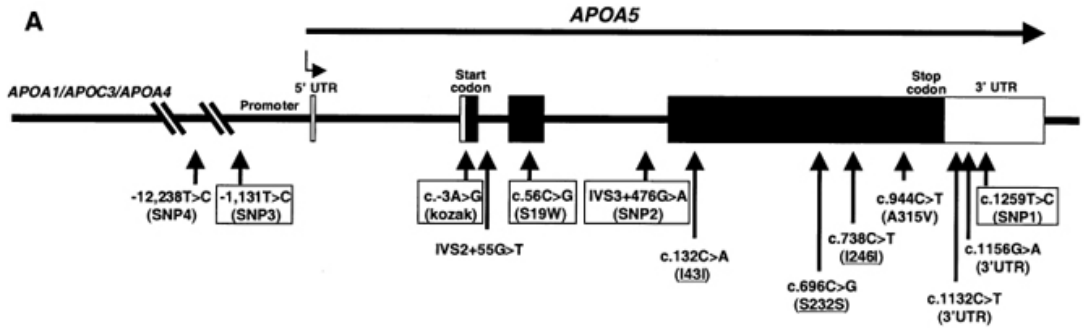
dependent on smoking status (increase of 13.7% in triglycerides −482T allele in current smokers, 8.6% in exsmokers, and - 7.4% in those who never smoked).

1.1.3.1.2 Apolipoprotein A5 gene

1.1.3.1.2.1 Structure and function of Apolipoprotein A5

Pennacchio and colleagues (2001) discovered the *APOA5* gene by sequencing of the *APOA1-CIII-AIV* gene region in mice and humans. The gene is located 27kb distal to apoA-IV in apoA1/C3/A4/A5 gene cluster on chromosome 11q23. The function of *APOA5* gene was determined in *APOA5* transgenic mice and *APOA5* knockout mice. The human APOA5 transgenic mice (overexpression of APOAV) had low levels of plasma triglyceride (0.32 ± (SD) 0.11 mg/ml) compared to APOA5 knockout mice (1.53 ± 0.77 mg/ml). Figure 3 presents the structure of *APOA5* gene and location of SNPs within the gene (Pennacchio et al., 2002).

figure 3 Structure of APOA5 gene



The gene consists of 4 exons (black boxes) which encode a 366 amino acid protein. Position of the most common SNPs within APOA5 gene are shown here. The SNP of interest for this thesis is -1131T>C located within the promoter region of APOA5 gene.
Reproduced from Pennacchio, 2002, p.3033

1.1.3.1.2.2 Single nucleotide polymorphisms of Apolipoprotein A5 gene and levels of triglycerides and high density lipoprotein cholesterol

Chandak et al., (2006) investigated the prevalence of *APOA5* genetic variations and its association to triglyceride levels in 557 Indian adults from Pune, India and 237 White adults in the UK. The minor allele frequency for *APOA5* -1131C allele was more common amongst Indians from Pune compared to UK White subjects (20% vs. 4%, p =0.00001). The percentage increase in triglyceride levels in subjects with -1131C allele was 19%, p = 0.0003. In 2808 healthy middle aged men, subjects with CC genotype of *APOA5* -1131T>C polymorphism had 40% higher triglyceride compared to those with TT genotype (Talmud et al., 2002).

Jiang et al., (2010) demonstrated in two Chinese populations that APOA5 -1131 T>C was strongly related to triglyceride levels in Hong Kong subjects (β =0.192, p=2.6 × 10(-13) and Gangzhou subjects (β = 0.159, p = 1.3 × 10 (-12). The levels of triglyceride were 36.1% higher in CC compared to TT genotype. The odds for elevated triglycerides (≥ 1.7 mmol/l) in subjects with TC and CC genotypes respectively were 1.81 (1.37-2.39) and 2.22 (1.44-3.43) in Hong Kong subjects and 1.27 (1.05-1.54) and 1.97 (1.42-2.73) in Guangzhou subjects. In the study by Aouizerat et al., (2003), the C allele of the *APOA5* -1131 T>C had elevated triglyceride by 21mg/dl, p=0.009, VLDL cholesterol by 8 mg/dl, p=0.0001 and reduced HDL cholesterol by 2 mg/dl, p=0.017.

Pennacchio (2001) showed in 500 Caucasian men and women that a minor haplotype of APOA5*2 defined by three polymorphisms, 1259T>C, IVS3+ 476G>A and -1131T>C was associated with a 20-30% elevation in triglyceride levels. In 2002, the same group showed that a minor haplotype of *APOA5*3* containing a rare allele of the 56C>G is associated with high plasma triglyceride levels. The *APOA5*3* haplotype was almost three fold more prevalent in

Caucasian men and women with plasma triglyceride concentrations above the 90th percentile compared to those with levels below the 10th percentile (Pennacchio, 2002).

Dorfmeister et al., (2007) examined the association of *APOA5* -1131 T>C with lipids and diabetic parameters in 931 Europeans and 610 Indian Asians with T2DM. Carriers of the minor allele, C allele had higher triglyceride levels in both groups but was significant only in Europeans (p=0.04) and was not associated with parameters of diabetes.

1.1.3.1.3 Genetic variants of *Apolipoprotein C3* and *Apolipoprotein A5* and disease associations

1.1.3.1.3.1 Cardiovascular disease and metabolic syndrome

Oliviero et al., (2002) investigated the link between *APOC3* 455T>C genetic polymorphisms and CHD in 549 patients who had angiographically documented CHD and 252 CHD free patients. The prevalence of CC genotype was higher in CHD patients compared to CHD-free subjects, 18.6% vs. 9.2% (P<0.001). The odds for increased risk of CHD in patients with CC genotype after adjusted for all major risk factors were 2.18. In 2003, Olivier investigated in the same cohort a possible link between *APOC3* 455T>C polymorphism and the metabolic syndrome. Metabolic syndrome was more prevalent amongst CHD patients compared to CHD free patients (38.1% vs. 15.3%) and the odds for CHD in patients with metabolic syndrome was 3.39 (95% CI, 2.35-4.90).

Bhaskar et al., (2011) investigated the association of *APOA5* -1131T>C in a cohort of 520 South Indians of whom 250 were CHD patients (160 with T2DM and 90 without diabetes), 150 T2DM subjects and 120 age and sex matched healthy subjects. The frequency of *APOA5* -1131 CC

genotypes and C allele was higher in CHD patients with T2DM compared to CHD patients without diabetes and healthy controls (OR =1.71: 95% CI 1.0-2.67, p = 0.012).

In the Italian study of early-onset MI which comprised of 1864 patients with first MI above 45 years and 1864 age and sex matched controls, *APOA5* -1131T>C was associated with risk of early-onset MI after adjustment for triglyceride (p= 0.006). The odds of having early MI in subjects with C allele was 1.44 (95% CI 1.23 – 1.69). *APOA5* -1131 T>C was significantly associated with raised plasma triglyceride levels in healthy subjects compared to non-carriers. The percentage increase per C allele was 11.4% (95% CI 4 -19%) equivalent to 0.15 mmol/l (95% CI 0.11-0.20 mmol/l) (De Caterina et al., 2011). Evans et al., (2011) investigated the development of atherosclerosis and genetic variations in the *APOA5* gene in 60 patients with type III hyperlipidaemia. The odds for the development of atherosclerosis in patients with type III hyperlipidaemia with the C allele was 3.69.

1.1.3.1.4 Common genetic variants that contribute to circulating lipid levels and risk of coronary artery disease

It is beyond the scope of this thesis to mention all the genetic markers that are related to triglycerides and low HDL-c levels. However, this section summarises a genome wide association study conducted by Waterworth et al., (2010) to identify novel/known genetic determinants of triglycerides, low HDL-c and LDL-c. The genome wide association study was performed in three stages initially on 17 723 participant from 8 studies (stage 1), then replicated in 37 774 participants from 8 populations and in a population of Indian Asian descent (stage 2), and association studies between SNPs at lipid loci and risk of CHD in 9633 cases and 38 684 controls (stage 3).

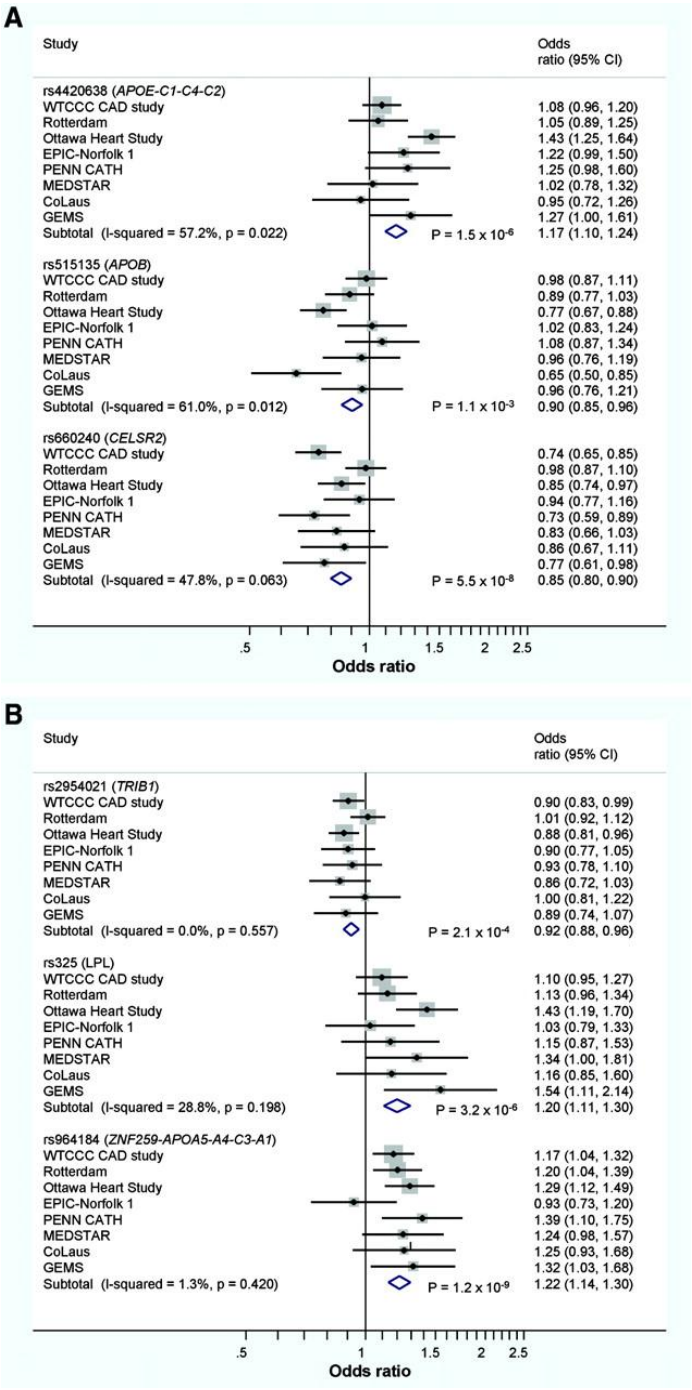
In the genome wide association meta-analysis, the following genes showed strong association with LDL-c, *APOB*, the *APOE-C1-C4-C2* cluster, *CELSR2*, EGF LAG seven-pass G-type receptor 2), *HMGCR* (3-hydroxy-3-methylglutaryl-CoA reductase), *LDLR*, *PCSK9 (*proprotein convertase subtilisin/kexin type 9) and *CILP2* (cartilage intermediate layer protein 2). The genes that showed strong association with HDL-c were *CETP*, *LIPC* (lipase hepatic), *LIPG* (lipase endothelial), *LPL*, *ABCA1*, *LCAT*, *GALNT2 (*UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 2) and *MMAB/MVK*

*(*methylmalonic aciduria (cobalamin deficiency) cblB type/mevalonate kinase). Whilst the genes that were strongly associated with triglyceride levels were *ZNF259-APOA5-A4-C3-A1* cluster, *LPL*, *ANGPTL3* (angiopoietin-like 3), *GCKR* (glucokinase (hexokinase 4) regulator), TRIB1 (tribbles homolog 1) and MLXIPL (MLX interacting protein-like). A SNP in APOE-*C1-C4-C2* cluster showed strong association with all 3 lipid traits (Waterworth, 2010).

In the Indian Asian population, the following loci were associated with lipid traits, PPP1R3B (SNP associated was rs2126259, β-coefficient –0.023, $p = 9.6\times10^{-4}$) for circulating LDL-c levels, FADSI (SNP associated was rs174548, β-coefficient -0.017, $p=1.1\times10^{-4}$) for circulating HDL-c and (β-coefficient 0.041, $p=2.6\times10^{-5}$) triglyceride levels and AFF1 (SNP associated was rs442177, β-coefficient 0.027, $p=2.9\times10^{-4}$) for circulating triglyceride levels (Waterworth, 2010).

Positive association with CHD risk were found within genetic variations in *CELSR2*, *APOB* and *APOE-C1-C4-C2* cluster genes that influenced LDL-c levels. SNPs at APOA5-A4-C3-A1 cluster which contributed strongly to triglyceride levels in stage 1 were also associated with risk of CHD (Waterworth, 2010). Figure 4 shows the association between SNPs that contributed to lipid traits and risk of coronary heart disease.

figure 4 *Association between SNPs that contributed to lipid traits and risk of coronary heart disease*



Meta-analysis included 8 studies comprising 7,018 cases and 20 765 controls

- *A- Shows SNPs that contributed to LDL-c levels that were associated with the risk of CHD*
- *B- Shows SNPs that contributed to triglycerides and HDL-c levels that were associated with the risk of CHD. Reproduced from Waterworth, 2010, p.2273*

1.2 Characterisation and validity of cardiovascular biomarkers

Biomarkers are an efficient tool for identifying vulnerable subjects at the risk of developing CVD, where these signature molecules could aid clinicians in the diagnosis, evaluation or monitoring of patients with CVD effectively and efficiently. According to the NIH working group on definitions, 2001 (Biomarkers Definitions Working Group, 2001), biomarker is "a characteristic that is objectively measured and evaluated as an indicator of normal biological process, pathogenic processes, or pharmacologic response to a therapeutic intervention". Definitions for different types of biomarkers are shown in Table 3 (Vasan, 2006; Shorr et al., 2008).

Table 3 Type of Biomarkers

Type 0 biomarker: 'A marker of the natural history of a disease and correlates longitudinally with known clinical indices'.
Type 1 biomarker: 'A marker that captures the effects of a therapeutic intervention in accordance with its mechanism of action'.
Type 2 biomarker (Surrogate end-point): 'A marker that is intended to substitute for a clinical endpoint; a surrogate endpoint is expected to predict clinical benefit on the basis of epidemiological, therapeutic, pathophysiological, or other scientific evidence'.

Definitions taken from Shorr, 2008

A biomarker can be any measurement or test obtained from a patient, it can be measured on a biological substance such as blood, urine, tissue, it can be a recording obtained from patient such as blood pressure, ECG, arterial stiffness or can be an imaging test such as echocardiogram, angiography. Biomarkers can be broadly classified as antecedent biomarkers –those risk factors or risk markers that identifies the risk of developing a disease, screening biomarkers –those markers that identifies the presence of clinical or preclinical disease, diagnostic biomarkers- those markers that identifies overt disease, staging or prognostic biomarkers –those markers that categorise disease severity or evaluate responses to therapeutic intervention (Biomarkers Definitions Working Group, 2001; Vasan, 2006).

Biomarkers can serve as surrogate end points but only a few biomarkers achieve the surrogate endpoint status. A surrogate marker is a marker that is intended to substitute for a clinical endpoint; a surrogate endpoint is expected to predict clinical benefit on the basis of epidemiological, therapeutic, pathophysiological, or other scientific evidence. It is extremely useful when it can be easily measured and highly correlated with true clinical endpoints for example ejection fraction being surrogate marker for heart failure (HF). One of the benefits of surrogate endpoints is; it can be evaluated for safety and efficacy of a particular therapy within a short time frame in small studies rather than distant clinical events such as mortality or clinical outcomes, which requires larger clinical trials. The utility of surrogate endpoint in clinical settings requires demonstration of accuracy- highly significant correlation with the true endpoint, reproducibility, easily measurable and cost effective. However, if numerous factors affect the clinical outcome of interest along with the surrogate endpoint then its validity is drastically reduced (Wittes et al., 1989; Vasan, 2006).

1.2.1 Characteristics of an ideal biomarker

A biomarker can be applied in a clinical setting when it is accurate, reproduceable, easy to measure, has high sensitivity and specificity for the endpoint and can be easily interpreted by clinicians. Examples for such biomarkers that have enabled clinicians in CV disease management are troponin T which indicates the extent of myocardial damage and identifies patients who had a MI from those who had an unstable angina, BNP (brain natriuretic peptide) which indicates progression of HF, D-dimer which identifies patients with pulmonary embolism and coronary angiography which evaluates the severity of CHD (Vasan, 2006). The desirable properties of

CVD biomarkers of screening, diagnosis and prognosis are shown in Table 4 (reproduced from Vasan, 2006, p.2337).

Table 4 Desirable properties of CVD biomarkers

All biomarkers	Screening biomarkers to identify vulnerable patients	Diagnostic biomarkers to identify ischemia or injury	Prognostic biomarkers
General features	Known reference limits	High myocardial specificity	Known reference limits
Measure a specific pathology	Add to known CHD index such as the FHS risk score	Not present in normal serum/ non-cardiac tissue	Add to known prognostic index
Add to clinical assessment	Change in c statistic or AUC (discrimination)	Zero baseline, immediate release (early detection)	Change in marker alters management
Acceptable to patient	Acceptable calibration	Long half life	Affect choice of drug
Linear relation between change in marker and change in pathology	High specificity to avoid mislabelling asymptomatic individuals	Permit long time window for diagnosis	Change dose of drug
Stable product	Account for a moderate or greater proportion of CHD in the community	But <24 h to permit diagnosis of recurrent ischemia	Indicate tolerance
Single measure representative	Change management	Release proportional to injury size or ischemic burden	Indicate safety margin
Applicable to men and women, different ages, different ethnicities	Reclassify risk in patients at intermediate risk	Convenience for point-of-care testing	Used for monitoring progression of disease
Replication in multiple studies	Target individuals with increased levels of biomarker superior to conventional risk factors for reducing risk	Rapid test available in <1 h	Trajectory of marker correlates with disease progression
	Cost-benefit ratio favourable	No special sample preparation needed	High specificity to avoid mislabelling asymptomatic individuals
Assay/measurement features		Inexpensive	Cost effective
Internationally standardised		Readily available	REMARK guidelines (McShane *et al*; 2005)

Accuracy Precision *Assay application features* Tested in a spectrum of people with varying degrees of pathology High sensitivity and Specificity *Laboratory features* Automation High throughput/short turnaround time Connectivity to laboratory information management systems Compatibility with existing laboratory processes *Desirable features for in vitro diagnostic industry* Address unmet patient needs Return on investments Requirements for research and development phases Manufacturability Marketability including barriers to entry		Diagnostic cut-off well defined and accepted Known discrimination limits or action thresholds Change management, triage or specific treatment "Rule out" strategy With high sensitivity; more important to avoid missing disease Cost-effective STARD guidelines (Bossuyt et al, 2003)	

Post sales customer support Other market features (acceptance, competition, regulatory issues, reimbursement, third party patent rights)			

Reproduced from Vasan, 2006, p.2337

1.2.2 Development of biomarkers in cardiovascular disease

There are mainly two approaches for the development of biomarkers in CVD i) "knowledge based" approach depends on the understanding of the biological processes involved in the development of the disease ii) "unbiased" approach uses current technologies such as genomics, proteomics bioinformatics to study tens of thousands of molecules to identify biomarkers that relates to different stages of the disease (Vasan, 2006).

The various stages involved from the discovery of a biomarker to its application in clinical practice are shown in figure 5. Before considering a biomarker in clinical practice, clinician should consider some key questions relating to its measurement, validation, whether the new biomarker adds new information diagnostically or prognostically and its cost is justifiable to the healthcare system. Table 5 shows some key questions before considering a new biomarker in practice.

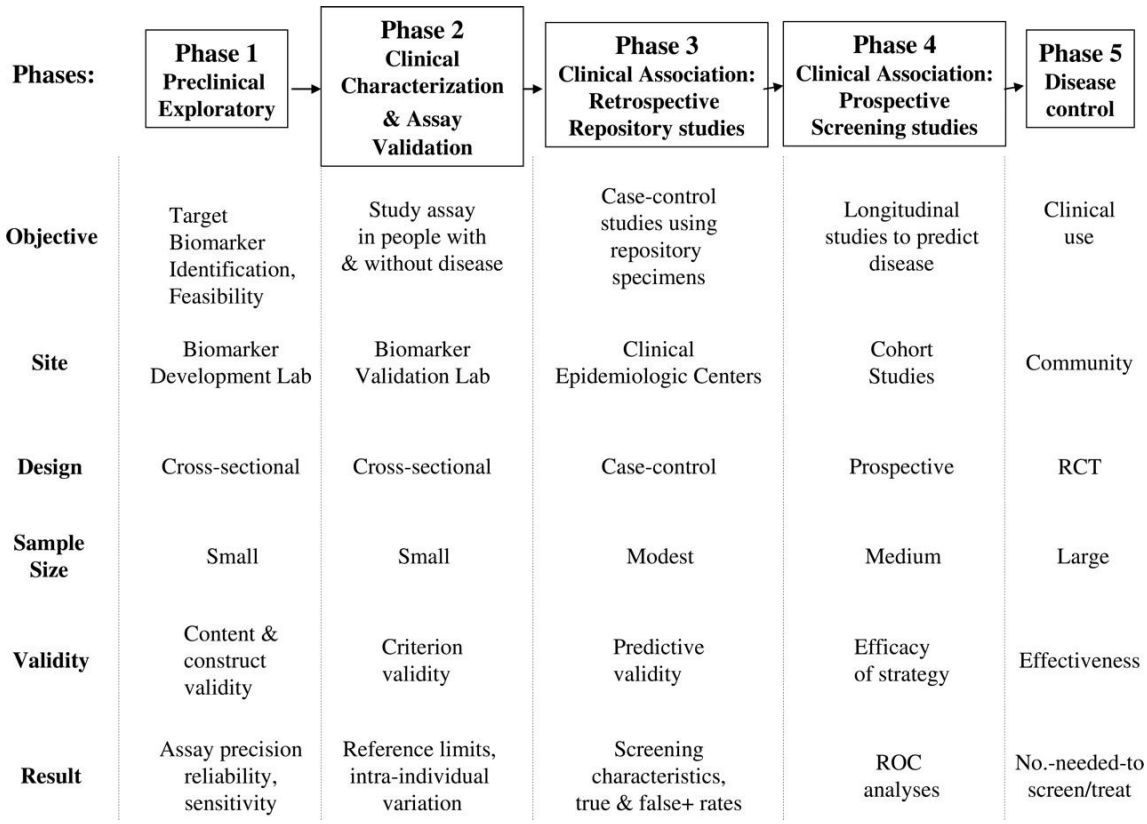
Table 5 Some key questions that need to be considered before using a new biomarker in practice

Has the assay been standardized?
Is the assay reproducible, accurate, and available?
Is the distribution of biomarker values in the general population and in select demographic subsets well known?
What are abnormal levels (reference limits and discrimination limits)?
Do biomarker levels correlate with known CVD risk factors?
Does a new biomarker reveal novel mechanisms of CVD initiation or progression?
Does the biomarker predict the outcome of interest?
Has residual confounding been excluded as an explanation for the observed association of a marker with CVD risk?

Is the new biomarker better than or does it have incremental utility over currently established biomarkers considered together? Will the use of a multimarker strategy using a new biomarker in combination with known biomarkers improve overall testing for CVD? Do the biomarkers add to the established risk prediction algorithms? Can a therapeutic course of action or the likelihood of response to an agent be determined with the use of a new biomarker? Will clinical practice change as a result of use of a new biomarker for screening, diagnosis, prognostication, or treatment? Is use of the biomarker shown to be cost-effective?

Reproduced from Vasan, 2006, p.2349

figure 5 *Five phases of biomarker development: from discovery to application in clinical practice*



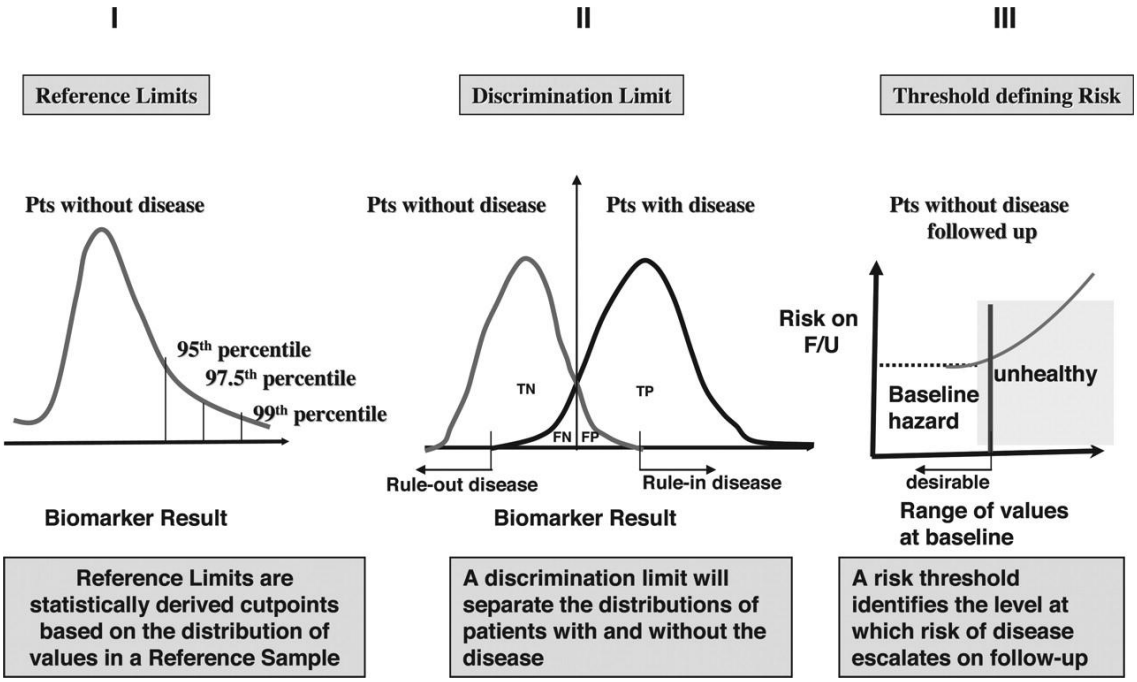
Reproduced from Vasan, 2006, p.2348
RCT- randomized controlled trial, ROC –receiver operating curves

1.2.2.1 Defining abnormal biomarker levels

In order to apply a biomarker in a clinical setting, it is important to characterise the distribution of the biomarker initially in a healthy population free of the disease of interest and then in patient

population. There are 3 approaches for defining abnormal biomarker levels (Vasan, 2006). Figure 6 shows the approaches for defining abnormal biomarker levels.

figure 6 Approaches for defining abnormal biomarker levels



In approach 1, reference limits are generated in a healthy sample free of the disease of interest in cross-sectional studies and a percentile cut point mostly between the 95th or 97.5th percentile is chosen to define abnormality. In approach II, discrimination limits are generated by evaluating the degree of overlap between patients with and without disease in cross sectional studies. Finally in approach III, threshold defining risk is generated in prospective cohort studies and relates levels of biomarkers to the incidence of disease and establishes a threshold beyond which risk escalates (Definitions taken from Vasan, 2006).

Reproduced from Vasan, 2006, p.2338
FN- false-negative, FP-false positive, TN- true negative, TP- true positive, Pts-patients, F/U- follow-up

1.2.2.2 Evaluation of the performance of biomarkers

There are several factors that lead to variability in biomarkers and this should be considered in the early phases of biomarker development. Table 6 shows sources of biomarker variability and recommended steps to reduce variability.

Table 6 Sources of biomarker variability

Preanalytical Variability	Analytical Variability	Steps to Reduce Variability
Sample and assay related	Interlaboratory variability	For clinical chemistry laboratory
Type of specimen	Analytical platforms	Use of a reference lab and reference standard
Type of sample	Lot-lot variability	Replicate measurements
24-h vs. single morning void	Reagents	Add phantoms (dummy Ids) to samples
Sample processing	Calibration functions	Limit multiple lots
Anticoagulant		Freeze samples immediately
Stabilizing agent	Intralaboratory variability	Avoid repeated freeze and thaw cycles
Temperature	Personnel-related	Regular calibration of instruments
Endogenous degrading enzymes	Interreader and intrareader	Assess interassay and intra-assay precision at low and high levels
Freeze-thaw cycles	Temporal drifts	Optimal is <1/4 (CV_I; within-subject variability)
Sample storage	Lot-lot variability	Desirable is <1/2 CV_I
Assay related		Minimal acceptable is <3/4 CV_I
Minimal detection limit		Assess bias (based on CV_I and CV_G where CV_G is between-subject variability) Optimal is <0.125 $(CV_I+CV_G)^{1/2}$
Image acquisition		Desirable is <0.25 $(CV_I+CV_G)^{1/2}$
Interobserver		Minimal acceptable is <0.375 $(CV_I+CV_G)^{1/2}$
Intraobserver		Regular laboratory supervision and assessment of drifts
Biological (subject related)		Develop reference ranges
Intraindividual		Assess impact of covariates on analyte values
Diurnal Day-to-day Seasonal Menstrual Fasting state Beat-to-beat (for imaging studies)		*For imaging studies* Standardized reading protocol Centralized reading Multiple beats measured Blind duplicate readings to evaluate interobserver and intraobserver variability
Interindividual Age Gender		Periodic recertification of Observers

Race Pregnancy Menopausal status Drugs Diet Exercise		*For genotyping studies* Check for mendelian consistent and inconsistent errors (spurious excess of recombinants) Hardy-Weinberg equilibrium checks Analyses of repeats *For microarray data* Minimum Information About a Microarray Experiment (MIAME) standards Includes use of normalization controls (housekeeping genes, RNA spiking)
Metrics of analytical variability **Accuracy**: The degree of agreement of a test result with an accepted reference standard or true value. **Bias**: A quantitative measure of inaccuracy or departure from accuracy. A signed difference between 2 values, T, usually expressed as the difference between 2 values, X−T, or the difference as a percentage of the reference or true value, 100 (X−T)/T. **Precision**: *C*loseness of agreement between independent test results obtained under stipulated conditions; indicates freedom from inconsistency or random error. **Coefficient of variation (CV)**: A measure of precision calculated as the standard deviation of a set of values divided by the average. It is usually multiplied by 100, to be expressed as a percentage. Repeatability relates to essentially unchanged conditions and is often termed *within-series precision* or *within-run precision*. Intermediate precision refers to conditions in which there is variation in 1 or more of the factors time, calibration, operator, and equipment, usually within a laboratory. Reproducibility relates to change in conditions, i.e., different laboratories, operators, and measuring systems (including different calibrations and reagent batches) and is often termed *interlaboratory precision*. **Limit of detection (LoD)**: Smallest amount or concentration of analyte that can be distinguished from background at a stated confidence level. **Limit of quantitation (LoQ)**: Lowest amount of analyte in a sample that can be quantitatively determined with (stated) acceptable precision and (stated, acceptable) accuracy, under stated experimental conditions. **Reference method**: A thoroughly investigated measurement procedure, clearly and exactly describing the necessary conditions and procedures, for the measurement of 1 or more property values that has been shown to have trueness of measurement and precision of measurement commensurate with its intended use. **Reference materials:** Substances with properties that are established for the use as standards, calibrators, controls, the verification of a measurement method, or the assignation of values.		

Reproduced from Vasan, 2006, p.2343

In this thesis, the performances of blood biomarkers that have been extensively studied in the general population were evaluated in South Asian population. Receiver operating characteristics (ROC) curves were used to evaluate the accuracy of the biomarkers in terms of its sensitivity and its specificity. Table 7 shows evaluation of the performance of biomarkers by receiver operating characteristics curves.

Table 7 Evaluation of the performance of biomarkers by receiver operating characteristics curves

An ROC curve is a plot of the sensitivity versus (1−specificity) of a diagnostic test, in which the different points on the curve correspond to different cut points used to determine whether the test results are positive. Sensitivity is defined as the ability of a test to detect disease (condition of interest) when it is truly present, i.e., it is the probability of a positive test result given that the patient has the disease. Specificity is the ability of a test to exclude the disease (condition of interest) in patients, who do not have disease, i.e., it is the probability of a negative test result given that the patient does not have the disease. Predictive value tells us how good the test is at predicting the true positives or true negatives, i.e., the probability that the test will give the correct diagnosis. The positive predictive value is the probability that a patient has the disease given that the test results are positive. The negative predictive value is the probability that a patient does not have the disease or condition given that the test results are indeed negative.

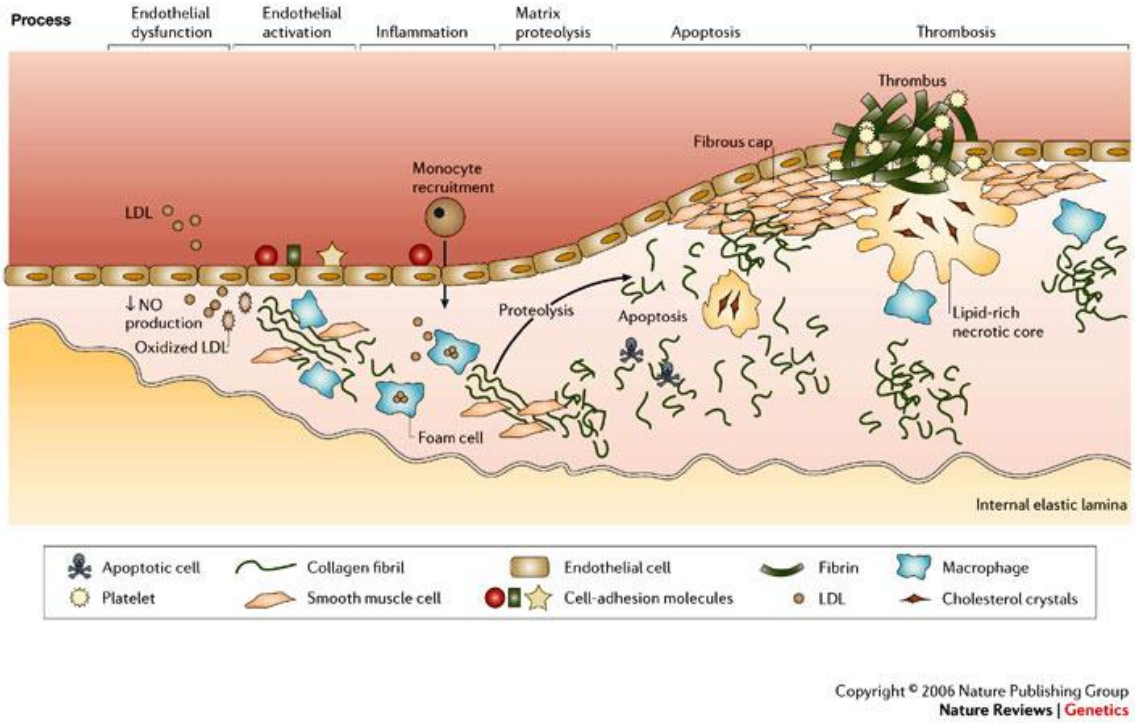
Reproduced from Vasan, 2006, p.2338

1.3 Biomarkers of cardiovascular disease

Blood borne biomarkers in CVD are developed based on the understanding of their role in the pathogenesis of CVD. Atherosclerosis has been implicated in the pathogenesis of ischemic cardiovascular and cerebrovascular diseases (Ross, 1999; Libby et al., 2002). Clinical manifestations of atherosclerosis comprise of CHD, ACS, peripheral artery disease (PAD) and stroke (Lusis, 2004). Figure 7 summarises the pathobiology of cardiovascular disease (Watkins and Farral, 2006).

It is beyond the context of this thesis to list all the biomarkers of CVD, this section mainly concentrates on biomarkers of inflammation and haemostasis which will be measured in our study cohort. Table 8 presents potential blood borne biomarkers of cardiovascular disease.

figure 7 Pathophysiology of cardiovascular disease



Low density lipoprotein (LDL) cholesterol enters dysfunctional endothelium (which is damaged by smoking or diabetes, for example and this is reflected by decreased nitric oxide (NO) production) and is oxidized by macrophage

and smooth muscle cells. Release of growth factors and cytokines, and upregulation of adhesion molecules, attract further monocytes. Foam cells (arising from lipid-laden macrophages) accumulate and smooth muscle cells proliferate, which results in the growth of the plaque. Inflammatory cell infiltrate, smooth muscle cell death through apoptosis, and matrix degradation through proteolysis (by matrix metalloproteinases- MMPs) generate a vulnerable plaque with a thin fibrous cap and a lipid-rich necrotic core. Plaque rupture can cause thrombosis which might be sufficient to cause vessel occlusion.

Reproduced from Watkins and Farral, 2006, p.164

Inflammatory mechanisms play a key role in all stages of the disease from the initial recruitment of circulating leucocytes to the arterial wall to the eventual rupture of the unstable plaque. (Blake and Ridker, 2002; Paffen and DeMaat, 2006). The onset of inflammation within the arterial wall begins with the accumulation of lipids which creates a diseased endothelium (Blake and Ridker, 2002; Ridker, 2005). Adhesion molecules such as P-selectin, vascular cell adhesion molecule –1(VCAM-1) induced by CRP mediates the process of endothelial attachment resulting in the migration of inflammatory cells into the sub endothelial space. Accumulation of foam cells and proliferation of smooth muscle cells results in the growth of the plaque. Apoptosis of smooth muscle cells and infiltration of inflammatory cytokines results in vulnerable plaque with a thin fibrous cap and a lipid-rich necrotic core, rupture of which can cause thrombus or blood clot (Watkins and Farral, 2006).

Table 8 Blood borne biomarkers of cardiovascular disease

Biomarker	**CVD pathophysiological process**
Acute coronary syndrome and silent myocardial ischemia/injury	
Ischemia-modified albumin Glyocogen phosphorylase isoenzyme BB Choline Unbound free fatty acids Cytokines and chemokines Heat shock proteins Autacoid mediators: adenosine, nitric oxide, angiotensin-II, prostanoids, neuro-peptides Vasoactive peptides: endothelin (vasoconstrictor), adrenomedullin, kinins, Nt-pro-BNP/BNP, ANP, calcitonin gene-related peptide, substance P (vasodilator peptides)	Myocardial ischemia
Serum glutamate oxaloacetate transferase Myoglobin CK-MB cTns T and I Glyocogen phosphorylase isoenzyme BB Heart fatty-acid binding protein Heat shock proteins	Myocyte necrosis / irreversible injury
Pregnancy-associated plasma protein A Exracellular matrix molecules (versican), matrix metalloproteinases Myeloperoxidase, other reactive oxygen species (ROS) Cytokines (ICAM-1, VCAM-1, sCD40L, interferon-γ, TNF-α, IL-1; chemokines: MCP-1, MIP-1, IL-8) Platelet-endothelial cell adhesion molecule (PECAM) Placental growth factor	Plaque destabilization
Atherosclerosis (plaque formation and progression)	
Oxidized LDL-C, apoA, apoB, particle size, particle density CRP	Inflammation

Cytokines (IL-6, IL-1β, -10, -12- 18, TNF-α, transforming growth factor-β (TGF-β), sCD40L) and their receptors Chemokines (MCP-1, IL-8) and their receptors Lp-PLA2 Serum amyloid A PECAM Heat shock proteins Adiponectin	
Growth factors (fibroblast growth factor, platelet-derived growth factor, TGF-β) ROS	Smooth muscle cell proliferation, migration and differentiation
Cell adhesion molecules (E-, P-, L-selectins, P-selectin glycoprotein ligand-1, ICAM-1, VCAM-1) Fibrinogen, t-PA, PAI-1, tissue factor, D-dimer, vWF, TAFI sCD40L Homocysteine Lp(a)	Thrombogenesis/lysis
LDL-C, oxidized LDL-C Cell adhesion molecules ROS	Endothelial dysfunction/damage
Oxidized LDL-C Heat shock proteins Microbe antigens Neopterin Interferon-γ Inflammatory cytokines	Immune activation
Oxidized LDL-C ROS: myeloperoxidase, glutatione peroxidase, superoxide dismutase	Oxidative stress
Endopeptidases (matrix metalloproteinase)	Elastin and collagen breakdown
Haemodynamic stress and left ventricular remodelling	
B-type natriuretic peptide (BNP), pro-BNP peptide	Stretch of ventricular myocardium
Atrial natriuretic peptide	Atrial stretch

NT-proBNP – N-terminal pro- B-type natriuretic peptide; BNP – B-type natriuretic peptide; ANP – atrial natriuretic peptide; CK-MB – creatine kinase MB fraction; cTns – cardiac troponins; ROS - reactive oxygen species; ICAM-1 – intercellular adhesion molecule 1; VCAM – vascular cell adhesion molecule 1; sCD40L – soluble CD40 ligand; TNF-α – tumor necrosis factor α; IL-1, -6, -8, -10, -12, -18 – interleukin –1, -6, -8, -10, -12, -18; MCP-1 - monocyte chemoattractant protein-1; MIP-1 - fractalkine macrophage inflammatory protein; PECAM - platelet-endothelial cell adhesion molecule; LDL-C – low density lipoprotein cholesterol; CRP – C-reactive protein; TGF-β - transforming growth factor-β; lipoprotein-associated phospholipase A2 – Lp-PLA2; t-PA – tissue type plasminogen activator; PAI-1 – plasminogen activator inhibitor 1; vWf – von Willebrand factor; TAFI – thrombin activatable fibrinolysis inhibitor; Lp(a) – lipoprotein (a)
Reproduced from Dotsenko et al., 2008

1.3.1 Markers of Inflammation

Inflammation plays a major role in all stages of atherosclerosis i.e., initiation, progression and thrombotic complication of the atherosclerotic plaque. Table 9 shows the inflammatory markers considered as predictors of prevalent or incident CVD by The Centers for Disease Control and Prevention and The American Heart Association (AHA) (Pearson et al., 2003).

Table 9 Inflammatory markers considered as predictors of incident or prevalent cardiovascular disease

Adhesion molecules
Cytokines
Acute phase reactants- Fibrinogen, Serum amyloid A, C-reactive protein
White Blood Cell count
Erythrocyte sedimentation rate

Adapted from Pearson, Circulation 2003, p.501

The following sections summarize some of the prominent inflammatory biomarkers in clinical practice and evidences from epidemiological and clinical studies.

1.3.1.1 C-reactive protein

C reactive protein is the widest in use inflammatory biomarker. Healthy subjects have serum CRP levels of <5 mg/L and levels above these are considered abnormal. Plasma concentration of CRP can raise 10000 fold in 24 hours in an event of tissue injury. For the purpose of CVD screening, it is recommended to use high sensitivity assay for CRP (Hs-CRP). The assay can detect very low levels of CRP in blood down to 0.3 mg/L in apparent healthy subjects and was introduced after a standardization program at the Centers for Disease Control and Prevention. In recent years, the acute phase reactants such as CRP have been studied as potential markers of more subtle and persistent systemic variables loosely termed as low grade inflammation. The detection of this biomarker is easy and cost effective with commercially available Hs-CRP assay kits. The

coefficient variation of this assay is generally <10%. There is a considerable intraindividual variability in the levels of CRP even in the absence of underlying inflammatory conditions (Pearson, 2003). Table 10 shows the biological states that are associated with variation in CRP levels.

Ridker et al., (2001a) showed in the AFCAPS/TexCAPS trial that the levels of CRP could remain stable over a long period of time in 2834 metabolically stable subjects; there was no difference in the median levels of CRP at baseline and follow up at 1 year. Similarly Meier-Ewert et al., (2001) and Ockene et al., (2001) showed that CRP levels were not subjected to circadian variations and exhibited high reproducibility.

Table 10 Biological states associated with variations in CRP levels

Increased levels of CRP	Age Elevated blood pressure Elevated body mass index Cigarette smoking Metabolic syndrome/ diabetes mellitus Low HDL and high triglycerides Estrogen/progesterone hormone use
Decreased levels of CRP	Moderate alcohol consumption Physical activity Weight loss

Adapted from Pearson 2003, p.503

According to the recommendations of American Heart Association, Hs-CRP assay should be performed on metabolically stable subjects without obvious inflammatory conditions and expressed as mg/L only. The cut-off values for low risk (<1.0 mg/L), average risk (1.0 to 3.0 mg/L), and high risk (>3.0 mg/L) are based on the distribution of hs -CRP in the adult population (Pearson, 2003; Fortmann et al., 2004; Myers et al., 2004).

1.3.1.1.1 Association of C-reactive protein with cardiovascular disease

Prospective studies have demonstrated the role of CRP in primary and secondary prevention of CVD. This section summarizes the findings from major epidemiological and clinical studies that associated CRP with incident CVD events, recurrent events and mortality rates.

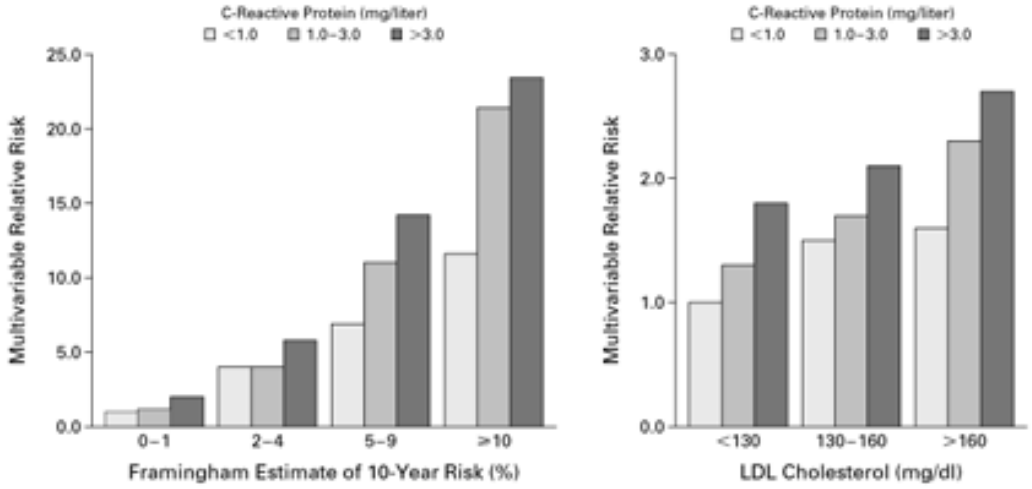
1.3.1.1.1.1 C-reactive protein and prediction of incident cardiovascular events

Prospective studies conducted in healthy subjects showed a dose-response relationship between the levels of CRP and the risk of incident or first CVD event. Ridker (1997) showed that healthy men with high CRP levels had three times the risk of MI (relative risk, 2.9; 95% confidence interval, (1.8-4.6); P<0.001) and twice the risk of stroke (relative risk, 1.9; 95% confidence interval, (1.1-3.3); P=0.02) compared to men with low CRP levels.

A similar nested case control study in 28,263 healthy women showed that the relative risk for cardiovascular events in women in the highest quartile to be 1.5; (95% confidence interval, (1.1 - 2.1); P<0.001) compared to those in the lowest quartile of hs- CRP. In this study, Ridker (2000a) demonstrated that prediction models that incorporated both hs- CRP and lipid levels were better at predicting the risk of CVD compared to the model based on lipid levels alone (P<0.001). In the ROC analysis, the addition of CRP levels to total cholesterol improved the area under the curve (AUC) from 0.59 to 0.66 (P<0.001) and the addition to the ratio of total cholesterol to HDL- c from 0.64 to 0.68 (P<0.001). The risk of CVD was significantly higher among women who had high levels of CRP and targeted low levels of LDL cholesterol. These findings suggest that incorporation of CRP can significantly improve models for the prediction of cardiovascular risk and may lead to better clinical identification of patients who might benefit from primary prevention.

Later Ridker (2002) demonstrated in 27,939 healthy women followed up for a mean of 8 years, that plasma levels of CRP are a better predictor of future CVD events at all levels of estimated 10-year risk for events according to the Framingham risk score and LDL-c. Figure 8 shows multivariable-adjusted Relative Risks of Cardiovascular Disease based on the levels of C - reactive protein (AHA guild lines) and the Estimated 10-Year Risk Based on the Framingham Risk Score; and levels of C - reactive protein and categories of LDL Cholesterol.

figure 8 *Multivariable-adjusted Relative Risks of Cardiovascular Disease based on the levels of C-Reactive Protein (AHA guildlines) and the estimated 10-Year Risk Based on the Framingham Risk Score; and levels of C-Reactive Protein and categories of LDL Cholesterol.*

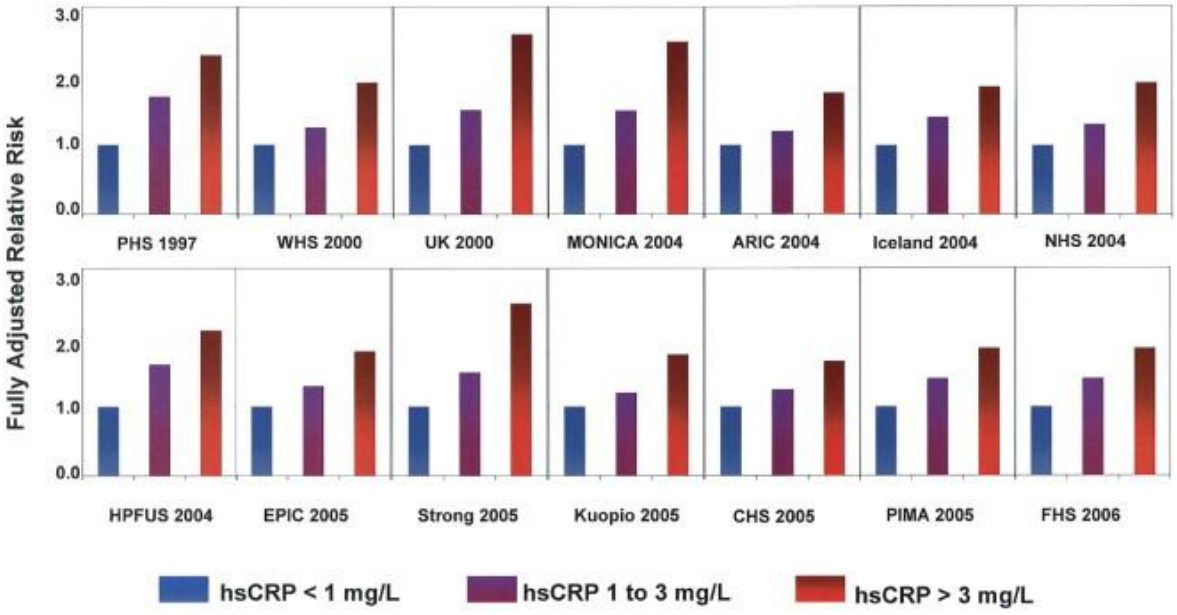


Reproduced from Ridker, 2002, p.1564

A number of prospective studies have demonstrated the ability of CRP to predict incident CVD events in diverse populations which included the women's health study (Ridker et al., 1998a; Ridker, 2000a; Ridker, 2002), the Honolulu Heart study (Curb et al., 2003), the MONICA (Multinational MONInitoring of trends and determinants in CArdiovascular disease)- Ausberg cohort, the ARIC (Atherosclerosis Risk in Communities) study. Meta analyses of prospective population studies (Danesh, 1998) compared individuals with CRP values in the upper tertile

with those in the lower tertile at baseline showed a combined risk ratio 1.7 (95% CI, 1.4-2.1) for CVD. These prospective studies recorded CVD events some years after baseline blood collection, thus CRP measurement preceded the onset of disease. The study design allows to estimate relative risk across various strata of hs- CRP (Pearson, 2003). The relative risk of future cardiovascular events in 14 healthy populations with baseline CRP levels of <1 mg/L, 1 to 3 mg/L and >3 mg/L after adjustment for traditional risk factors (Figure 9). The findings of these studies are highly consistent with no evidence of heterogeneity.

figure 9 Relative risk of future cardiovascular events in 14 healthy populations with baseline Hs-CRP levels of <1 mg/L, 1 to 3 mg/L and >3 mg/L after adjustment for traditional risk factors.



Reproduced from Ridker, 2007a,

In the Cardiovascular Health Study, Tracy et al., (1997) reported higher levels of CRP in subjects with subclinical disease at baseline in 5201 healthy elderly men and women. The presence of subclinical disease was defined based on the resting electrocardiogram, echocardiogram, carotid artery duplex scanning, ankle-brachial blood pressure, history of CVD, Rose questionnaire,

cardiovascular surgery, and use of selected medications consistent with the diagnosis of angina or congestive HF. Mean baseline levels of CRP in women with subclinical disease compared to control subjects were 3.33 and 1.90mg/L respectively (p=<0.05). There was a 2.3 fold (95% CI, 0.9-6.1) increase in the risk of incident MI among women with subclinical disease with CRP levels in the upper quartile (2.79 -46.04 mg/L). The overall odds ratio (OR) for incident MI in both men and women was 2.67 (95% confidence interval [CI], 1.04-6.81), with a stronger relationship in women (4.50 [0.97 to 20.8]) than in men (1.75 [0.51 to 5.98]).

Epidemiological studies have also demonstrated a link between inflammatory markers and incident HF. In the Framingham Heart Study, 732 patients (mean age 78 years, 67% women) free of prior MI and HF were followed up for a mean of 5.2 years. There was a 2.8-fold increase in the risk of HF amongst those with a serum CRP level ≥5 mg/dL (95% CI, 1.22 -6.50, *P*=0.02) (Vasan et al., 2003).

CRP has not been a good measure for the extent of atherosclerosis in studies by Redberg et al., (2000); Folsom et al., (2001) and Hunt et al., (2001). The marker showed poor association with tests that measure the extent of atherosclerosis such as Doppler ultrasound scans of carotid arteries and electron beam computerized tomography for coronary calcium. On the contrary, some studies have shown positive association between CRP and atherosclerotic burden, further research is required to establish this relationship (Tataru et al., 2000; Gronholdt et al., 2000).

1.3.1.1.1.2 C reactive protein and prediction of cardiovascular disease risk factors

Apart from prediction of incident CVD events, CRP has shown to predict incident T2DM, hypertension and modify risk associated with metabolic syndrome. In the women's health study, the relative risks of T2DM for women in the highest vs. lowest quartiles of CRP were 4.2 (95%

CI, 1.5-12.0; P=0.001) independent of BMI and insulin (Pradhan, 2001). Similar findings were reported in middle aged men in the West of Scotland Coronary Prevention study, whereby men in the upper quintile of CRP (>4.18 mg/L) had threefold risk for T2DM compared to those in the lower quintile (<0.66 mg/L) after adjustment for established CVD risk factors including blood pressure and lipid levels (Freeman et al., 2002).

In the nondiabetic population of the Insulin Resistance Atherosclerosis Study (IRAS), 1008 participants aged 40 -69 years, of whom 33% had impaired glucose tolerance were studied (Festa et al., 2000). A linear relationship between the CRP levels and an increase in the number of metabolic disorders (dyslipidaemia, body adiposity, insulin resistance and hypertension) was observed, in the presence of 0, 1, 2, 3, or 4 metabolic disorders, the mean log of CRP levels (±SE) were 0.075 ± 0.06, 0.511 ± 0.06, 0.845 ± 0.07, 1.34 ± 0.10 and 1.39 ± 0.17 respectively. CRP was strongly associated with BMI, waist circumference, insulin sensitivity, fasting insulin and proinsulin (all correlation coefficients > 0.3, P<0.0001).

Sesso et al., (2003) showed CRP to predict incident hypertension in women, the relative risks of developing hypertension from the lowest to the highest levels of CRP were 1.00, 1.07 (95% CI, 0.95-1.20), 1.17 (95% CI, 1.04-1.31), 1.30 (95% CI, 1.17-1.45), and 1.52 (95% CI, 1.36-1.69) (P<.001) respectively after adjustment for established CVD risk factors. Studies have also reported an association between elevated CRP levels and incident ischemic stroke (Rost et al., 2001), PAD (Ridker et al., 2001b) and sudden death (Albert et al., 2002).

1.3.1.1.1.3 Utility of c-reactive protein in clinical practice over established cardiovascular disease risk factors

Although prospective studies have demonstrated independent predictive power of CRP (Ridker, 2002; Cushman et al., 2005; Folsom et al., 2006), there is a growing concern regarding the

usefulness of CRP measurement to improve CVD risk prediction beyond those provided by classical risk factors. Prospective studies conducted to determine the role of CRP in everyday clinical practice suggest that CRP measurements may have limited clinical utility as a biomarker for CVD risk assessment. (van der Meer et al., 2003; Miller et al., 2005; Wang et al., 2006). In the third National Health and Nutrition Examination Survey, the elevated levels of CRP in the general population were attributed to the presence of traditional CVD risk factors mainly measure of adiposity and high levels of CRP rarely occurred in their absence (Miller, 2005).

The incorporation of CRP to global CVD model to assess future CVD events in a cohort of 24 558 healthy women, followed up for more than 10 years resulted in the reclassification of 40% - 50% of women at intermediate risk based on Adult Treatment Panel III (ATP-III) score to higher or lower risk categories (Ridker et al., 2007b). This finding is in conflict with the Framingham heart study by Wang, (2006) which showed a minimal contribution of CRP to CVD risk assessment.

1.3.1.1.1.4 C-reactive protein and prediction of recurrent cardiovascular events and deaths

Prospective studies suggest that CRP may have a role in the risk stratification of patients with already established CVD. In the European Concerted Action of Thrombosis and Disabilities (ECAT) Angina Pectoris study**,** hs- CRP was a predictor of coronary events in 2121 patients with angina, followed up for two years. The risk of coronary event in patients whose CRP levels were >3.6mg/L was almost doubled compared to those with lower levels of CRP (Haverkate, 1997).

Speidl (2002) studied a group of 125 patients with premature CHD for 54 months. Clinical end points were defined as death, MI, need for coronary revascularization, or admission to hospital with angina pectoris. There was a 3.8-fold (risk ratio 3.82, 95% CI 1.19-12.17) increased risk of

future CVD events in patients in the highest tertile of CRP levels compared to the patients in the lowest tertile.

Similarly in patients with unstable CHD, followed up for almost 36 months, CRP was a predictor of mortality. The mortality rates were 5.7% among 314 patients with CRP levels of <2 mg/L, 7.8% among 294 patients with CRP levels of 2 -10 mg/L and 16.5% among 309 patients with CRP levels >10 mg/L (P= 0.29 and P=0.001, respectively) (Lindahl, 2000). In the lowa 65+ Rural Health study, higher CRP levels (≥ 2.78 mg/L) were associated with increased risk of death (RR=1.6; 95% CI, 1.0-2.6) in 1293 elderly subjects (mean age of 77.8 (3.2) years) (Harris, 1999).

Biasucci et al., (1999a) showed in 53 patients with unstable angina who were followed up for 1 year that those who had elevated CRP ≥ 3 mg/L were at increased risk of recurrent CVD events. At discharge, 27 patients had normal CRP levels and 26 patients had elevated CRP levels, median CRP levels were 2.4 mg/L (range, 0.6-2.9 mg/L) and 9.9 mg/L (3.3-9 mg/L), (p<0.001) respectively. During the follow up, only 15% (4 patients) with discharge CRP levels of <3mg/L but 69% (18 patients) with elevated CRP had coronary events, 4 had MI and 14 showed phases of instability. The odds ratio for recurrent instability at 1 year in those with elevated CRP levels >3mg/L was 8.57 (95% CI, 1.66-44.2) p=<0.01. New phases of instability occurred in 13% of patients in the lower tertile of CRP (≤2.5 mg/L), in 42% of those in the intermediate tertile (2.6 to 8.6 mg/L), and in 67% of those in the upper tertile (≥8.7 mg/L,). During follow up, the recurrence of coronary events were significantly higher in patients in upper tertile of CRP (≥8.7 mg/L) compared to patients in intermediate (2.6 to 8.6 mg/L) and lower (≤2.5 mg/L) tertiles (*P*<0.001).

A similar study in 32 patients with chronic stable angina, 31 with severe unstable angina, and 29 with acute MI showed that elevated CRP levels at the time of hospital admission predicts poor outcomes in patients with unstable angina. Twenty patients with unstable angina had elevated levels of CRP $\geq$ 0.3 mg per decilitre and experienced more ischemic episodes in the hospital than those with levels <0.3 mg per deciliter (mean [±SD] number of episodes per patient, 4.8 ±2.5 vs. 1.8 ±2.4; P = 0.004). Moreover, 22 patients with MI who had levels of CRP $\geq$ 0.3 mg per decilitre had unstable angina (Liuzzo et al., 1994).

1.3.1.2 Interleukin 6

IL-6 is considered to be less superior to CRP in the assessment of inflammatory response and the risk of CVD. IL-6 is subjected to diurnal variations and is a less stable when compared to CRP. The expression of CRP is directly regulated by IL-6 while the synthesis of IL-6 is by diverse stimuli (Vickers et al., 2002).

1.3.1.2.1 Association of interleukin 6 with cardiovascular disease

This section summarizes the findings from major epidemiological and clinical studies that associated IL-6 with incident CVD events, recurrent events and mortality.

1.3.1.2.1.1 Interleukin 6 and prediction of incident cardiovascular events

A prospective study conducted by Ridker (2000a) in 28,263 healthy women showed that the baseline levels of IL-6 were significantly higher in women with CVD events (median (IQR), 1.65 (1.14-2.62) pg/ml) compared to women free of CVD events (1.30 (1.00-2.03) pg/ml, P=0.003). The relative risk for future cardiovascular events in women in the highest quartile compared to those in the lowest quartile of IL-6 was 2.2; (95% confidence interval, (1.1 -4.3); P=0.02). The prediction models that incorporated IL-6 along with lipid levels were better at predicting the risk

of CVD compared to the model based on lipid levels alone (P<0.01). The addition of IL-6 to the lipid measurements in the ROC analysis increased the AUC from 0.59 to 0.64 (P<0.003).

In the same year again, Ridker (2000b) showed in the Physician's health study that plasma levels of IL-6 predicts the risk of future MI in 14,916 healthy men who were followed up for 6 years. During the follow up, 202 men developed MI and had higher levels of IL-6 compared to those who were free of CVD (1.81 versus 1.46 pg/mL; *P*=0.002). The relative risk of MI in men in the highest quartile compared to those in the lowest quartile was 2.3 (95% CI 1.3 to 4.3, *P*=0.005). There was a 38% increase in the risk of MI for each quartile increase of IL-6 (95% CI, 15% - 66%, *P*=0.001).The association between plasma levels of IL-6 and the risk of future MI remained significant after adjustment for CVD risk factors and CRP (*P*<0.001).

Epidemiological studies have demonstrated a link between IL-6 and incident HF. Vasan (2003) demonstrated IL-6 to be a predictor of HF in the Framingham heart study, where there was a 68% increase in the risk of HF across the increasing tertiles of serum IL-6 levels (P=0.03). In the Multi-Ethnic study of Atherosclerosis, levels of IL-6 showed a strong independent inverse association with prognostic measure of HF (regional left-ventricular (LV) function) in 894 healthy individuals without documented evidence of CVD. In this prospective community based study, 44.6% were women and the median age was 67 (IQR, 59-74), the participants were assessed for the presence of subclinical atherosclerosis and CVD risk factors. High levels of IL-6 were independently associated with reduced systolic function after adjustment for demographic factors, CVD risk factors and measures of subclinical atherosclerosis, in the LV septum [regression coefficient = 1.03, 95% CI, 0.26-1.79, P = 0.008] and inferior wall (regression coefficient = 1.65, 95% CI 0.74-2.56, P < 0.001). Here regression coefficient of 1.03 means that 1.03 variation in the dependent variable is predicted by one unit increase in IL-6 levels. Elevated

levels of IL-6 showed a stronger independent association with reduced regional systolic function compared to CRP (Yan et al., 2010).

IL-6 has shown to predict incident T2DM. In the women's health study, the relative risks of T2DM for women in the highest vs. lowest quartiles of IL-6 were 2.3 (95% CI, 0.9 -5.6; P for trend =0.07) independent of BMI and insulin. Overall the RR for future diabetes increased by 28% per quartile increase in baseline IL-6 levels (P=0.07) (Pradhan, 2001).

1.3.1.2.1.2 Interleukin 6 and prediction of recurrent cardiovascular events and deaths

Prospective studies have reported elevated levels of plasma IL-6 in patients with ACS. Biasucci (1996) provided evidence for the role of IL-6 in unstable angina in a case control study. The levels of IL-6 were undetectable (<3pg/mL) in healthy subjects, were determined in only 6 (21%) out of 29 patients with stable angina but were detectable in 23 (61%) of 38 patients with unstable angina (P<0.01). The plasma levels of IL-6 were significantly different in both patient groups, median levels 5.25 pg/ml (range, 0-90pg/ml) in patients with unstable angina and below detection limit (0-7pg/ml) in patients with stable angina (p<0.005). Moreover, 19 (83%) of 23 patients with unstable angina who had detectable levels of IL-6 had a complicated in hospital outcome. IL-6 was positively correlated with CRP (r=0.4, p=0.013), that means the levels of IL-6 were directly related to levels of CRP. A better positive correlation between IL-6 and CRP (r=0.96, p=0.002) was shown by Sturk et al., (1992).

Biasucci (1999b) again showed higher levels of cytokines in patients with unstable angina who had an in-hospital CVD event after 48 hours. In 43 patients aged 62±8 years admitted with unstable angina who were given the same medical therapy, 26 patients had a CVD event. In patients who were free of any in-hospital event, the levels of IL-6 decreased at 48 hours from

(median (IQR)) 4.7 pg/ml (0.1-12.6) to 0.94 pg/ml (0.05-10.4). In patients with a CVD event, the levels of IL-6 increased from 7.3 pg/ml (0.1-22.9) to 9.5pg/ml (0.1-47.5), $p<0.01$. The levels of IL-6 were significantly different between the two groups at admission ($p=0.018$).

Miyao et al., (1993) reported elevated levels of IL-6 in 23 patients with MI in a case control study. The subjects were followed for 4 weeks. The plasma levels of IL-6 in patients with MI were significantly higher (ranging from mean (SD), 28.5 (6.6) pg/ml at admission to 46.5 (7.8) pg/ml at discharge) compared to control subjects (11.4 (2.9) pg/ml, $p<0.01$).

In the lowa 65+ Rural Health study, 1293 healthy subjects were followed up for a mean of 4.6 years. The relative risk of death for the highest quartile of IL-6 ($\geq$ 3.19 pg/ml) compared with the lowest quartile of IL-6 was 1.9 (95% CI, 1.2-3.1). During follow up, subjects with both elevated IL-6 and CRP had almost a threefold greater risk of mortality compared to those with low levels of inflammatory markers. These findings were independent of age, sex, BMI, smoking status, diabetes and CVD (Harris, 1999).

1.3.2 Lipids and lipoproteins

In the 1940's the "lipid hypothesis" was a target of swirling controversy and it was only after the discovery of statin in the late 1980's that the causal relationship between cholesterol accumulation in the arterial wall and atherosclerosis was proven (Steinberg, 2004). The 'Atherogenic lipid triad' of high serum triglyceride levels, low serum HDL-c levels and high levels of LDL-c particles along with insulin resistance, visceral adiposity and a low grade inflammation are strong predictors of T2DM and cardiovascular complications (Reilly and Rader, 2003).

In plasma, lipoproteins mainly transport water-insoluble lipids such as triglycerides, cholesterol and cholesteryl esters. Apolipoprotein B containing lipoproteins are of two forms –apoB48, expressed in mammalian intestine and apoB100, expressed in mammalian liver. ApoB containing lipoproteins, VLDL, LDL and lipoprotein (a). (Lp (a)) are atherogenic and an elevated level of these lipoproteins in the plasma are associated with increased risk of CHD (Gusarova et al., 2003).On the contrary, apolipoprotein A1 (ApoA1), main protein of HDL particle is anti atherogenic and plays a major role in reverse cholesterol transport. This section briefly summarise the epidemiological studies that have investigated the association between lipids and lipoproteins with CVD.

1.3.2.1 Triglycerides and cardiovascular disease

The role of triglycerides in CVD is controversial (Hulley et al., 1980); however case control studies have demonstrated triglycerides as an independent risk factor for CVD (Brunner et al., 1977; Castelli et al., 1977; Reardon et al., 1985). Austin et al., (1988) examined the association between LDL subclass phenotypes and the risk of MI in 109 cases and 121 controls. Patients had more small dense LDL particles which were associated with 3 fold increase risk of MI. Triglycerides and low HDL-c were independent predictors for the increased risk of MI associated with small dense LDL phenotypes.

In a meta-analysis of population based cohort studies (Hokanson and Austin, 1996), the evidence for the association between triglycerides and CVD was weakened after adjustment for total cholesterol, LDL-c or HDL-c. The meta analysis was performed on 17 studies that involved 46413 men and 10864 women, the univariate relative risk per 1 mmol/l increase in triglyceride were 1.32 (95% CI, 1.26-1.39) and 1.76 (95% CI, 1.50-2.07) for men and women respectively.

After adjustment for HDL-c, the relative risk was reduced to 1.14 (95% CI, 1.05-1.28) and 1.37 (95% CI, 1.13-1.66) in men and women respectively, all $p<0.05$.

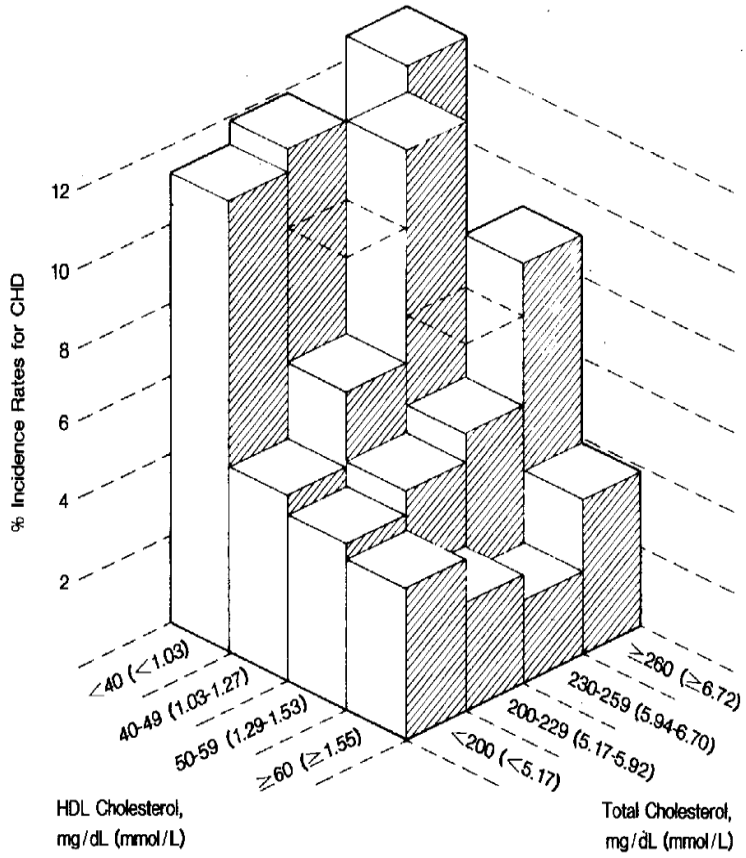
Sarwar et al., 2007 performed a meta-analysis consisting of 29 Western prospective studies involving 262,525 participants who were followed up for 12.1 years. The odds ratio for increased risk of CVD was 1.72 (95% CI, 1.56-1.90) in subjects in the upper tertile of triglycerides compared to the lower tertile. In 2009, the Emerging Risk factors Collaboration conducted meta-analyses of 68 prospective studies involving 302,430 participants. After adjustment for age and sex, the hazard ratio for CHD with triglyceride was 1.37 (95% CI, 1.31-1.42), however after adjustment for HDL-c and non-HDL-c, this was reduced to 0.99 (95% CI, 0.94-1.05).

1.3.2.2 Low density lipoprotein cholesterol, total cholesterol, high density lipoprotein cholesterol and cardiovascular disease

A prospective meta-analysis of 14 randomized trials of statins involving 90,056 participants demonstrated that a 1.0 mmol/l reduction in LDL-c over 1 year reduced coronary mortality by 19% (rate ratio, RR 0.81 (95% CI, 0.76-0.85), $p<0.0001$, all-cause mortality by 12% (RR 0.88 (95% CI, 0.84-0.91), $p<0.0001$, major vascular events by 21% (RR 0.79, 95% CI, 0.74-0.80), $p<0.0001$ (Baigent, et al., 2005). Further reduction in CV events was demonstrated by Cannon et al., (2006) with intensive statin therapy. In the Prospective Studies collaborative meta-analysis of 61 prospective observational studies, a 1mmol/l reduction in total cholesterol was associated with lower CHD mortality at all age groups (hazard ratio of 0·44 (95% CI, 0·42—0·48), 0·66 (0·65—0·68) and 0·83 (0·81—0·85) at ages 40—49, 50—69, and 70—89 years respectively) (Prospective Studies Collaboration, 2007).

HDL-c is inversely associated with the risk of CVD (Gordon, 1977; Gordon et al., 1989). Castelli et al., (1986) demonstrated in participants of the Framingham study who were followed up for 12 years that HDL-c is an independent determinant of CHD risk. Figure 10 shows the incidence of CHD by HDL-c and total cholesterol levels.

figure 10 *Incidence of coronary heart disease by high density cholesterol and total cholesterol level in the Framingham Study*



Dashed lines indicate two bars that are hidden from view: CHD rate for HDL-C less than 40 mg/dL (<1.03mmol/L), total cholesterol 230 to 259 mg/dL (5.95 to 6.70 mmol/L) is 10.7%;
CHD rate for HDL-C 40 to 49mg/dL (1.03 to 1.27 mmol/L), total cholesterol greater than or equal to 260 mg/dL (6.72 mmol/L) is 6.6%. CHD rate for those with HDL-C levels < 40 mg/dL (<1.03mmol/l) and normal Total-C < 200 mg/dL (<5.17 mmol/l) is 11.24% Reproduced from Castelli, 1986, p.2837

1.3.2.3 Apolipoproteins and cardiovascular disease

In the INTERHEART case-control study, McQueen et al., (2008) demonstrated in 9,345 MI patients and 12,120 age and gender matched controls that ApoB/ApoA1 ratio was a better discriminator than any of the lipid measures for the risk of MI. The odds ratio for MI per 1 SD (standard deviation) change in ApoB/ApoA1 ratio was 1.59 (95% CI, 1.52-1.64), in

ApoA1 0.67 (95% CI, 0.65-0.70), ApoB 1.32 (95% 1.28-1.36), in total cholesterol 1.16 (95% CI, 1.13-1.19), in HDL-c 0.85 (95% CI, 0.83-0.88), in total cholesterol/HDL-c 1.17 (95% CI, 1.13-1.20). Parish et al., (2009) demonstrated in a case-control study consisting of 3510 MI patients and 9805 controls that ApoA1 and Apo B were predictive of risk of MI. The relative risk of MI with ApoA1/ApoB ratio was 7.3 (95% CI, 5.8-9.2) when comparing those in the top with bottom decile of this ratio.

In the AMORIS (Apolipoprotein-related mortality risk) study, apolipoproteins were better predictors of fatal MI compared to normal lipid measures. The age adjusted risk ratios (RR) for fatal MI per 1SD change in ApoB was 1.43 (95% CI, 1.28-1.60) in men and 1.34 (95% CI, 1.10-1.62) in women whilst in ApoB/ApoA1 ratio, RR was 1.23 (1.18-1.27) in men and 1.38 (1.25-1.52) in women, all $p<0.05$.

1.3.3 Circulating cytokines, adhesion molecules and immune regulation molecules

Atherosclerosis is a chronic inflammatory disease and a complex network of factors: cytokines, chemokines, cell adhesion molecules, reactive oxygen species, immune regulation molecules, acute phase reactants to name a few mediate the underlying immunologic and inflammatory process. These mediators function as potential targets for monitoring the different stages of

atherosclerosis or the underlying inflammatory process and in providing prognostic information towards CVD risk. There is limited data on the predictive value of markers such as IL-8, IL-18, TNF-α, MCP-1, P-selectin, E-selectin, ICAM-1 and VCAM-1 in CVD. Most of the published research on these markers has been done in prospective nested case-control studies (Dotsenko, 2008).

1.3.3.1 Adhesion molecules

In a nested case-control study, Ridker et al., (1998b) measured soluble intercellular adhesion molecule 1 (sICAM-1) in 474 patients who developed their first MI and 474 controls after 9 years of follow up. The relative risk of future MI was 80% higher in subjects whose baseline sICAM-1concentration were in the highest quartile >260ng/ml (RR 1.8 [1.1-2.8], p=0.02). Luc et al., (2003) conducted a case-control study nested in the PRIME study where ICAM-1 was associated with the risk of future coronary events. The relative risk of future coronary events in patients who had high levels of ICAM-1 (>625 ng/ml) compared to those with low levels (<505 ng/ml) was 1.90 (95% CI, 1.21-2.96), p=0.005 after adjustment for major risk factors.

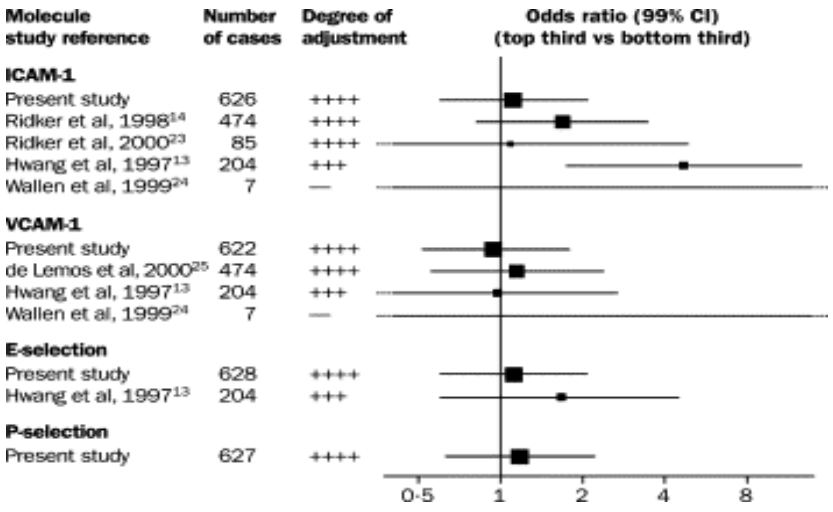
Pradhan et al., (2002) demonstrated in the Physician's Health Study, a nested case-control study consisting of 14,916 middle-aged men that levels of ICAM-1 at baseline predicted the development of PAD. After adjustment for lipid and non-lipid risk factors, the odds for the development of PAD in men in the top quartile of ICAM-1 levels compared to those in the bottom quartile was 3.9 (95% CI, 1.7-8.6), p=0.001. In the ARIC study (Hwang et al., 1997), CHD subjects were identified after a 5 year follow up period. Subjects in the highest quartile of baseline ICAM-1 levels had 5.53 times increased risk of developing incident CHD and 2.64 times greater chance of having carotid artery atherosclerosis compared to those in the lowest quartile

(95% CI, 2.51-12.21) and (95% CI, 1.40-5.01) respectively. Similarly the odds for carotid artery atherosclerosis amongst those in the highest quartile of E-selectin compared to those in the lowest quartile were 2.03 (95% CI, 1.14-3.62). However sVCAM-1 levels were not associated with CV risk in the same cohort (de Lemos et al., 2000).

In a prospective cohort of 1246 patients with angiographically documented CHD, there was a 2.8-fold increase in the risk of future fatal CV events amongst patients in the top quartile of baseline sVCAM-1 levels compared to the bottom quartile (95% CI, 1.4-5.4), p=0.003 after controlling for all major inflammatory markers (Blankenberg et al., 2001).

Malik et al., (2001) conducted a meta-analysis of prospective studies of soluble adhesion molecules and CHD. The odds for CHD after adjustment for major risk factors in men in the top third of baseline measurements of adhesion molecules compared to the bottom thirds were as follows for VCAM-1: 0.96 (0.66-1.40); E-selectin: 1.13 (0.78-1.62); and P-selectin: 1.20 (0.81-1.76) respectively. The study concluded that adhesion molecules do not add much predictive value compared to existing established risk factors. Figure 11 shows the prospective studies that were included in the meta-analysis and odds ratio for CHD in individual studies in comparison to men in the top thirds of baseline measurements of adhesion molecules with those in bottom thirds of measurements.

figure 11 *Meta-analysis of prospective studies of adhesion molecules and risk of coronary heart disease*



Reproduced from Malik, 2001, p.972

1.3.4 Markers of thrombosis, haemostasis and coagulation pathway

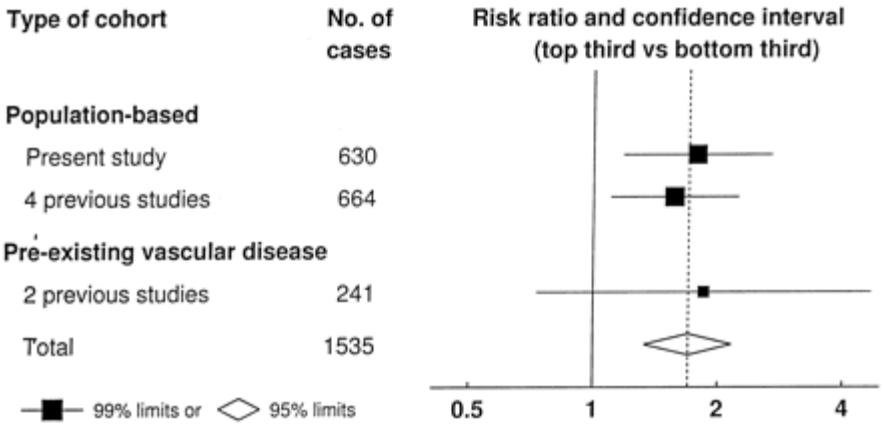
1.3.4.1 Thrombotic, haemostatic and coagulation markers and incident cardiovascular disease

1.3.4.1.1 Thrombotic markers

In the study by Danesh et al., (2001) which involved 630 cases and 1269 controls nested in a prospective cohort of 5661 men followed up for 16 years. The odds for CHD in men with baseline fibrin D-dimer in the top third compared to those in the bottom third was 1.79 (95% CI 1.36 -2.36) after adjustment for conventional risk factors. In a meta-analysis of seven prospective studies including the above mentioned study involving a total of 1535 CHD cases who were followed up for a mean of 5 years. The combined odds ratio in participants with baseline D-dimer

values in the top third compared to those in the bottom third was 1.7 (95% CI 1.3-2.2).Figure 12 shows the meta-analysis of prospective studies of D-dimer and coronary heart disease

figure 12 Prospective studies of D-dimer and coronary heart disease



Reproduced from Danesh, 2001, p.2326

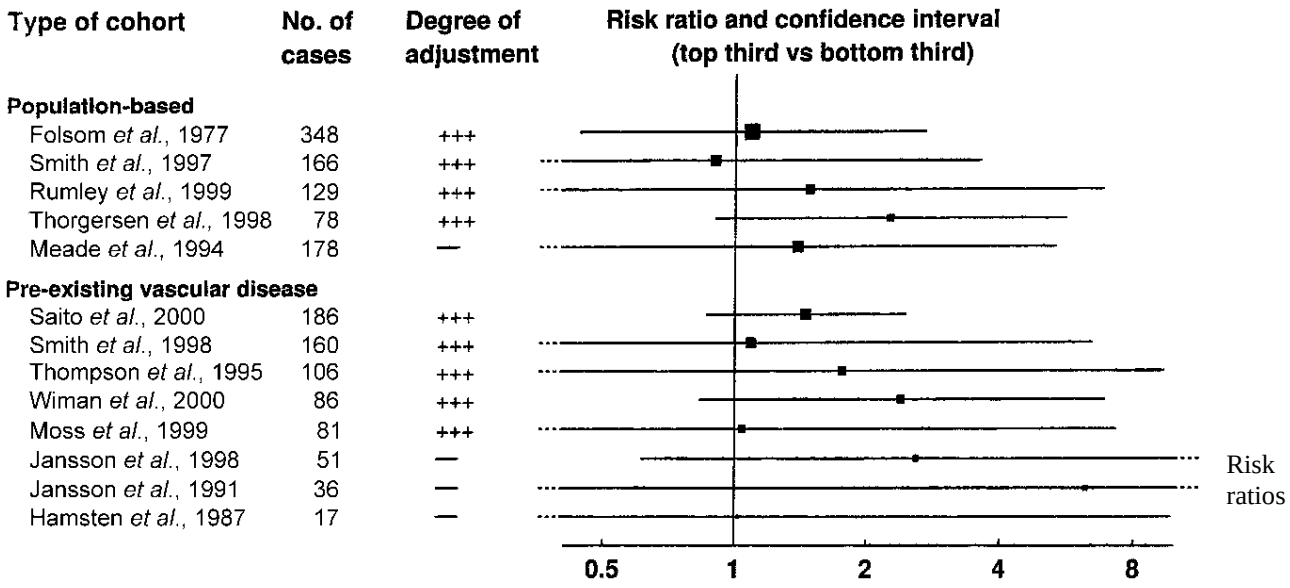
1.3.4.1.2 Markers of endothelial dysfunction

In a prospective incident case-control study from Sweden, von willebrand factor (vWF) was strongly associated with first MI, with an odds ratio of 2.52 (95% CI, 1.72-3.67) in patients with high levels of vWF at baseline (Wennberg et al., 2011).

Whincup et al., (2002a) investigated the relation between vWF and risk of CHD in 625 men with coronary events and 1226 controls nested in a prospective study of 5661 men aged 40-49 years, who were followed up for 16 years and conducted a meta-analysis of relevant studies at that time. Men who had baseline vWF concentration above 126 IU.dl had an increased risk of CHD, odds ratio 1.82 (95% CI, 1.37 -2.41) compared to those who had less than 90 IU.dl (-1), odds ratio, 1.53 (95% CI, 1.10 -2.12), p <0.001. In five prospective studies (Meade et al., 1994; Folsom et al., 1997; Smith et al., 1997; Rumley et al., 1999; Thorgersen et al., 1998) involving a total of

899 cases of MI and 18 553 controls with a mean follow up of 7 years. The combined odds ratio for CHD was 1.2 (95% CI 0.8-1.9) in subjects with vWF levels in the top third compared to those in the bottom third. Figure 13 summarises the prospective studies of vWF and CHD published before mid-2001.

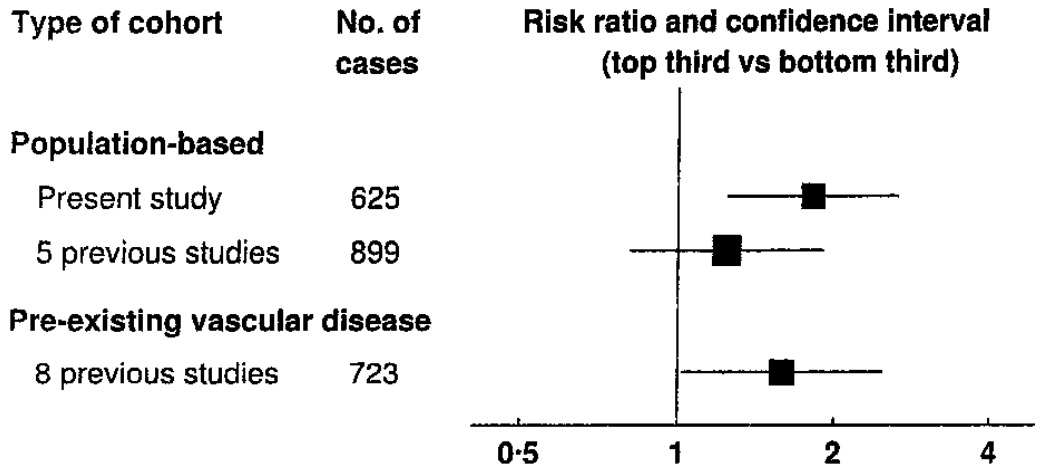
figure 13 Prospective studies of von Willebrand factor and coronary heart disease published before mid-2001



compare top and bottom thirds of baseline measurements. Black squares indicate the risk ratio in each study, with the square size proportional to the number of cases and the horizontal lines representing 99% confidence intervals.—, no adjustment reported for possible confounders; +, adjustment for age and sex only; ++ for these plus smoking; +++, for these plus some other classical vascular risk factors.
Reproduced from Whincup, 2002a, p.1765

A meta-analysis of all prospective studies before mid-2000 involved a total of 1524 CHD cases and 19 830 controls which yielded a combined odds ratio of 1.5 (95% CI 1.1 -2.0). By including in the meta analysis, prospective studies with pre-existing vascular disease (studies shown in figure 12) yielded an additional 723 CHD cases and 5720 controls. The combined odds ratio for CHD was similar 1.6 (1.0 -2.5) (figure 14).

figure 14 *A meta-analysis of prospective studies of von Willebrand factor and coronary heart disease*



Risk ratios compare top and bottom thirds of baseline measurements. Black squares indicate the risk ratio in each study, with the square size proportional to the number of cases and the horizontal lines representing 95% confidence intervals.
Reproduced from Whincup, 2002a, .p.1768

1.3.5 Cardiac natriuretic peptides

Groenning et al., (2004) evaluated the efficacy of N-terminal pro-brain natriuretic peptide (NT-proBNP) concentrations as a diagnostic and prognostic marker for left ventricular systolic dysfunction (LVSD) in the general population who were followed up for a median 805 days. NT-proBNP successfully discriminated patients with symptoms of HF and left ventricular ejection fraction (LVEF) ≤40% with an area under the curve of 0.94. NT-proBNP was the strongest independent predictor of mortality (hazard ratio, 5.7, $p<0.0001$) and hospital admissions for HF (13.83, $p<0.0001$). In the Breathing Not Properly study which involved 1586 patients admitted to emergency department with acute dyspnoea and without severe renal failure, the diagnostic accuracy of BNP as a marker of HF at a cut-off value of 100pg/ml was 83.4% (Maisel et al., 2002). In the same cohort, McCullough et al., (2002) demonstrated that the addition of BNP to clinical setting improved the diagnostic accuracy of chronic HF from 74% to 81%. The ESC

guidelines for the diagnosis and treatment of acute and chronic HF recommends the following in patients with acute dyspnoea, BNP values <100ng/L rules out HF, for values between 100-500 ng/L and above, diagnosis of HF should be confirmed by imaging (Task Force Members, 2010).

Berger et al., (2002) demonstrated in patients with chronic HF that BNP alone was the predictor of sudden death (p=0.0006). Furthermore, survival rates were significantly higher in patients with levels below 130 pg/ml compared to patients with values above 130 pg/ml (91% vs. 81%, p=0.0001). Harrison et al., (2002) evaluated the performance of BNP as a predictor of future cardiac events in patients with dyspnoea. A BNP value of 480 pg/ml significantly predicted HF endpoints –HF death or hospital admission with the AUC of 0.870 (95% CI 0.826-0.915). The incidence of HF endpoints in patients with BNP levels >480 pg/ml was 51% compared to 2.5% in patients with BNP levels less than 230 pg/ml. In patients with BNP levels > 230 pg/ml, the relative risk of 6 month HF death was 24.1 and 6 month non-cardiac death was 1.1.

Wallen et al., (1997) performed a similar study evaluating BNP in 85 years old from the general population in Sweden who were followed up for five years. Circulating BNP levels predicted five year mortality in the elderly population (p<0.001). BNP levels were associated with mortality independent of confounding factors such as IHD, HF, hypertension, atrial fibrillation in this elderly population (risk ratio 1.259 (95% CI 1.088 – 1.457), P=0.0020.

In the Framingham heart study (Wang, 2004), a 1 SD increase in log BNP levels was associated with a 27 percent increase in mortality (p=0.009), a 28 percent increase in the risk of first CVD event (p=0.03) and a 77 percent increase in the risk of HF (p<0.001). Hazard ratios for death, first CVD event and HF were 1.62, 1.76 and 3.07 respectively in subjects with BNP levels above the 80^{th} percentile (20.0 pg per millilitre for men and 23.3 pg per millilitre for women), all p<0.05.

In 2006, Wang and colleagues evaluated multiple biomarkers in the prediction of CVD events in

the Framingham Heart Study. BNP levels best predicted mortality and major CV events; hazard ratios were 1.40 and 1.25 respectively. BNP levels alone provided valuable information for CV risk stratification compared to the additional 10 biomarkers to conventional risk factors.

In a population-based prospective study of 658 older Dutch adults, NT-proBNP predicted mortality and first major CVD events. Adjusted Hazard ratio for mortality and first major CVD events were 1.96 (95% CI, 1,21 -3.19) and 3.24 (1.80-5.79) respectively in subjects with NT-proBNP values above the 80th percentile compared to those with values below the 80th percentile (Kistorp et al., 2005). In the LIFE sub study (Olsen et al., 2006); the incidence of CV events was 11.5 in participants with median NT-proBNP above 170pg/ml compared to 5.4% in those with low levels. NT-proBNP was superior to CRP as a predictor of composite endpoints and CVD mortality, (HR 1.9 [1.6–2.4] per SD 0.49 log (pg/ml and HR 2.5 [1.9–3.3] per SD, both P < 0.001). NT-proBNP added significantly to CVD risk stratification along with conventional risk factors in the prediction of composite CV events with an AUC of 0.75 and CVD mortality with an AUC of 0.83.

The utility of BNP levels in therapeutic interventions were evaluated in the STARS-BNP multicenter study where plasma BNP guided therapy was compared to medical treatment in 220 New York Heart Association functional classes II to III patients. During the follow up period of median 15 months, the HF end points- HF related death or hospital stay was fewer in the BNP guided therapy group compared to the clinical group (24% vs. 52%, p<0.001) (Jourdain et al., 2007).

The prognostic value of BNP has also been evaluated in patients with CHD. In the A to Z trial (Morrow et al., 2005), a prospective observational study of 4497 patients with non-ST elevation or ST-elevation ACS, BNP levels were evaluated at hospital discharge for ACS and during

follow up for up to 12 months. Patients who had BNP levels >80 pg/ml at baseline were associated with subsequent death or new HF (adjusted HR 2.5, 95% CI 2.0 -3.3), at 4 months (adjusted HR 3.9, 95% CI 2.6-6.0) and at 12 months (adjusted HR 4.7, 95% CI 2.5-8.9).The hazard ratio for patients who had newly elevated BNP levels at month 4 was higher 4.5 (95% CI 2.3 -8.6) compared to those who had elevated BNP levels both at baseline and 4 months.

1.4 Cardiovascular disease in South Asians in the United Kingdom: potential problems with cardiovascular disease risk assessment in South Asians

1.4.1 South Asians in the UK

South Asians generally refers to people originating from India, Pakistan, Bangladesh and Sri Lanka with distinct languages, religions and customs. The early settlement of South Asians in the UK started during the mass migration in the 1970's. According to the 2001 census, the total ethnic minority population in the UK was 7.9% (4.6 million) and South Asians accounted for 3.5% (2 million) of this population In Britain, the areas where the ethnic minorities are mainly concentrated are Greater London, West Midlands, West Yorkshire and Greater Manchester (Office for National Statistics, census 2001). In 2008, the ethnic minority population in the West Midlands was 12.3% Table 138 in appendix A 9.1.1 shows the proportion of ethnic minority population in the West Midlands. Table 139 shows the ethnic minority population in Birmingham (Race for opportunity, regional factsheet, 2010).

1.4.2 Cardiovascular disease in South Asians in the UK

The burden of CVD is most prominent amongst South Asians in the United Kingdom compared to the general population. The prevalence of CVD is reported to be 40% or higher in UK South Asians compared to White Europeans (Balarajan, 1996; Wild and McKeigue, 1997). The onset of the disease is also much earlier in life, where CVD mortality is at least 2-fold higher in young South Asian males than their counterparts (Balarajan, 1996). This section highlights the burden of CVD, factors that contribute to excess risk of CVD in South Asians and its consequences.

1.4.2.1 Incidence of cardiovascular disease amongst UK South Asians

Pedoe et al., (1975) studied 42,200 residents of the London Borough of Tower Hamlets below the age of 65 for nearly 3 years (1970-1972), of whom 3380 were South Asians. The number of coronary events observed amongst South Asian men mostly Bangladeshi was 40 and the expected rate for Asian born men in the Borough was 31.

Khattar et al., (2000) performed a longitudinal comparison of CV morbidity and mortality in 528 whites, 106 South Asians and 54 Afro-Caribbean's with essential hypertension who were followed up for 9.2 years. All cause event rate was highest among South Asians, 3.46 compared to 2.50 (ns) and 0.90 (p=0.002) events/100 patient-years for whites and Afro-Caribbean's respectively. Moreover, South Asians showed an excess of coronary events compared to whites, 2.86 vs. 1.32 events/100 patient-years, p=0.002. In this study, South Asian ethnicity was an independent predictor of CV outcomes, hazard ratio 1.79, p=0.008 compared to whites.

In the UK Prospective Diabetes Study, the incidence of MI was examined in 4974 patients (82% white, 10% South Asians and 8% Afro-Caribbean's) with newly diagnosed T2DM aged 25-65 years between 1977 and 1991. The age standardised rate per 1000 person years for MI was 14.6 (95% CI, 13.3 -15.9), 4.3 (2.5 -7.0) and 15.4 (10.6-21.4) for whites, Afro Caribbeans and South Asians respectively. On cox regression, the hazard ratio associated with Afro-Caribbean ethnicity for MI compared to whites was 0.3 (95% CI, 0.2-0.6) after adjusting for conventional risk factors, whilst for South Asians was 1.2 (95% CI, 0.9-1.7). In this study South Asians and whites showed a similar risk of MI (UK Prospective Diabetes Study group, 1998).

Patel et al., (2008a) investigated the incidence of CV events in 350 Caucasian and 104 South Asian patients with hypertension over a 5 year period. The MI event rate was 6.4 per 1000 patient years in Caucasians and 17.8 per 1000 patient years in South Asians. South Asians also had a lower event-free survival rate for MI compared to Caucasians, log rank chi square =4.12, p=0.04.

Fischbacher et al., (2007) conducted a retrospective cohort study (2001 to 2003) using linkage techniques that combined health and census data so that ethnicity is recorded accurately for the population of Scotland. The incidence rate ratio for MI was 1.45 (95% CI, 1.17 -1.78) for South Asian men and 1.80 (95% CI, 1.31 -2.48) for South Asian women compared to non-South Asians in Scotland. However, the study reported a better survival rate following incident MI in South Asians, hazard ratio for death was 0.59 (95% CI 0.43 -0.81). This is contrary to prior studies that have reported poorer survival rates in South Asians (Hughes, 1989; Wilkinson et al., 1996).

1.4.2.2 Prevalence of cardiovascular disease amongst UK South Asians

William et al., (1993) conducted a cross sectional survey amongst 159 South Asians and 319 White Europeans, mean age of 35 years in the city of Glasgow. CVD events were self-reported and a significant ethnic difference in the prevalence of CVD was reported in Asian women, not men compared to White European women (14% versus 6%, P<0.0005). Rudat (1994) conducted a similar survey in 1017 Indians, 927 Pakistanis and 665 Bangladeshis living in inner cities in the UK. Self-reported CVD events were 2%, 3% and 5% (percentage-standardized for age and sex) amongst Indians, Pakistanis and Bangladeshis respectively. Nazroo (1997) conducted a similar survey in 1273 Indians, 1185 Pakistanis, 591 Bangladeshis and 2867 White European who

provided self-reported data on heart disease. Adjusted prevalence ratios accounting for standard of living in Indian and African Asian groups combined and Pakistani and Bangladeshis combined were 0.67 and 1.24 respectively compared to the White population.

Bhopal et al., (1999) performed a cross sectional survey to investigate the prevalence of CVD in South Asians compared to the general population in Newcastle upon Tyne. Self-reported details on CVD and ECG were obtained from 259 Indian, 305 Pakistani, 120 Bangladeshi and 825 European men and women aged 25-74 years. South Asians showed a higher prevalence of CVD compared to the general population, age adjusted prevalence of CVD based on ECG was 6% in South Asians and 2% in Europeans.

McKeigue et al., (1993) conducted a similar survey in 1421 South Asian men and 1515 European men aged 40-69 years in Southall, London. Age-standardized prevalence rate for CVD based on ECG reports was 16.6% in South Asians compared to 12.1% in Europeans. Hughes et al., (1989) evaluated presentation of first MI in immigrant Asians and Whites in Britain. The relative rate of MI in immigrant Asians was 4.9% (95% CI 3.4-6.9) compared to White population. Moreover, the mean age of first MI in immigrant Asians was much lesser in 50.2 years compared to 55.5 years in Whites (mean difference 5.5 years (95% CI 2.5-7.1).

In 2004, the prevalence of CVD at all ages irrespective of gender was highest in the Irish population and the general population in the UK at around 15%. The next highest rates were observed amongst Indian men and Pakistani men at 11% and 12% respectively. The prevalence of CVD in Indian women and Pakistani women were 7%. The prevalence of CHD was similar in men of Irish origin (6%), Indian origin (6%) and of English descent (6%). Pakistani men had the highest prevalence rates of CHD at 8%. A similar trend but lower rates of CHD were observed in women compared to men (Ethnic differences in cardiovascular disease, 2010 online resource).

1.4.2.3 Cardiovascular mortality rates in UK South Asians

The population-based studies, the Southall study and Brent study in West London examined the risk of CHD mortality in 1420 South Asian and 1787 European men aged 40 to 69 years. On multivariable analysis after adjustment for age, smoking, and cholesterol, the CHD mortality rate almost doubled in South Asians compared to Europeans (hazard ratio, HR 2.14, 95% CI 1.56 - 2.94, P<0.001). South Asian men showed higher CHD mortality in all models after adjustment for conventional risk factors (Forouhi et al., 2006).

Balarajan et al., (1984) performed a proportional mortality analysis on immigrants of Indian subcontinent origin and English descent who died in England and Wales in 1975-77. A total of 3657 immigrants died in England and Wales, of whom 1667 died of circulatory diseases mainly IHD and cerebrovascular disease. A proportional mortality ratio of 100 meant that the proportion of total mortality for a specified condition in immigrants was equal to that of the reference population in England and Wales. The proportional mortality ratios for circulatory disease in immigrants of Indian subcontinent was calculated separately for men and women, 120 (number of deaths, 1341) and 117 (number of deaths, 326) respectively with deaths in English descent as standard.

Marmot et al., (1984) also performed mortality analysis on immigrants of Indian subcontinent based on the 1971 census in England and Wales and deaths in 1970-72. Standardised mortality ratios (SMR) for IHD in immigrants born in the Indian subcontinent were calculated separately for men and women, SMR (number of deaths) 115 (1533) and 115 (739) respectively, with deaths in England and Wales as standard. Mortality from CHD was 20% higher in men and women of South Asian origin compared to the general population in England and Wales in 1970-72. Wild

and McKeigue (1997) analysed mortality in adults aged 20-69 years in 1970-72 and 1989-92 using 1971 and 1991 censuses. SMR and number of deaths from IHD in South Asian men and women for the period 1989-92 were 146 (3348) and 151 (882) respectively. Young South Asian men aged 20-44 years showed a higher SMR of 169 compared to other age groups.

McKeigue and Marmot (1988a) examined CHD mortality in South Asians aged 20-64 years in four London Borough between 1979-1983. SMR for CHD amongst different Asian groups in four London Borough between 1979-83 are shown in table 11. The study concluded that mortality from CHD in South Asians has increased by 25% compared to the general population since 1970-72.

Table 11Mortality from coronary heart disease among Asians aged 20-64 in different London boroughs during 1979-83

London borough (predominant Asian ethnic group)	No of deaths	Standardised to average (100%) for England and Wales: Standardised mortality ratio	95% Confidence interval
		Men	
Brent and Harrow (Gujarati)	177	160	136 to 183
Ealing (Punjabi)	118	147	120 to 173
Tower Hamlets (Bangladeshi)	49	141	102 to 180
Waltham Forest (Pakistani)	36	156	105 to 207
		Women	
Brent and Harrow (Gujarati)	33	160	105 to 215
Ealing (Punjabi)	30	206	132 to 280
Tower Hamlets (Bangladeshi)	2	108*	
Waltham Forest (Pakistani)	7	217*	

*Ratio calculated from small numbers.

Adapted from McKeigue and Marmot, 1988a, p.903

Balarajan (1991) examined ethnic differences in mortality from IHD in England and Wales by country of birth of the deceased in 1979-83 and 1970-72. SMR were calculated for each ethnic group for IHD for the two periods with the five year age-sex specific rates for England and Wales for 1979-83 as standard. In 1979-83, SMR for IHD in men and women aged 20-69 years from the

Indian subcontinent was 136 and 146 respectively. The mortality ratio for IHD in young Indian men aged 20-29 and 30-39 years were 313 (21 deaths) and 210 (123 deaths) respectively. Compared to 1970-72, mortality rates from IHD increased in Indian men and women by 6% and 13% respectively.

Harding and Maxwell (1997) examined mortality by social class in migrants aged 20-64 years by country of birth in England and Wales based on 1991-93 mortality data. Men from the Indian subcontinent showed higher mortality rates from IHD, SMR 150 (number of deaths 1736) compared to the reference population. Wild et al., (2007) examined IHD mortality for people aged 20 years and over in England and Wales by country of birth based on the 2001 census and mortality data for 2001-2003. SMR for IHD (number of deaths) in men born in Bangladesh, India and Pakistan were 409 (175), 2528 (131) and 1044 (162) respectively and in women born in Bangladesh, India and Pakistan were 167 (97), 149 (1672), 174 (454) respectively. Excess mortality from IHD was found in young Pakistani adults aged 20-44 years, SMR 261 (95% CI 203-330) based on 70 deaths.

Bellary et al., (2010) investigated the effect of ethnicity on the risk of CV events and mortality in 1486 South Asian and 492 white Europeans with T2DM over a 2 year period. The adjusted odds ratio for CVD event or death from CVD was 1.4 (95% CI, 0.9-2.2) in South Asian compared to white European (p value-NS). The mean age of death was much younger for South Asian 66.8 years. (11.8) compared to 74.2 years. (12.1) in white Europeans, a mean difference of 7.4 years (95% CI 1.0-13.7), p=0.023.

The mortality rates for CHD fell by 29% in men and 17% in women in the general population (aged 20-69) from 1971 to 1991. A similar but slow pattern was observed amongst South Asian immigrants, whose rates fell by 20% in men and 7% in women (Wild and McKeigue, 1997).

Recent data from 1979 to 2003 also showed a steady decline in CHD mortality rates in South Asians (aged 39-69). However rate ratios for CHD mortality are still high amongst South Asians (Harding et al., 2008).

1.4.3 Heart failure in South Asians

South Asians have a higher prevalence of CHD and diabetes mellitus compared to the general population in the UK and hence a greater prevalence of HF is anticipated in this ethnic group. Prior epidemiological studies showed a threefold higher risk of hospitalization with HF amongst South Asians compared to white population. Moreover the onset of HF was much younger in South Asians and mostly in men compared to the White population in the UK (Lip et al., 1997; Davis et al., 2000; Blackledge et al., 2003; Newton et al., 2005; Gill et al., 2009).

Blackledge (2003) compared 5057 white patients and 336 South Asian patients aged 40 or over with newly diagnosed HF in Leicestershire between 1 April 1998 and 31 March 2001. The hospital incidence rates for HF in South Asian men and women were 2.2 and 2.9 respectively compared to the white population. Moreover South Asian HF patients were much younger, had a high prevalence of concomitant MI and diabetes mellitus compared to white HF patients (18.8% vs.10.7%) and (45.8% vs. 16.2%), all $p<0.001$ respectively. Despite an adverse risk profile, the hazard ratio for all-cause mortality was lower in South Asians compared to White HF patients (HR 0.82, 95% CI 0.68-0.99). Similarly findings were reported by Lip (1997) where South Asian HF patients had lower inpatient mortality compared to white European patients (13.2% vs. 20.7%). Davis (2000) followed up these patients for 8 years and the total mortality was 87.0% amongst South Asians and 90.5% amongst White European patients.

Bhopal and Fischbacher (2003) suggested that the young age of South Asian patients admitted with HF is an artefact and simply display the age distribution of the South Asian population in the UK. Newton (2005) used the same population in Leicestershire but this time age and gender matched South Asian and White patients hospitalized for the first time for HF and compared prognosis. South Asian HF patients showed a higher prevalence of prior hypertension and diabetes mellitus, (45% vs. 33%) and (46% vs. 18%), both $p<0.05$. South Asian HF patients showed more preserved left ventricular function (38% vs. 23%, $p=0.002$) and less advanced HF (18% vs. 28%, $p=0.025$) compared to white HF patients. The odds ratio for all-cause mortality amongst South Asian HF patients was 0.71 (95% CI 0.53-0.96), $p=0.02$.

In the study by Galasko et al., (2005) in Harrow, UK between January 2000 and May 2001, the prevalence of LVSD was similar in White and South Asian patients but a significant difference was observed in the underlying aetiology. South Asian patients had a higher prevalence of prior CHD, findings similar to those reported by Blackledge (2003) and Gill et al., (2011). Bhopal et al., (2012) conducted a retrospective cohort study in Scotland comprising of 4.65 million subjects to examine the ethnic variations in the incidence of HF between May 2001 and April 2008. The incidence of first hospitalization for HF was modestly higher amongst Pakistani men (RR 134.9) compared to white British men (RR 86.4) and Chinese men (RR 54.2). Similar findings were observed amongst women with higher incidence rates among Pakistani women compared to white British women (RR 86.0) and Chinese women (RR 62.9). In the E-ECHOES, the prevalence of HF amongst South Asians and Afro-Caribbeans groups was 1.1%, similar to those reported in the general population (Gill, 2011; Davies et al., 2001)

The prevalence of HF is more likely to be similar in South Asians and Whites in the UK (Gill, 2011). South Asians with HF are more likely to have concomitant MI, hypertension and diabetes

mellitus which might predispose this group to diastolic HF with preserved LV function hence the lower prevalence of LVSD (Galasko, 2005). Better survival in South Asian HF patients are mainly due to preserved left ventricular function, less advanced HF and younger age at first presentation (Blackledge, 2003; Newton, 2005; Sosin et al., 2004).

1.4.4 Traditional Cardiovascular risk factors in South Asians

Several hypotheses exist relating to ethnic differences and the excess of CHD in South Asians. Table 12 shows some of the plausible hypotheses that have been suggested (Kuppuswamy and Gupta, 2005; Patel and Bhopal, 2004).

Table 12 Hypotheses relating to excess coronary heart disease in South Asians

1.	Cardiovascular risk profiles amongst South Asians living abroad are likely to have deteriorated with migration from countries of origin to Western Countries, specifically from changes in dietary exposures.
2.	The prevalence of non-conventional risk factors such as high levels of lipoprotein a and homocysteine amongst South Asians
3.	South Asians are more susceptible to cardiovascular disease due to genetic reasons
4.	The increased prevalence of traditional risk factors comprising of insulin resistance, diabetes mellitus, hypertension, central obesity, hyperlipidaemia, lack of physical activity and smoking amongst South Asians.
5.	The classic risk factors may interact differently and hence cause more adverse events in South Asians
6.	People with ancestral origins in hostile tropical environments such as people from the Indian subcontinent exhibit increased pro-inflammatory responses compared to those living in a temperate environment. However, when migrating to temperate environment the balance between the benefit and the risk of these increased pro inflammatory responses is altered, resulting in an increased incidence of inflammatory related diseases.
7.	South Asians show elevated subclinical inflammation and endothelial dysfunction and the manifestations of these factors are much earlier in life

Of all the above mentioned hypotheses, the higher prevalence of metabolic syndrome is the most convincing one and this has been proven in research in South Asians so far. This section will summarise studies that have examined traditional and non-traditional cardiovascular risk profiles in South Asians.

1.4.4.1 Insulin resistance and diabetes mellitus

Whincup et al., (2002b) conducted a cross sectional study to examine insulin resistance in 73 British South Asian and 1287 White children aged 8-11 years. Mean glucose concentrations were similar in both groups. However, insulin concentrations were higher in South Asian children compared to White children, percentage difference 53% (95% CI, 14-106) after fasting and 54% (95% CI, 19-99) after glucose load, and differences remained after adjustment for adiposity and other confounders. Cruickshank et al., (1991) compared 107 Indian Gujaratis and 101 White Europeans in West London and found the prevalence rate for diabetes was 30% and 3% respectively.

Mckeigue et al., (1988b) compared the prevalence of diabetes mellitus and hyperinsulineamia in 253 men and women aged 35-69 in East London, UK. The prevalence of diabetes was three times higher in Bangladeshis men and women, 22% and 23% compared to European men and women, 10% and 4% respectively. Again serum insulin concentrations measured after a glucose load were higher amongst Bangladeshis men and women 65 mU/l and 57mU/l compared to European men and women 32mU/l and 27 mU/l respectively, $p<0.05$. Insulin levels were associated with high triglyceride levels and low HDL cholesterol levels in Bangladeshis.

In the cross sectional study by Bhopal (1999) in Newcastle upon Tyne, the prevalence of diabetes was higher amongst Pakistani and Bangladeshi men 22.4% and 26.6 % respectively compared to 15.2% in Indians. In the Whitehall II study, the prevalence of diabetes mellitus was higher in South Asians, the odds of being diabetic was 4.2 (95% CI 3.0-5.8) compared to the White population (Whitty et al., 1999). In the Wandsworth Health Authority population based study in

South London between 1994 to 1996, age and sex adjusted prevalence ratio for diabetes mellitus was 3.8 (95% CI 2.6 -5.6) in South Asians compared to Whites (Cappuccio et al., 1997).

Mckeigue et al., (1991) examined metabolic disturbances related to insulin resistance in a population survey of 3193 men and 561 women aged 40-69 years in London. South Asians showed a higher prevalence of diabetes mellitus compared to the European group (19% vs. 4%). The prevalence of diabetes mellitus in European men and women were 4.8% (95% CI 3.7-5.8) and 2.3% respectively compared to 19.6 (17.5-21.7) and 16.1 (11.7-20.5) in South Asian men and women. Compared to the European men, mean serum insulin levels were 1.4 higher in the fasting state and 2.1 times higher 2h after a glucose load in South Asian men, this remained after adjustment by plasma glucose levels. South Asians also had elevated blood pressure, higher triglyceride levels and lower HDL-c compared to Europeans.

Dhawan et al., (1994) performed a case-control study to compare the prevalence of diabetes and hyperinsulinaemia in 83 British Asians, 30 Indian Asians and 87 White men, all with angiographically proven CHD with age matched controls. British Asians and Indian Asians exhibited a higher prevalence of diabetes and impaired glucose tolerance compared to White British men. Amongst British Asian patients and Indian patients, only 52% and 43% were normoglycaemic whilst 75% of White patients were normoglycaemic. Table 13 summarises the prevalence of diabetes, impaired glucose and normoglycaemia for this study. Fasting insulin concentrations were higher in patients of all ethnic groups compared to controls ($p<0.001$) with British Asian patients having higher levels compared to white patients (28.2µ/ml vs. 12.7µ/ml). Again at one hour of glucose challenge, insulin concentrations were higher in all patients compared to controls ($p<0.001$), with British Asian patients showing a high level of 122.0µ/ml compared to 72.7µ/ml in White patients.

Table 13Prevalence of diabetes, impaired glucose and normoglycaemia

	White subjects		British Asians		Indian Asians	
	Patients (n = 87)	Controls (n = 81)	Patients (n = 83)	Controls (n = 80)	Patients (n = 30)	Controls (n = 30)
Diabetic	7 (8)	2 (2)	21 (25)	9 (11)	10 (33)	5 (17)
Impaired glucose tolerance	15 (17)	6 (7)	19 (23)	16 (20)	7 (23)	5 (17)
Normoglycaemic	65 (75)	73 (90)	43 (52)	55 (69)	13 (43)	20 (67)

χ^2 (5) = 30·6; P = 0·00001.

Adapted from Dhawan, 1994, p.416. Values are number (%)

In a longitudinal study, Patel, (2008a) showed that the prevalence of diabetes was 22.9% higher in hypertensive South Asians compared to hypertensive Caucasians. Diabetic hypertensive patients showed a lower event-free survival rate for MI compared to non-diabetic hypertensive patients (log rank =12.2, p=<0.001). Of all the conventional risk factors, the onset of MI was associated only with diabetes in South Asians, odds ratio 3.77 (95% CI, 1.55-9.15, p=0.003). In South Asians, the onset of diabetes mellitus is much earlier in life and the disease is more progressive resulting in higher rates of nephropathy and retinopathy compared to European diabetic patients (Burden et al., 1992; Chandie Shaw et al., 2006; Stolk et al., 2008; Raymond et al., 2009).

1.4.4.2 Obesity

Population studies in the United Kingdom have shown central adiposity reported as waist to hip ratio and insulin resistance to be the underlying factors that predispose South Asians to increased risk of CVD (McKeigue, 1991; Dhawan, 1994). A pro inflammatory state has been associated with these factors and the increased risk of CVD, but the precise mechanisms in South Asians is unclear. South Asians have a greater waist-to-hip ratio compared to whites which has been associated with atherogenic lipid profile and dysglycemia despite having similar BMI. This is

mainly due to under development of the superficial subcutaneous adipose tissue in South Asians which results in fat deposition in the visceral adipose tissue (Patel et al., 2012a).

The Southall and Brent studies in West London (Forouhi, 2006) showed that South Asians were younger (51.2 years versus 52.9 years, $p<0.001$)), had low BMI (25.9 kg/m^2 versus 26.2 kg/m^2) and lower cholesterol (5.8 mmol/l versus 5.9 mmol/l), $p<0.001$) than European men. The components of metabolic syndrome were more prevalent among South Asians compared to Europeans, waist circumference (93.3 cm versus 92.0 cm), fasting glucose (6.0 mmol/l versus 5.6 mmol/l), low HDL-c (1.1 mmol/l versus 1.2 mmol/l), high triglyceride (1.7 mmol/l versus 1.4 mmol/l) and more hypertensive (52% versus 39.9%), all $p<0.001$.

In the study by Mckeigue (1991) mean waist-hip circumference ratio was higher in South Asians compared to Europeans in the UK. Waist hip circumference ratio in South Asian men and women were 0.98 (95% CI 0.97-0.98) and 0.85 (0.84-0.86) compared to 0.94 (0.93-0.94) and 0.76 (0.75-0.77) in European men and women respectively. In South Asians, waist-hip girth ratio positively correlated with insulin, glucose tolerance, blood pressure and triglyceride. The study concluded that the higher prevalence of insulin resistance syndrome along with central obesity amongst South Asians in the UK might predisposes this group to diabetes and excess risk of CHD. Dhawan (1994) showed similar results in their study cohort. Waist to hip ratio was positively correlated to insulin and triglyceride levels and negatively to low HDL- c. Waist to hip ratio was the strongest independent predictor of CHD in British Asians, relative risk 4.1 (95% CI 2.2 -7.7), $p<0.0001$.

1.4.4.3 Hypertension

Cruickshank et al., (1983) investigated ethnic differences in blood pressure by studying factory workers aged 15-64 years in Birmingham, England. The prevalence of hypertension was 22% in Whites and 17% in South Asians. South Asian men had lower systolic (128.7 mmHg vs. 134.2 mmHg) and higher diastolic blood pressure (79.9mmHg vs. 78.7mmHg) compared to Whites. In the Birmingham factory screening survey conducted by Lane et al., (2002), the prevalence of hypertension was similar in South Asian men and Whites (16% vs. 19.4%).

In the study by Mckeigue (1988b) mean blood pressure were lower in Bangladeshis 119/78 mmHg compared to 129/81 mmHg in Whites. Cruickshank (1991) compared 107 Indian Gujaratis and 101 White Europeans in West London and found the prevalence rate for hypertension was 32% and 20% respectively.

Bhopal (1999) conducted a cross sectional study, the Health Survey of England to examine the prevalence of hypertension in 259 Indian, 305 Pakistani, 120 Bangladeshi and 825 European men and women aged 25-74 years in Newcastle upon Tyne. The prevalence of hypertension was lower in South Asians, 10% compared to 18% in Europeans. Amongst South Asians, Indians were more hypertensive compared to Pakistanis and Bangladeshis, 14% vs. 9% and 6% respectively. In the Whitehall II study (Whitty, 1999), the prevalence of hypertension was higher in South Asians, the odds of being hypertensive was 2.3 (95% CI 1.6-3.3) compared to the White population.

In the Health Surveys for England study (Primatesta et al., 2000); between 1991-1996 the prevalence of hypertension amongst South Asians 40 years and above was 51% compared to 36% in Whites. The odds of being hypertensive was 1.9 (95%CI 1.4-2.4), $p<0.001$ in South Asian men compared to their White counterparts. In the Wandsworth Health Authority study (Cappuccio,

1997) in South London between 1994 to 1996, age and sex adjusted prevalence ratio for hypertension was 2.5 (95% CI 2.1 -3.2) in South Asians compared to Whites. The prevalence rate for hypertension in South Asians was 44% compared to 28% in Whites (Cappuccio et al., 1998). There are inconsistency in blood pressure and prevalence rates of hypertension in UK studies that have compared South Asians to White population. This is mainly attributed to the differences in age, sex of the samples, method of measuring blood pressure and definition of South Asians. The Health Survey of England study 1999 clearly showed the difference in hypertension within the South Asian group, with Indians having higher blood pressure compared to Pakistanis, and Bangladeshis having the lowest blood pressure (Bhopal, 1999; Agyemang and Bhopal, 2002).

1.4.4.4 Smoking

In the UK, smoking is more common amongst Bangladeshi men (49%) compared to Whites (29%), Pakistani (28%) and Indian (19%). In the community based qualitative study conducted by Bush et al., (2003) in 87 Bangladeshi and 54 Pakistani aged 18-80 years in Newcastle, 60% of Bangladeshi men and 51% of Pakistani men smoked. Smoking is considered to be socially acceptable in these groups.

1.4.4.5 Abnormal lipid profiles

The ‘Atherogenic lipid triad’ of high serum triglyceride levels and low serum HDL-c levels occur commonly in South Asians compared to Whites. More than half of the HDL in South Asians consist of dysfunctional pro-inflammatory HDL and have been associated with sub-clinical CHD (Dodani et al., 2008).

In the Whitehall II cohort study (Whitty, 1999) , the odds for being in the adverse quartile of risk for lipids amongst South Asian men were 0.89 (95% CI 0.71-1.0) for cholesterol, 2.1 (95% CI 1.7-2.8) for HDL-c, 1.55 (95% CI 1.3 -1.9) for triglycerides and amongst South Asian women were 0.60 (95% CI 0.4-0.9) for cholesterol, 2.73 (95% CI 1.8-4.3) for HDL-c, 1.55 (95% CI 1.0-2.3) compared to White subjects. The levels of ApoB were also higher in South Asians compared to Whites, odds ratio being 1.33 (95% CI 1.1-1.7) in South Asian men and 1.26 (95% CI 0.8-1.9) in South Asian women.

In the Child Heart and Health Study in England (CHASE), Donin et al., (2010) assessed blood lipid patterns in 2026 UK children who consisted of 285 black Africans, 188 black Caribbeans, 534 South Asians and 512 white Europeans children. Compared to white Europeans, South Asian children had lower HDL-c, -0.7 mmol/l (95% CI, -0.11 - -0.03 mmol/l) and elevated triglyceride levels (proportional difference 0.14 mmol/l (95%CI, 0.09 – 0.20 mmol/l) and similar total and LDL cholesterol levels.

Palaniappan et al., 2007 demonstrated in South Asian women that a highly atherogenic lipid profile was associated with insulin resistance. Insulin resistant women had higher levels of triglycerides (mean (SD) 151 (70) mg/dL vs. 93(42) mg/dL), lower HDL-c (44 (9) mg/dL vs. 53 (9) mg/dL), lower HDL-c classes and smaller and dense LDL particle size compared to insulin sensitive women with no differences in total cholesterol and LDL-c between the two groups. Gasevic et al., (2012) investigated the clinical utility of triglyceride to HDL-c ratio (TG/HDL-C) in predicting insulin resistance in 4 ethnic groups – Aboriginals, Chinese, Europeans and South Asians. The performance of TG/HDL-C as a discriminator of insulin resistance on ROC analysis was area under curve (95% CI) 0.777 (0.707 -0.847) in Aboriginals, 0.723 (0.647-0.798) in Chinese, 0.752 (0.675-0.828) in Europeans and 0.676 (0.590-0.762) in South Asians. The

TG/HDL-C ratio was not a good marker for the identification of insulin resistance in South Asians compared to the other groups.

Patel et al., (2010) demonstrated that fasting serum triglycerides were the best discriminator of metabolic and CHD risk in South Asians of all lipid and lipoprotein variables. Fasting triglycerides discriminated South Asians with obesity, raised blood pressure, fasting glycaemia, hyperinsulinaemia, metabolic syndrome and raised CHD risk score from healthy subjects with a mean area under curve of 0.683 (0.564-0.879), all $p \leq 0.001$.

1.4.4.6 Lack of physical activity

Williams et al., (2011a) compared physical activity levels on the Health Survey for England (1999-2004) participants, 5421 South Asians and 8974 Whites aged 18-55 years. The total physical activity metabolic equivalents of task scores were lower in South Asians compared to White participants in the UK, 973 vs.1465 –min/week, $p<0.001$, this was irrespective of individual sociodemographic factors. The same group then performed a longitudinal observational study with follow up mortality data on 13,293 Whites and 2120 South Asians aged ≥ 35 years who participated in the Health Survey for England study. South Asians were more physically inactive compared to White participants (28.1% vs. 47%). Mortality data showed deaths from CHD occurred more in UK South Asians, mainly in those of Pakistani and Bangladeshi origin compared to UK Whites (HR 2.87, 95% CI 1.74 - 4.73). Lack of physical activity explained >20% of the excess CHD mortality observed amongst UK South Asians even after adjustment for potential confounding factors (William et al., 2011b)

1.4.5 Problems and challenges for cardiovascular disease risk assessment in South Asians

The burden of CVD is more prominent amongst South Asians compared to the general population in the UK where the rates of CVD mortality is 40% higher in South Asians compared to the indigenous white population (Gill et al., 2007). The underlying pathophysiology for this increased risk of CVD is not clear. The higher prevalence of diabetes mellitus and elevated levels of triglycerides do reflect an increased risk of CVD in South Asians (Patel, 2008a; Patel, 2010). However the onset of CVD events is much earlier in life amongst South Asians and is often accompanied by the absence of traditional CVD risk factors such as hypertension and elevated serum lipids (Patel, 2008a; Patel, 2012a). Karthikeyan et al., (2009) demonstrated in the INTERHEART study that the risk of MI is higher in South Asians despite optimal levels of LDL-c and suggested a lower threshold for LDL-c for this ethnic group. In this study apolipoproteins which are not included in the CVD risk assessment showed strongest association with the risk of MI in South Asians.

The primary prevention of CV events using established risk estimation strategies based on the Framingham equation underestimates risk in South Asians. Cappuccio et al., (2002) demonstrated in a population based cross sectional study in the UK that the 10 year CHD risk score based on Framingham equation underestimated CVD risk in South Asians when compared to the general population and suggested a lower threshold for the CHD risk score in South Asians. In the Newcastle Heart Project, Bhopal et al., (2005) explored the predictive ability of the Framingham and FINRISK risk prediction scores in South Asians where their performance were similar and agreed with the observed CHD SMR in South Asians as a combined group but with substantial heterogeneity in sub groups. The risk prediction models overestimated CHD mortality rates in

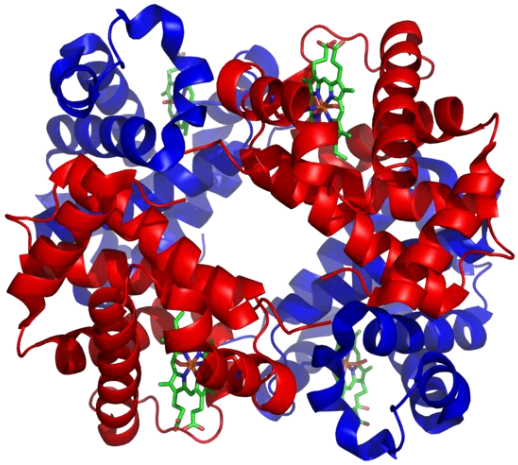
Indian men and underestimated CHD mortality rates in Pakistani and Bangladeshi women suggesting the need to address heterogeneity of the South Asian population in cardiovascular healthcare.

The Framingham based risk score has been recalibrated in order to extend its application to ethnic minority groups for instance ETHRISK, a web based risk score was proposed by Brindle et al., (2006) but it requires further validation in South Asians. The QRISK2 score has been recommended in South Asians where CVD risk estimated is multiplied by a factor of 1.4 (National Institute for Health and Clinical Excellence, 2008). However CV events occur in South Asians at clinically insignificant levels of CVD risk scores and adjustment of CVD risk prediction scores based on ethnicity can lead to identifying high risk South Asians. Health inequalities in cardiovascular healthcare are further widened by cultural and social barriers faced by South Asians in the UK (Patel, 2012a). There is a need for exploring biomarkers that can identify South Asians at increased risk of CVD early.

1.4.6 Haemoglobinopathies in South Asians

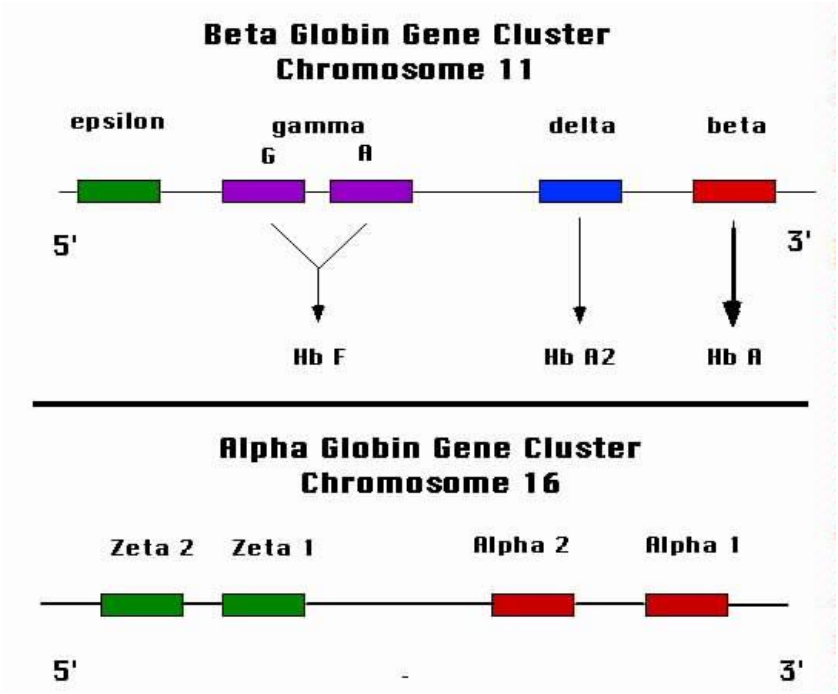
Haemoglobin is an iron containing protein in red blood cells that delivers oxygen from the lungs to peripheral tissues and is a tetramer made up of two pairs of alpha and non-alpha globin chains (β, δ, γ). The globin chain is coded by the *HBA1* and *HBA2* genes on chromosome 16p13.3 while the non-alpha globin chains are coded by the *HBB* gene on chromosome 11p15.5 (Trent, 2006). Figure 15 shows the structure of haemoglobin and figure 16 shows the globin gene loci. In a normal adult, ~95% of the haemoglobin is HbA ($\alpha_2\beta_2$), 2-3% is HbA_2 ($\alpha_2\delta_2$) and less than 1% is HbF ($\alpha_2\gamma_2$) (Ryan et al., 2010).

figure 15 *Structure of haemoglobin*



Haemoglobin consists of two pairs of α and β globin chains made of 141 and 146 amino acid residues respectively (in red and blue). The globin chains are coordinated to four oxygen binding iron containing haem groups (in green). Reproduced from Protein database ID- 1GZX

figure 16 *The globin gene loci*



Beta globin locus-The two gamma genes produces Hb F & is active only during fetal growth, after birth beta gene is activated. Alpha globin locus- All four genes contribute to the production of alpha globin chains. Reproduced from http://sickle.bwh.harvard.edu/hbsynthesis.html

Haemoglobinopathies are inherited autosomal recessive disorders and are due to mutations in genes coding for the globin proteins which either results in the underproduction of normal globin chains known as the thalassemia syndromes or synthesis of structurally abnormal globin chain known as variant Hbs. In very few cases haemoglobin disorders can be characterized by both phenotypes for example Hb E. (Old et al., 2003).

Thalassemias are further classified based on the genes involved into α and β thalassemias and sub divided based on whether some or no globin proteins are produced (α^+, β^+ or α^o, β^o) (Trent, 2006). In beta thalassmia, reduced or no synthesis of beta globin chains leads to excess synthesis of alpha chains (Marengo-Rowe, 2007). Beta thalassemia is particularly common in subjects from the Indian subcontinent with a carrier rate of 3-17% while alpha thalassemia is less prevalent in this region (Panigrahi and Marwaha, 2007).

1.4.6.1 Common haemoglobinopathies in South Asians

It is beyond the scope of this thesis to go into details on all the haemoglobin disorders found in the Indian subcontinent. Common clinically significant variants Hbs and beta thalassemia mutations are shown in table 14.

Table 14 Common haemoglobinopathies in the Indian subcontinent

Variants Hb
Hb S Hb C Hb D-Punjab Hb E Hb J Hb H Hb O-Arab Hb Lepore (αδ)
Beta thalassemia mutations
IVS1-5 (G-C) (frequency 63.17%) 619 base pair deletion (3.75%) IVS 1-1 (G-T) (2.71%) CD 41/42 (-CTTT) (3.49%)

CD 15 (G-A) (7.83%) CD 16 (-C) (A-T) CD 17 CD 30 (G-C) (4.33%) CD 8/9 (+G) (1.42%) Capsite + (A-C) Poly A (T-C) (3.55%)

(Old, 2003; Edison et al., 2008; Sachdev et al., 2010)

1.4.6.2 Diagnosis of haemoglobinopathies

Various tests involved in the diagnosis of haemoglobinopathies are shown in table 15.

Table 15Diagnosis of haemoglobinopathies

Full blood count	Thalasssemias reflected by low MCV (mean corpuscular volume) and MCH <27pg (mean corpuscular haemoglobin). Variants Hb normally have normal MCV and MCH for e.g. Hb S which will require a blood film. *Note: Iron deficiency anaemia should be excluded. Abnormal FBC and/or family history should be further investigated*
Haematological tests	➢ HbEPG test involves electrophoresis of globin proteins on gel, membrane based kits or HPLC. Abnormal bands help to identify variants Hb. ➢ HbA_2 test involves globin electrophoresis and quantitation of HbA_2 peak on HPLC. Elevated HbA_2 indicate β thalassemia ➢ HbF test involves globin electrophoresis and quantitation of HbF peak on HPLC. Slightly raised HbF indicate silent β thalassemia. ➢ Mass spectrometry is ideal for population screening and characterise various variant Hbs.
DNA testing	This is done when haematological tests fail to identify haemoglobinopathies or to confirm a diagnosis by identifying specific mutation. Chan and colleagues (2010) described a two stage DNA based test for the identification of the most prevalent β thalassemia mutations.

Trent, 2006

1.4.6.3 Evolution of haemoglobinopathies in South Asians

The prevalence of haemoglobinopathies is mostly concentrated in malarial and tropical regions such as the Indian subcontinent and has been associated with an evolutionary benefit of protection against malaria in carriers or heterozygotes. According to the "malaria hypothesis" 'this could result in a "balanced polymorphism" where the homozygote's haematological

disadvantage is balanced by the resistance to malaria displayed by the heterozygote' (Haldane, 1949: Lopez et al., 2010). Resistance to malaria is mainly due to enhanced immune response to parasite infected erythrocytes and erythrocyte abnormalities provide poor environment for parasite invasion and growth (Pasvol et al., 1978; Ayi et al., 2004).

On the other hand, carriers of beta thalassemia trait exhibit aberrant erythropoiesis and haemolysis which leads to mild anaemia and iron loading (Weatherall, 2000) which could potentially increase the risk of CVD in this group (Sullivan, 1981). However, studies by Maioli et al., (1989 & 1997) demonstrated that carriers of beta thalassemia trait exhibit lower total cholesterol, LDL-c and ApoB levels and hence lower risk of CVD. Case-control studies in non-Asian populations (Wang and Schilling, 1985; Crowley et al., 1987) have demonstrated a lower prevalence of beta thalassemia trait in patient with MI compared to controls. However the link between haemoglobinopathies such as Hb variants, beta thalassemia trait and risk of CVD has not been explored in South Asian population.

CHAPTER 2 AIMS AND HYPOTHESIS

2.1 General aims and hypothesis

CVD is the leading cause of death in Britain, but compared to the general population, the burden is more prominent amongst the country's ethnic minorities (Gill, 2007). The classic cardiovascular risk factors – hypertension, diabetes and smoking play a central role in the prevention of CVD, however many individuals susceptible to heart disease exhibit one or none of the above mentioned risk factors (Gerszten and Wang, 2008). On-going Government initiatives highlight an urgent need to have targeted CVD risk management for these groups. Even though we can diagnose CVD using clinical tools, our prediction of CVD risk remains poor (Raleigh et al., 1997; Vasan, 2006) and is likely to be underestimated in high-risk groups such as South Asians (McEwan et al., 2004; Brindle et al., 2005: Patel et al., 2007). In recent years, there has been a surge in the evaluation of the prognostic value of circulating biological markers or 'biomarkers'–measurable and quantifiable biological parameters that can aid clinicians in CVD disease management, risk stratification and prevention. The main aim of this thesis is to evaluate the performance of such blood biomarkers and genetic based markers in identifying South Asians with an increased risk of CVD from those who are asymptomatic for CVD. Secondly, to determine if haemoglobinopathies which are highly prevalent amongst the South Asian population contribute to increased risk of CVD.

2.1.1 Hypothesis 1- The blood biomarker study

The study hypothesis is that South Asians with CVD risk factors and existing CVD events will have high levels of biomarkers of inflammation (CRP, IL-6), endothelial dysfunction (vWF), thrombosis (D-dimer) and coagulation (P-selectin) compared to healthy South Asians. The study also hypothesized that South Asians with HF will have abnormal levels of biomarker of cardiac function (BNP) compared to healthy South Asians. The performance of each biomarker will be evaluated using ROC analysis. ROC curve analysis will be performed initially in South Asians asymptomatic for CVD and then will be validated in a group of South Asians with CVD risk factors (on medication for anti-hypertensive, anti –diabetic or lipid lowering therapy) and existing CVD events.

2.1.2 Hypothesis 2 -The genetic study

This study investigates the genetic susceptibility of South Asians towards phenotypic markers of inflammation and metabolic disorders and also to increased risk of CVD. The study hypothesis is that abnormal levels of biomarkers of inflammation (CRP, IL-6) and metabolic disorder (lipid profiles) are associated with specific genetic polymorphisms -390C>T>A (rs3091244) within the promoter region of *CRPgene,* -174G>C (rs1800795) within the promoter region of *IL-6 gene,* -455T>C (rs2854116) and -482C>T (rs2854117) within the insulin response element on *APOC3 gene* and -1131T>C (rs662799) on *APOA5 gene*. The prevalence of these genetic polymorphisms is different from those reported in other ethnic groups.

CHAPTER 3 METHODS

3.1 Study population

This section gives an overview of the study population for the two main studies in this thesis –the blood biomarker study and the genetic study.

3.1.1 The blood biomarker study

The study population for the blood biomarker study consisted of subjects of South Asian origin in the UK originating from India, Pakistan and Bangladesh. A total of 1222 South Asians aged 45 years and over were recruited through the Ethnic-Echocardiographic Heart of England Screening Study (E-ECHOES) (Gill, 2009).

3.1.1.1 The Ethnic-Echocardiographic Heart of England Screening Study (E-ECHOES)- The blood biomarker study

The E-ECHOES (Gill, 2009) is a population based epidemiology study which was conducted within the West Midlands in the UK from September 2006 to August 2009. The aim of the study was to establish the prevalence and severity of LVSD and HF amongst South Asian and Black African-Caribbean ethnic groups within the West Midlands. This study was purely observational. A sub study –blood biomarkers was conducted to explore 2 aspects of HF physiology, which have pathophysiological and prognostic implications. Firstly, BNP testing has been proposed as a screening test for cardiac and HF and a risk marker for CVD, but there are no substantial population-based data relating to South Asians. Secondly, abnormal inflammatory indices (IL-6 and CRP), endothelial dysfunction (vWF), coagulation (P-selectin) and thrombosis (D-dimer) are present in HF, and have prognostic implications. Again, there are no substantial population-based

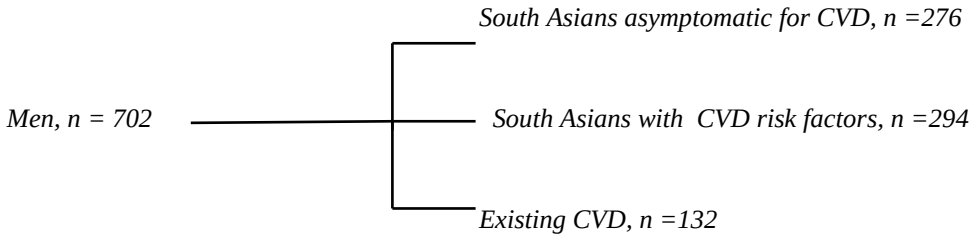
data relating to non-white ethnic groups, nor any exploration of the inflammatory stimuli of the endothelium and thrombogenesis or the genetic susceptibility to these disorders (British Heart Foundation grant application, PG/05/036). The study protocol (05/Q2708/45) was approved by the Walsall Local Research Ethics Committee and a written consent was obtained from all study participants.

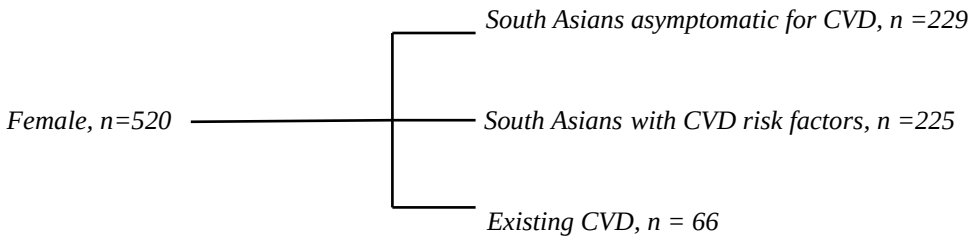
Note: The study protocol and all study related documents were prepared by the Principal and Chief investigators for the E-ECHOES.

Detailed description of the E-ECHOES including subject recruitment, study protocol, subject characteristics: demographic details, clinical and medication history are shown in section 3.1.1.1.1.

In the total of 1222 subjects, 505 were South Asians asymptomatic for CVD (this included high risk subjects but who were unaware of it until the assessment), 519 South Asians were symptomatic for CVD risk (on medication for modifiable CVD risk factors namely for high blood pressure, diabetes mellitus and abnormal lipid profiles) and 198 subjects were with existing CVD events (figure 17).

figure 17 Subjects in the blood biomarker study



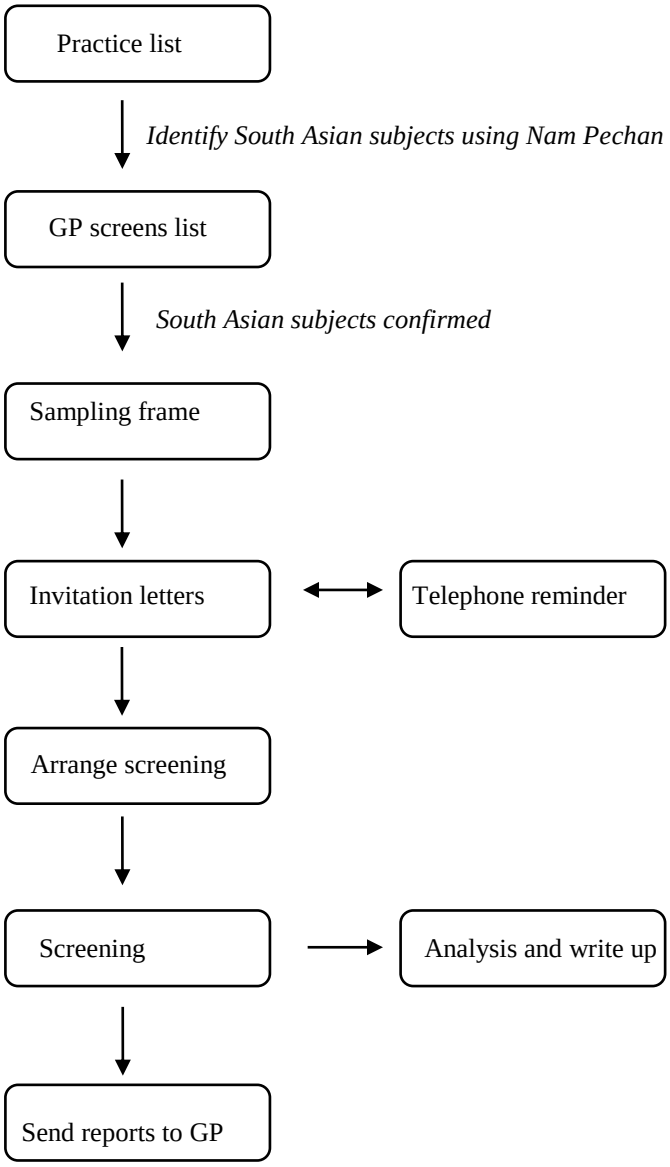


South Asians asymptomatic for CVD were South Asians who were not on any medication for modifiable CVD risk factors at recruitment. South Asians with CVD risk factors were South Asians on medication for modifiable CVD risk factors such as on anti hypertensive, anti-diabetic and lipid lowering therapies.

3.1.1.1.1 Subject recruitment

General practices within Birmingham with a high proportion of ethnic minority patients were identified and eligible subjects were selected by sex and aged 45 years and over from the practice age-sex registers. South Asian subjects were identified from the practice list using the Nam Pechan software which screens for South Asian names. The general practitioner then reviewed the list to ensure the ethnicity of the selected subjects and excluded anyone whom they consider inappropriate for the study. Invitation letters were then sent to potential subjects inviting them to attend their practice. The ethnicity of the selected subject was again confirmed before booking an appointment with the help of an interpreter where required (Gill, 2009). Figure 18 shows the recruitment process for the E-ECHOES.

figure 18 *Recruitment process for the E-ECHOES*



Adapted from Gill, 2009

3.1.1.1.2 Study Protocol

Subjects were invited to participate in the study at their local general practice and written informed consent was obtained. At the clinic, the research team collected data using a standardised protocol comprised of a questionnaire which incorporated details on demography

and clinical history, physical examination, and electrocardiography and echocardiography examination. Blood sampling was done for those who gave consent for the blood sub-study.

Note: Clinical research fellows were actively involved in the recruitment for this study and provided me the samples for my thesis.

Table 16Study protocol

	Details obtained
Patient information sheet and consent form	Trained research fellows obtained written consent from all study subjects
Questionnaire	Included details on age, date of birth, address, post-code, self-determined ethnicity, religion, place of birth, migration history, languages spoken, level of education, alcohol consumption, cigarette smoking, tobacco use, exercise assessment, history of illness/ co-morbidity (myocardial infarction, angina, hypertension, heart failure, stroke, diabetes) in self and family and current medication
Physical examination	Standardised anthropometric measurement–Height & weight (Seca Ltd, Birmingham UK), body mass index and waist measurement. Blood pressure were undertaken three times using the OMRON 705CP (Omron Healthcare Europe, Mannheim, Germany).
Electrocardiography (ECG) and echocardiography Examination	Performed by trained research fellows For ECG investigation, a resting 12 lead ECG (Mortara ELI 150) was recorded & coded independently by two cardiologist using the Minnesota criteria. Measurement of left ventricular ejection fraction and function by echocardiography using a portable VIVID I machine (GE Healthcare, Chalfont St Giles, UK). The presence and degree of left ventricular hypertrophy was assessed from chamber dimensions. Left ventricular function was measured objectively using an area-length method from the apical four-chamber view. In cases where an objective measurement of left ventricular ejection fraction is not possible, a qualitative assessment was made, that is, definite impairment (LVEF <40%), borderline (40-50%) and preserved (>50%), consistent with the investigators' normal clinical practice
Blood sampling with consent	Samples were collected in EDTA treated, citrate treated and serum blood tubes and were transported to Centre for Cardiovascular sciences, City Hospital for processing and storage at -70^{o}c for batch analysis. (HbA1c & full blood count analysis were done on the same day of sampling.

	Plasma and serum samples for lipid profiles, liver enzymes, blood biomarkers and genetic analysis were stored at -70^{o}c for batch analysis)

3.1.1.1.3 Response rate

The overall response rate to the E-Echoes was 56% and response rate to blood sampling was 50%.

3.1.1.1.4 Subject characteristics:demographic details, clinical and medication history

3.1.1.1.4.1 Demographic details

A total of 1222 subjects were randomly selected from a large sample of 3441 South Asian for my thesis, of them 702 were men (mean age 58.0 years) and 520 were women (mean age 57.0 years). Details of their ethnicity (self-determined) are shown in table 17. There were six ethnic groups, namely Indian or British Indian, Pakistani or British Pakistani, Bangladeshi or British Bangladeshi, East African Asian, Sri Lankan and other Asian background. The mean age for the study subjects when they migrated to the UK was mean (SD), 21.3 (12.0) years for men (n=688) and 25.0 (12.1) for women (n=507). The mean age for subjects when they left school was mean (SD), 15.3 (2.4) years for men (n=615) and 14.2 (3.0) years for women (n =352). Details on alcohol consumption and smoking are shown in table 18.

Table 17Ethnic groups of the study participants

Male, n = 702 (mean age 58 years)	
Ethnic groups	Number of subjects (%)
Indian/British Indian	328 (46.7)
Pakistani/British Pakistani	257 (36.6)
Bangladeshi/British Bangladeshi	67 (9.5)
East African Asian	43 (6.1)
Other Asian background	7 (0.9)
Female, n = 520 (mean age 57 years)	

Indian/British Indian	270 (51.9)
Pakistani/British Pakistani	166 (31.9)
Bangladeshi/British Bangladeshi	52 (10)
East African Asian	28 (5.4)
Sri Lankan	1 (0.2)
Other Asian background	3 (0.6)

Table 18Alcohol consumption and smoking for the study cohort

Male, n = 702 (mean age 58 years)	
No, always a non-drinker	351 (28.7)
No, used to drink but stopped (over 12 months ago)	105 (8.6)
Yes, but very occasionally	175 (14.3)
Yes	71 (5.8)
Current smokers	116 (16.5)
Female, n = 520 (mean age 57 years)	
No, always a non-drinker	471 (38.5)
No, used to drink but stopped (over 12 months ago)	7 (0.6)
Yes, but very occasionally	39 (3.2)
Yes	3 (0.2)
Current smokers	8 (1.5)

3.1.1.1.4.2 Details on Medication

The questionnaire used during the screening process included details on current medication. The medication were broadly categorised into diuretics, beta blockers, ACE inhibitors, anti-arrhythmic drugs, positive inotropic drugs, lipid lowering drugs, calcium channel blockers, anti thrombotic agents, anti-coagulants, angiotensin-II receptor antagonists, alpha-adrenoceptor blocking drugs, nitrates and anti-diabetic drugs.

Table 19Medication history for the study population

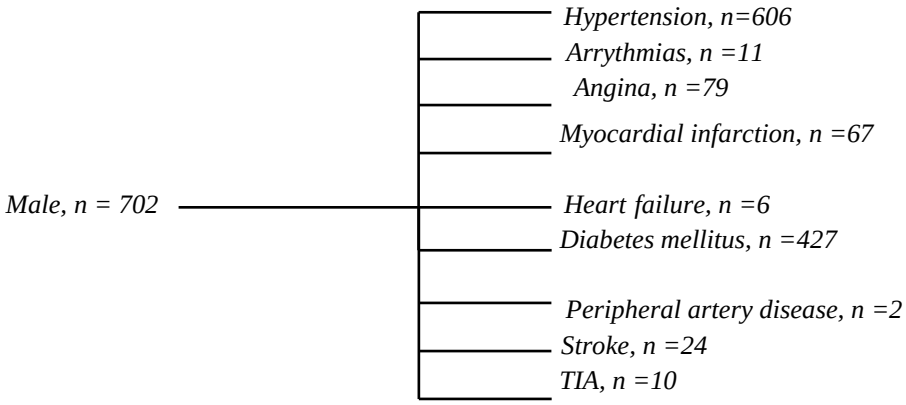
Male, n = 702 (mean age 58 years)	
Drug category	Number of subjects (%)
Diuretics	94 (13.4)
Beta blockers	95 (13.5)
ACE inhibitors	180 (25.6)
Anti-arrhythmic drugs	1 (0.14)
Positive inotropic drugs	1 (0.14)
Lipid lowering drugs	301 (43.0)
Calcium channel blockers	119 (17.0)
Anti thrombotic agents	118 (16.8)
Anti-coagulants	0
Angiotensin-II receptor antagonists	55 (7.8)

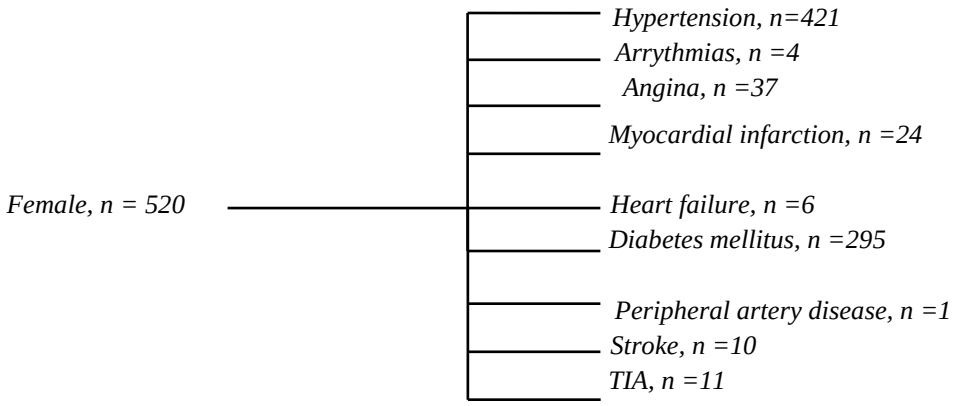
Alpha-adrenoceptor blocking drugs	22 (3.1)
Nitrates	31 (4.4)
Anti-diabetic drugs	159 (23.0)
Female, n = 520 (mean age 57 years)	
Diuretics	85 (16.3)
Beta blockers	60 (11.5)
ACE inhibitors	85 (16.3)
Anti-arrhythmic drugs	0
Positive inotropic drugs	2 (0.4)
Lipid lowering drugs	180 (34.6)
Calcium channel blockers	64 (12.3)
Anti thrombotic agents	53 (10.2)
Anti-coagulants	1 (0.2)
Angiotensin-II receptor antagonists	52 (10)
Alpha-adrenoceptor blocking drugs	6 (1.2)
Nitrates	11 (2.1)
Anti-diabetic drugs	100 (19.2)

3.1.1.1.4.3 Clinical History

The history of co-morbidity in self and family was recorded. The number of subjects with co-morbidities namely hypertension (130/85 mmHg and/or anti-hypertensive drugs), arrhythmias, angina, MI, HF, diabetes mellitus (HbA1c >6.5% and/or anti-diabetic drugs), peripheral artery disease, stroke and TIA (self-reported) are shown in figure 19.

figure 19 Details on co-morbidities-(E-ECHOES -The Biomarker study)





3.1.1.2 Power calculation

Sample sizes for ROC analysis were estimated from published tables (Obuchowski, 2000). At a 5% type I error rate, and 80% power, we estimated that 229 patients would be sufficient to detect moderate differences [10% difference in area under the curve (AUC)] in the performance of blood biomarkers at discriminating those with increased risk of CVD, assuming the curves to have a moderate accuracy (sensitivity of 0.60 and at a false-positive rate of 0.10).

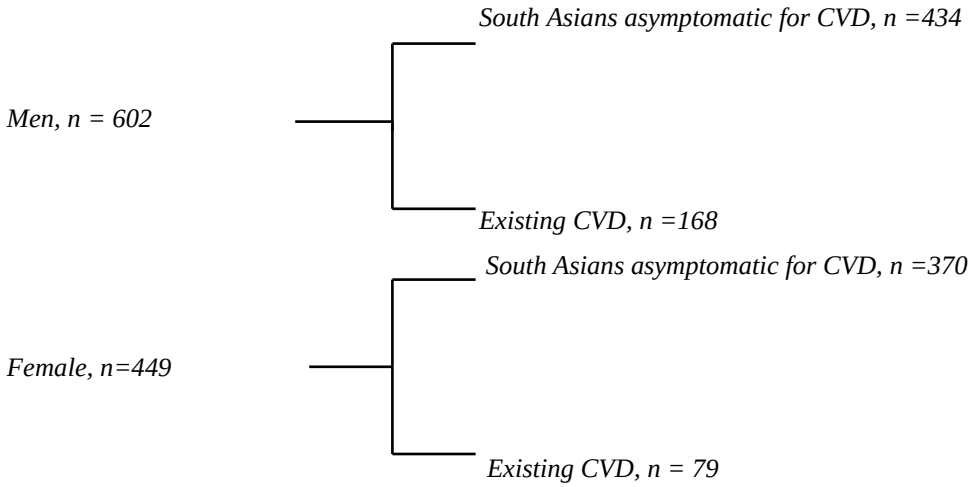
3.1.2 The genetic study

The study population for the genetic study were from two main studies involving South Asian population in the department, namely E-ECHOES and Niaspan: Indo-Asian Stroke patient Atherosclerotic Regression trial (NIASTAR). Detailed description of the Niastar trial and subject characteristics from each study: demographic details, clinical and medication history are shown in section 3.1.2.1.

A total of 1053 were included in the genetic study, 804 were South Asians asymptomatic for CVD (this included high CVD risk subjects on medication for modifiable CVD risk factors

namely for high blood pressure, diabetes mellitus and abnormal lipid profiles) and 247 South Asians were with existing CVD events (figure 20).

figure 20 Subjects in the genetic study



South Asians asymptomatic for CVD where South Asians who were not on any medication for modifiable CVD risk factors at recruitment.

3.1.2.1 Subject characteristics:demographic details, clinical and medication history- E-ECHOES study

3.1.2.1.1 Demographic details

A total of 954 subjects were randomly selected for the genetic study, of them 532 were men (mean age 58.0 years) and 419 were women (mean age 57.0 years). Details of their ethnicity (self-determined) are shown in table 20. There were six ethnic groups, namely Indian or British Indian, Pakistani or British Pakistani, Bangladeshi or British Bangladeshi, East African Asian, Sri Lankan and other Asian background.

Table 20Ethnic groups of the study participants

Male, n = 532 (mean age 58.0 years)	
Ethnic groups	Number of subjects (%)
Indian/British Indian	253 (48.0)
Pakistani/British Pakistani	190 (36.0)
Bangladeshi/British Bangladeshi	53 (10.0)

East African Asian	32 (6.0)
Other Asian background	4 (0.8)
Female, n = 419 (mean age 57.0 years)	
Indian/British Indian	213 (51.0)
Pakistani/British Pakistani	143 (34.1)
Bangladeshi/British Bangladeshi	39 (9.3)
East African Asian	21 (5.0)
Sri Lankan	1 (0.2)
Other Asian background	1 (0.5)

3.1.2.1.2 Details on Medication

The questionnaire used during the screening process included details on current medication. The medication were broadly categorised into diuretics, beta blockers, ACE inhibitors, anti-arrhythmic drugs, positive inotropic drugs, lipid lowering drugs, calcium channel blockers, anti thrombotic agents, anti-coagulants, angiotensin-II receptor antagonists, alpha-adrenoceptor blocking drugs, nitrates, anti-hyprtensive and anti-diabetic drugs.

Table 21Medication history for the study population

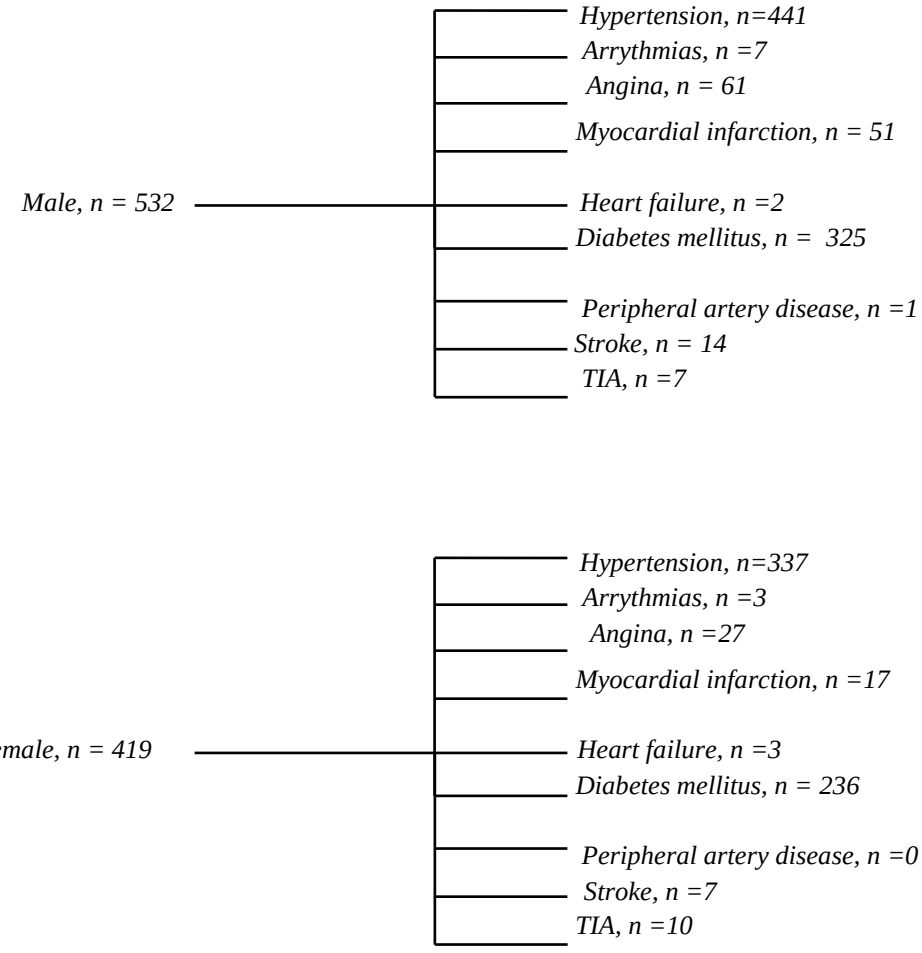
Male, n = 532 (mean age 58.0 years)	
Drug category	Number of subjects (%)
Diuretics	67 (13.0)
Beta blockers	71 (13.3)
ACE inhibitors	131 (24.6)
Anti-arrhythmic drugs	0
Positive inotropic drugs	0
Lipid lowering drugs	175 (33.0)
Calcium channel blockers	84 (16.0)
Anti -platelet agents	83 (16.0)
Anti-coagulants	0
Angiotensin-II receptor antagonists	39 (7.3)
Alpha-adrenoceptor blocking drugs	20 (4.0)
Nitrates	23 (4.3)
Anti-diabetic drugs	93 (18.0.)
Female, n = 419 (mean age 57.0 years)	
Diuretics	64 (15.3)
Beta blockers	43 (10.3)
ACE inhibitors	65 (16.0)
Anti-arrhythmic drugs	0
Positive inotropic drugs	2 (0.5)
Lipid lowering drugs	121 (29.0)
Calcium channel blockers	55 (13.1)
Anti platelet agents	39 (9.3)

Anti-coagulants	1 (0.2)
Angiotensin-II receptor antagonists	44 (11.0)
Alpha-adrenoceptor blocking drugs	5 (1.2)
Nitrates	7 (2.0)
Anti-diabetic drugs	63 (15.0)

3.1.2.1.3 Clinical History

The history of co-morbidity in self and family was recorded. The number of subjects with co-morbidities namely hypertension (blood pressure 130/85mmHg and/or on anti-hypertensive medications), arrhythmias, angina, MI, HF, diabetes mellitus (HbA1c >6.5% and/or on anti-diabetic medications), peripheral artery disease, stroke and TIA (self-reported) are shown in figure 21.

figure 21 Details on co-morbidities (E-ECHOES -The Genetic study)



3.1.2.2 NIASTAR TRIAL: Efficacy and Safety of Niaspan in South Asian Stroke Survivors

The NIASTAR trial was a randomised, double blind, placebo-controlled study comparing the efficacy and safety of co-administration of Niaspan (1000-2000 mg) with Simvastatin (10-40 mg) versus placebo with Simvastatin (10-40 mg) amongst 280 stroke patients of South Asian origin. The study was authorised by Medicines and Healthcare products Regulatory Agency (MHRA) (Eudract no. 2005-004080-28). The protocol was approved by the Oxfordshire Research Ethics Committee and written informed consent was obtained from all study participants. All participants were of South Asian origin in the UK originating from India, Pakistan and Bangladesh with a prior history of stroke or TIA. The participants were recruited from the stroke unit at Sandwell and West Birmingham Hospitals, NHS trust.

Note: The study participants for the NIASTAR trial were recruited by the research team at Sandwell Medical Research Unit

3.1.2.2.1 Subject characteristics: demographic details, clinical and medication history- NIASTAR TRIAL

3.1.2.2.1.1 Demographic details

A total of 102 South Asian subjects with stroke were selected from the NIASTAR trial for the genetic study, of them 71 were men (mean age 62.0 years) and 30 were women (mean age 66.0 years). Details of their ethnicity (self-determined) are shown in table 22.

Table 22Ethnic groups of the study participants

Male, n = 71 (mean age 62.0 years)	
Ethnic groups	Number of subjects (%)
Indian/British Indian	43 (69.4)
Pakistani/British Pakistani	17 (27.4)
Bangladeshi/British Bangladeshi	2 (3.23)
Female, n = 30 (mean age 66.0 years)	

Indian/British Indian	17 (71.0)
Pakistani/British Pakistani	5 (21.0)
Bangladeshi/British Bangladeshi	2 (8.33)

Ethnicity - Self reported or based on names, was available only for 62 men and 24 women

3.1.2.2.1.2 Details on Medication

All participants provided details on their current medications as per the study protocol. The medication were broadly categorised into diuretics, beta blockers, ACE inhibitors, anti-arrhythmic drugs, positive inotropic drugs, lipid lowering drugs, calcium channel blockers, anti thrombotic agents, anti-coagulants, angiotensin-II receptor antagonists, alpha-adrenoceptor blocking drugs, nitrates, anti-hypertensive and anti-diabetic drugs. Table 23 shows the medication history for the study population.

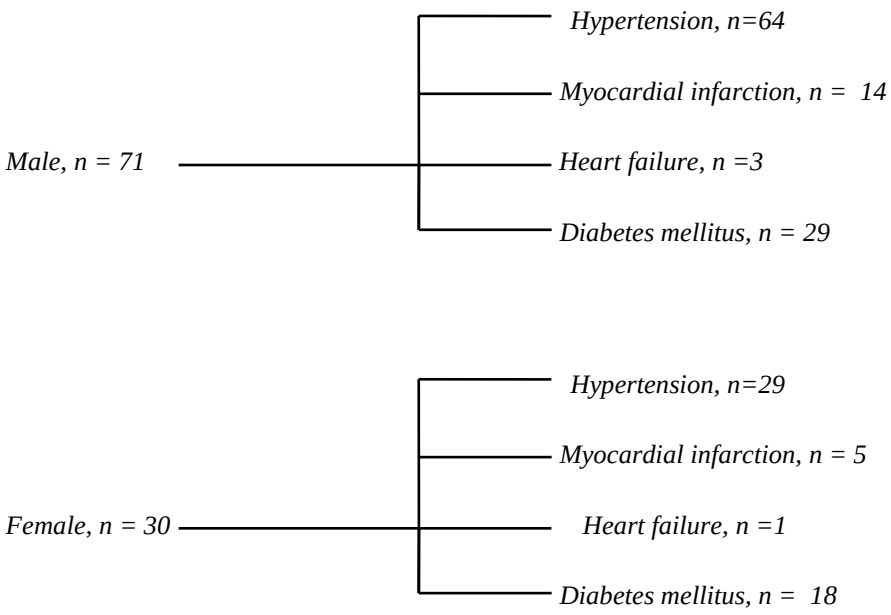
Table 23Medication history for the study population

Male, n = 71 (mean age 62.0 years)	
Drug category	Number of subjects (%)
Diuretics	13 (19.0)
Beta blockers	13 (19.0)
ACE inhibitors	43 (61.4)
Anti-arrhythmic drugs	1 (1.4)
Positive inotropic drugs	0
Lipid lowering drugs	48 (70.0)
Calcium channel blockers	15 (21.4)
Anti -platelet agents	61 (87.1)
Anti-coagulants	4 (6.0)
Angiotensin-II receptor antagonists	0
Alpha-adrenoceptor blocking drugs	5 (7.2)
Nitrates	4 (6.0)
Anti-diabetic drugs	28 (41.0)
Female, n = 30 (mean age 66.0 years)	
Diuretics	12 (40.0)
Beta blockers	10 (33.3)
ACE inhibitors	18 (60.0)
Anti-arrhythmic drugs	0
Positive inotropic drugs	0
Lipid lowering drugs	24 (80.0)
Calcium channel blockers	10 (33.3)
Anti platelet agents	26 (87.0)
Anti-coagulants	4 (13.3)
Angiotensin-II receptor antagonists	0
Alpha-adrenoceptor blocking drugs	2 (7.0)
Nitrates	3 (10.3)
Anti-diabetic drugs	18 (60.0)

3.1.2.2.1.3 Clinical History

The history of co-morbidity in self was recorded. The number of stroke subjects with co-morbidities namely hypertension (blood pressure 130/85mmHg and/or on anti-hypertensive medications), MI, HF, diabetes mellitus (HbA1c >6.5% and/or on anti-diabetic medications) and peripheral artery disease are shown in figure 22.

figure 22 Details on co-morbidities-(Niastar trial- The Genetic study)



3.1.3 Pilot data on abnormalities of beta globin gene and cardiovascular disease

3.1.3.1 Subjects characteristics: demographic details, medication and clinical history

3.1.3.1.1 Demographic details

A total of 156 South Asian subjects were included in this pilot study, of whom 84 control subjects were selected from E-ECHOES and 72 ischaemic stroke patients from NIASTAR trial

for this thesis, of them 98 were men (mean age 59.2 years) and 57 were women (mean age 58.1 years). Details of their ethnicity (self-determined) are shown in table 24.

Table 24Ethnic groups of the study population

Male, n = 98 (mean age 59.2 years)	
Ethnic groups	Number of subjects (%)
Indian/British Indian	49 (51.0)
Pakistani/British Pakistani	32 (33.3)
Bangladeshi/British Bangladeshi	9 (9.4)
East African Asian	6 (6.3)
Female, n = 57(mean age 58.1 years)	
Indian/British Indian	26 (53.1))
Pakistani/British Pakistani	14 (29.0)
Bangladeshi/British Bangladeshi	7 (14.3)
East African Asian	2 (4.1)

Ethnicity was missing for8 female subjects and 2 male subjects

3.1.3.1.2 Details on Medication

The medication were broadly categorised into diuretics, beta blockers, ACE inhibitors, anti-arrhythmic drugs, lipid lowering drugs, calcium channel blockers, anti-platelet therapy and anti-diabetic drugs.

Table 25Medication history for the study population

Male, n = 98 (mean age 59.2 years)	
Drug category	Number of subjects (%)
Diuretics	54 (55.1)
Beta blockers	17 (17.3)
ACE inhibitors	20 (20.4)
Anti-arrhythmic drugs	26 (26.5)
Lipid lowering drugs	72 (73.5)
Calcium channel blockers	45 (46.0)
Anti platelet drugs	59 (60.2)
Anti-diabetic drugs	17 (17.3)
Female, n = 57 (mean age 58.1years)	
Diuretics	26 (45.6)
Beta blockers	16 (28.1)
ACE inhibitors	19 (33.3)
Anti-arrhythmic drugs	10 (17.5)
Lipid lowering drugs	28 (49.1)
Calcium channel blockers	23 (40.3)
Anti-platelet drugs	26 (46.0)
Anti-diabetic drugs	9 (15.8)

3.2 Preparation of plasma and serum from whole blood

Blood samples were obtained from patients who gave written informed consent for E-ECHOES sub-study –blood biomarkers. Blood were collected in EDTA treated, citrate treated and serum tubes (Sarstedt, Germany) and were stored at 4^{o}C prior to transportation from the General Practices within West Midlands to the laboratory at the Centre for Cardiovascular Sciences, City Hospital by the research team at the end of each day. The blood tubes were spun at 3000 rpm for 15 minutes in a refrigerated centrifuge at 4^{o}C. Serum and plasma were then aliquoted into eppendorfs, labelled appropriately with patient's unique study ID, date of collection and nature of sample i.e. serum, EDTA treated plasma, citrated plasma. The serum and plasma samples were then stored at -70^{o}C freezer at the Centre for Cardiovascular sciences, City Hospital until batch analysis for blood biomarkers. Full blood count was analysed on fresh EDTA treated whole blood samples before storage at -70^{0}C for DNA extraction.

3.3 Measurement of biochemical measures, lipid profiles and plasma biomarkers on automated systems

3.3.1 Basic biochemical tests

All biochemical measures which included liver enzymes (alanine aminotransferase (ALT), aspartate aminotransferase (AST), γ-glutamyltransferase (GGT) & alkaline phosphatise), kidney function test (creatinine), lipid profiles (ApoAI, ApoB, total cholesterol, HDL-c, LDL-c & triglycerides), iron and glycated haemoglobin (HbA1c) were determined in serum samples batch wise on automated auto-analyser (COBAS Integra 400 plus, Roche Diagnostics, UK) as per manufacturer's intructions. Full blood count was measured on fresh EDTA treated whole blood samples on ADVIA 120 Haematology system (Siemens, UK).

3.3.1.1 Calculated Triglycerides

Calculated triglyceride was determined using the Friedwald formula (Friedewald, 1972);

Triglycerides = 2.2 × (CHOL-HDL-C-LDL-C)

3.3.2 Plasma biomarkers

3.3.2.1 High sensitivity C-reacyive protein

The acute phase reactant was determined by particle enhanced turbidimetric assay (COBAS Integra 400 plus, Roche Diagnostics, UK). The assay quantitatively determines human CRP concentration in serum, human CRP agglutinates with latex particles coated with monoclonal anti-CRP antibodies. The precipitate is determined turbidmetrically at 552 nm. The lower detection limit for this assay is 0.71 mg/L. The test was calibrated with Calibrator f.a.s. proteins (Roche Diagnostics, UK) and CRP T- control (Roche Diagnostics, UK) was used as QC, the inter assay CV was 5.0%.

3.3.2.2 D-dimer

The biomarker was determined by particle enhanced immunoturbidimetric assay (COBAS Integra 400 plus, Roche Diagnostics, UK) in citrated plasma. The assay quantitatively determines D-dimer epitopes in plasma, D-dimer agglutinates with latex particles coated with monoclonal antibodies to the D-dimer epitope. The precipitate is determined turbidmetrically at 659 nm. The lower detection limit for this assay is <0.08 μg FEU/ml. The test was calibrated with D-dimer calibrator (Roche Diagnostics, UK), D-dimer control I/II (Roche Diagnostics, UK) were used as QC, the inter-assay CV were 1.2% and 2.1% respectively.

3.3.2.3 Brain natriuretic peptide

Brain natriuretic peptide was determined in EDTA treated plasma samples on ADVIA Centaur automated system (Bayer Diagnostics, Germany). The priniciple behind the assay is two site sandwich immunoassay using direct chemiluminescent technology. The analyser detects relative light units produced by chemiluminescence reactions using acridinium ester as the substrate. Two antibodies, an acridinium ester labelled monoclonal mouse anti-human BNP F (ab') 2 fragment specific to the ring structure of BNP and a biotinylated monoclonal mouse anti-human antibody specific to the C-terminal portion of BNP which is coupled to streptavidin paramagnetic beads reacts with the analyte in the sample to form immune-complex, the chemiluminescent reaction is started by addition of acid and base. Unbound acridinium labelled antibody is removed. The amount of relative light units detected by the system is directly proportional to the amount of analyte present in the sample. Bayer BNP Calibrator and Control material was used in each run. The manufacturer's overall (including inter- and intra-assay variability) coefficient of variation was <5% at all tested BNP concentrations.

3.4 Measurement of plasma biomarkers by enzyme linked immunoassay

Plasma biomarkers IL-6, P-selectin and vWF were measured by sandwich enzyme linked immunoassay (ELISA) technique. ELISA technique for each assay are described in this section.

3.4.1 Interleukin 6

Interleukin 6 was determined in EDTA plasma. The Duoset ELISA development kit (R & D systems, UK) used two antibodies, one a mouse anti-human IL-6 as the capture antibody and the other biotinylated goat anti-human IL-6 linked to a peroxidase conjugate as a detection antibody.

Microtitre plates were coated with 100µl of capture antibody (working concentration-2µg/ml), incubated overnight at room temperature. After washing the plates with PBS buffer, 100µl of neat EDTA samples and standards were added to the plates and incubated for 2 hours at room temperature. Again after washing the plates with PBS buffer, 100µl of detection antibody (working concentration -200ng/ml) was added and incubated for 2 hours. After washing the microtitre plates with PBS buffer, 100µl streptavidin-HRP conjugate was added for 20 minutes. The addition of substrate (hydrogen peroxide and tetramethylbenzidine) promoted the colorimetric reaction which was stopped with 50µl stop solution and plates were read at 492nm (Dynatec 500). The lower detection limit for this assay is 9.375 pg/ml. The inter assay CV was 13.0% and intra assay CV based on standards was 11.0% (Table 26).

Table 26Inter-assay coefficient of variation for Interleukin-6

No:	Concentration of controls 1 (pg/ml)	Concentration of controls 2 (pg/ml)	Concentration of controls 3 (pg/ml)	Concentration of controls 4 (pg/ml)
1	55.69	635.18	1415.71	1529.85
2	45.22	588.08	1460.71	1553.32
3	56.83	497.85	1172.71	1281.03
4	46.19	455.71	1545.30	1631.44
5	-	-	1177.629	1320.28
6	-	-	1499.94	1759.62
	CV -12.0%	CV – 15.0%	CV -12.0%	CV -12.1%

Plasma samples of different IL-6 concentrations were used as quality control for IL-6 assays(in singlets) because IL-6 levels were undetectable in 174 healthy subjects and wanted to make sure the assay was working at various concentrations.

3.4.1.1 Interleukin 6 ELISA assay reagents and buffers

3.4.1.1.1 Phosphate buffer

Phosphate buffer saline tablets (5 tablets) and 0.5 ml Tween were dissolved in one litre of water (pH-7.4).

3.4.1.1.2 Antibodies

The capture antibody for the IL-6 ELISA assay was prepared by reconstituting the vial with 1ml PBS which gave a stock of 360µg/ml. A working concentration of 2µg/ml was obtained by adding 10ml of PBS to 55µl (i.e. 20µg) of stock.

The detection antibody was prepared by reconstituting the vial with 1ml PBS which gave a stock of 36µg/ml. A working concentration of 200ng/ml was obtained by adding 10ml of PBS to 55µl (i.e. 20 µg) of stock.

3.4.1.1.3 Standards

The standards were prepared by adding 500µl of PBS into the vial to give a stock of 35ng/ml (or 35000pg in 500µl). A working top standard concentration of 600pg/ml was obtained by adding 1961µl of PBS-T to 39.29 µl of stock (1200 pg or 1.2 ng/2ml). This is enough for 4 plates.

3.4.1.1.4 Preparation of samples

100µl of neat EDTA plasma was added to each well.

3.4.1.1.5 Conjugate and substrate solution

The conjugate, streptavidin-HRP was prepared by adding 10ml of PBS-T to 50µl.

Substrate solution was prepared by mixing equal volumes of colour reagent A, hydrogen peroxide and colour reagent B, tetramethylbenzidine (R & D systems, UK) (5 ml of each is enough for one 96-well plate).

3.4.1.1.6 Stop solution

To obtain 1mol/L of hydrochloric acid, 913.8ml of deionised water was mixed with 86.2 ml of HCl (11.6(M).

3.4.2 P-selectin

The Duoset ELISA development kit (R & D systems, UK) was used to determine the levels of P selectin in EDTA plasma. The kit used two antibodies, one a mouse anti-human P-selectin as the capture antibody and the other a biotinylated sheep anti-human P-selectin linked to a peroxidase conjugate as a detection antibody. Microtitre plates were coated with 100µl of the capture antibody (working concentration 2µg/ml), incubated at room temperature overnight. Plates were washed with PBS buffer (pH-7.4) and 100µl of 1:5 diluted EDTA plasma and standards were added to the microtitre plates for 2 hour at room temperature. Plates were again washed with PBS buffer and 100µl detection antibody (working concentration 20µg/ml) was added and incubated at room temperature for 2 hours. After washing the microtitre plates with PBS buffer, 100µl streptavidin-HRP conjugate was added for 20 minutes. The addition of substrate (hydrogen peroxide and tetramethylbenzidine) promoted the colorimetric reaction which was stopped with 50µl stop solution and plates were read at 492nm (Dynatec 500). The lower detection limit for this assay is 125.0 pg/ml. Precinorm U (Roche Diagnostics,UK) were used as QC, the inter assay CV was 13.0% and intra assay CV was 8% based on standards (Table 27).

Table 27Inter assay coefficient of variation for P-selectin assay

No:	Concentration of Precinorm U (ng/ml)
1	35.85
2	35.82
3	35.77
4	28.17
5	281.4
6	35.92
7	31.00
8	35.54
9	35.60
10	27.82
11	22.07
12	29.66
13	31.77
CV = 13.0%	

Quality controls and samples were analysed in singlets

3.4.2.1 P-selectin ELISA assay reagents and buffers

3.4.2.1.1 Phosphate buffer

Phosphate buffer saline tablets (5 tablets) and 0.5 ml Tween were dissolved in one litre of water (pH 7.4).

3.4.2.1.2 Antibodies

The capture antibody was prepared by reconstituting the vial with 1ml PBS which gave a stock of concentration 360µg/ml. A working concentration of 2µg/ml was obtained by adding 10ml of PBS to 55µl (i.e. 20µg) of stock.

The detection antibody was prepared by reconstituting the vial with 1ml PBS which gave a stock of 3.6µg/ml. A working concentration of 20µg/ml was obtained by adding 10ml of PBS to 55µl (i.e. 20 µg) of stock.

3.4.2.1.3 Standards

The standards were prepared by adding 500µl of PBS into the vial to give a stock of 130ng/ml (or 130,000pg in 500µl). A working top standard concentration of 8000pg/ml was obtained by adding 1877µl of PBS-T to 123 µl of stock (16000pg/2ml). This is enough for 4 plates.

3.4.2.1.4 Preparation of samples

EDTA plasma was diluted by mixing 50µl to 200µl of PBS to obtain a 1:5 dilution and 100µl was then added to each well.

3.4.2.1.5 Conjugate and substrate solution

The conjugate, streptavidin-HRP was prepared by adding 10ml of PBS-T to 50µl. Substrate solution was prepared by mixing equal volumes of colour reagent A, hydrogen peroxide and

colour reagent B, tetramethylbenzidine (R & D systems, UK) (5 ml of each is enough for one 96-well plate).

3.4.2.1.6 Stop solution

To obtain 1mol/L of hydrochloric acid, 913.8ml of deionised water was mixed with 86.2 ml of HCl (11.6(M).

3.4.3 Von willebrand factor

vWF was determined in citrated plasma using an in house ELISA developed by Dr Andrew Blann (Research Scientist, Centre of Cardiovascular sciences, City Hospital, Birmingham, UK). The ELISA used rabbit anti-human vWF polyclonal antiserum as the capture antibody and rabbit anti-human vWF polyclonal antiserum conjugated to horse radish peroxidase as the detection antibody. Microtitre plates were coated with 100µl of the primary antiserum (30µl in 20.5ml coating buffer, pH-9.6), incubated at room temperature overnight. Plates were washed with PBS buffer (pH-7.4) and 100µl of 1/40 diluted citrated plasma and standards were added to the microtitre plates for 1 hour at room temperature. Plates were again washed with PBS buffer and 100µl secondary antiserum-the peroxidise-labelled conjugate (30µl in 20.5 ml PBS buffer) was added and incubated at room temperature for 45 minutes. After washing the microtitre plates with PBS buffer, 100µl substrate (OPD, hydrogen peroxide, pH 5.3 citrate buffer) was added. The colorimetric reaction was stopped immediately with 50µl stop solution and plates were read at 492nm (Dynatec 500). The lower detection limit for this assay is 65 IU/dL. Universal controls were used as QC, the inter assay CV was 9.5% and intra assay CV was 3.0% (Table 28).

Table 28 Coefficient of variation for von willebrand factor assay

No:	Concentration of universal controls (IU/dL)	Concentration of universal controls (IU/dL)	Mean concentration of universal controls (IU/dL)
1	114.00	105.00	110.00
2	100.00	95.00	98.00
3	89.00	95.00	92.00
4	86.00	83.00	85.00
5	87.00	86.00	86.00
6	76.00	74.00	75.00
7	77.00	81.00	79.00
8	99.00	104.50	102.00
9	89.60	85.70	87.65
10	95.00	88.00	91.50
11	80.50	78.50	79.50
12	82.90	82.40	82.65
13	81.00	82.30	81.65
14	83.95	81.95	82.95
15	82.36	82.08	82.22
16	83.17	83.98	83.57
17	77.23	69.63	73.43
18	78.42	79.29	78.85
19	81.66	81.38	81.52
20	79.99	79.17	79.58
21	81.70	79.68	80.69
22	81.05	80.0	80.48
23	80.10	80.79	80.44
24	83.28	82.40	82.83
25	81.02	81.13	81.08
26	82.11	81.65	81.88
27	82.15	81.10	81.62

3.4.3.1 Von willebrand factor ELISA assay reagents and buffers

3.4.3.1.1 Coating buffer

Coating buffer was prepared by dissolving 0.795g of sodium carbonate and 1.465g of sodium hydrogen carbonate in 500ml of distilled water (pH-9.6). The buffer was stored at 4^0C.

3.4.3.1.2 Phosphate buffer

Wash buffer was prepared by dissolving phosphate buffer saline tablets (5 tablets) and 0.5 ml Tween in one litre of water (pH 7.4).

3.4.3.1.3 Antibodies

The capture antibody was prepared by adding 30 µL of rabbit anti-human vWF polyclonal antiserum to 20.5 mls coating buffer and the detection antibody by adding 35 µL anti-human vWF polyclonal antiserum conjugated to horse radish peroxidase to 20 ml of wash buffer. Both antibodies were commercially available from Danish company Dako.

3.4.3.1.4 Standards

The standards are referenced against a WHO standard from the National Institute for Biological Standards and Controls (NIBSC), Blanche Lane, South Mimms, Potters Bar, Hertfordshire EN6 3QH. Tel 01707 646 399, fax 01707 646 977. They provided a lyophilised ampoule of plasma with a defined mass of vWF which was made up in water to a desired concentration. The standards were prepared as follows;

The top standard was created by adding 80 µL of plasma to 780 µL of dilution buffer. This gave a result of 180 IU/dL. The second standard was prepared by adding 60 µL of plasma to 780 µL of dilution buffer which gave a concentration of 130 IU/dL. The middle standard was obtained by adding 30 µL of plasma to 780 µL of dilution buffer which gave a concentration of 90 IU/dL. Finally the bottom standard was obtained by adding 10 µL of plasma to 780 µL of dilution buffer and this this gave a concentration of 65 IU/dL. Blank of concentration 0 IU/dL was made up of 780 µL of dilution buffer.

3.4.3.1.5 Substrate solution

Substrate solution was prepared by adding one OPD tablet and 10 µl hydrogen peroxide to 20ml of citrate phosphate buffer. Citrate phosphate buffer was prepared by adding 3.65 g citric acid

and 4.73 g sodium hydrogen phosphate to 500 ml distilled water, pH was about 5.3. If not, the pH was adjusted with concentrated acid or concentrated sodium hydroxide solution.

3.4.3.1.6 Stop solution

To obtain 1mol/L of hydrochloric acid, 913.8ml of deionised water was mixed with 86.2 ml of HCl (11.6(M).

3.5 Isolation of deoxyribonucleic acid from human whole blood/ serum and taqman single nucleotide polymorphism genotyping

To study the genetic markers in this thesis, deoxyribonucleic acid (DNA) was extracted from whole blood or serum samples using Agencourt genfind blood & serum genomic isolation kit (Beckman Coulter, High Wycombe, UK) or QIAamp DNA blood mini kits (Qiagen, West Sussex, UK). Wet DNA was then used in taqman single nucleotide polymorphism genotying on StepOne real time and 7500 PCR systems (Applied Biosytems, UK).

3.5.1 Isolation of deoxyribonucleic acid

Whole blood treated with EDTA and serum samples were stored at -70°C at the Centre for Cardiovascular Sciences, City Hospital until the performance of DNA extraction. Genomic DNA was isolated from frozen whole blood or serum samples by Agencourt's SPRI paramagnetic bead technology or Qiagen's silica membrane technology. Both protocols involved the addition of lysis buffer to rupture cell membranes and proteinase k to remove any enzymes that might break apart DNA. In one of the protocols, DNA is immobilized on magnetic particles by the addition of a magnetic binding reagent. This differential binding allows the DNA to be easily separated from contaminants using a magnetic field, whilst in the second protocol DNA is adsorbed onto the QIAamp silica membrane. Contaminants which can inhibit polymerase chain reaction (PCR)

were then rinsed away using a simple washing procedure, and the genomic DNA was eluted from the magnetic particles and the silica membrane in sterile water.

NOTE: The DNA isolation for 60% of the samples was performed using QIAamp DNA blood mini kits. The extraction was completed by Dr Jonathan James at Functional Genomic and Proteomic services, School of Biosciences, University of Birmingham. The rest of the DNA was extracted using taqman sample to SNP kit (Applied Biosystems, UK). Details of the taqman sample to SNP kit are shown in appendix B 9.2.1.

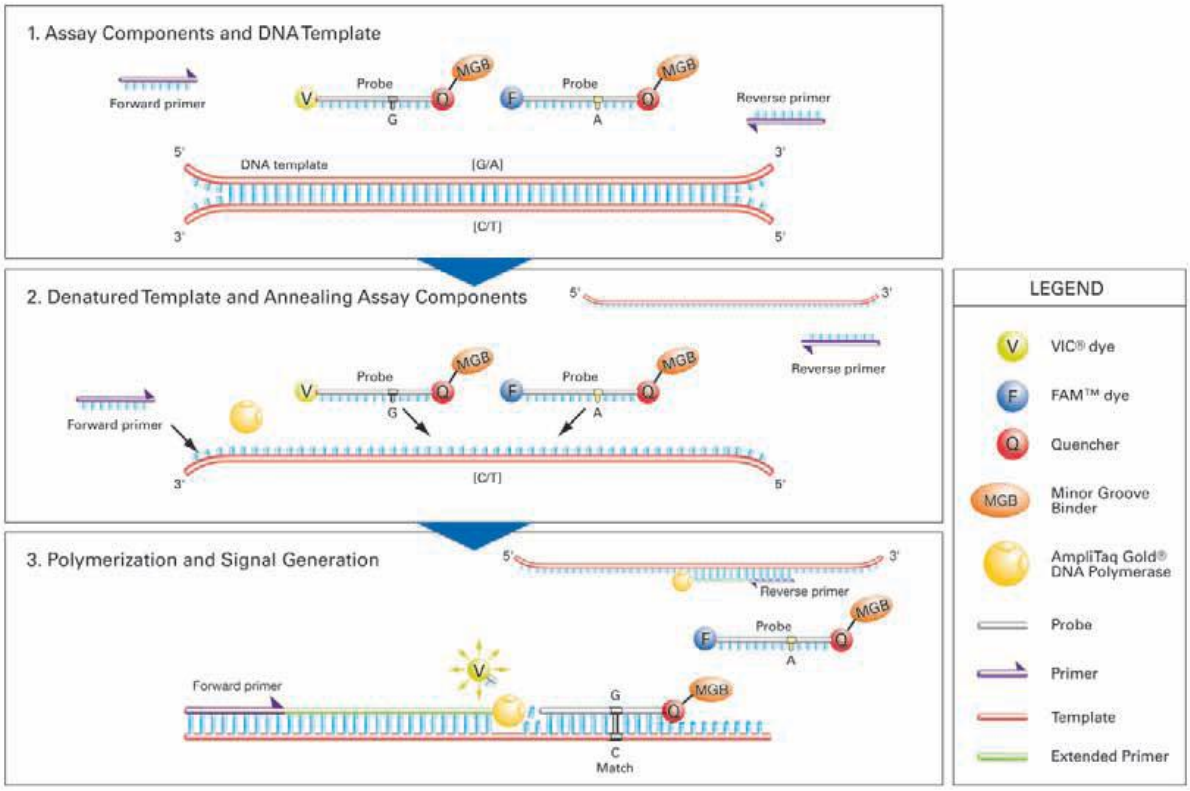
3.5.2 Taqman single nucleotide polymorphism genotyping

The taqman is a hybridisation based assay and utilizes taqman probes which incorporate minor groove binder (MGB) at the 3' end which confers a superior allelic discrimination. The goal of the MGB molecule is to bind to the minor groove of the DNA helix and thus stabilise the MGB-probe/template complex. The probe can be as short as 13 bases which ensure specificity and stabilisation. Furthermore, primers are used in the assay for PCR specificity.

The detection of SNP is achieved by 5' nuclease chemistry, where exonuclease cleavage of a 5' allele-specific dye label generates the permanent assay signal. All MGB probes include a nonfluorescent quencher (NFQ) that virtually eliminates the background fluorescence associated with traditional quenchers and provides a greater signal-to-noise ratio for superior assay sensitivity (Taqman Probe-based assay chemistry, www.appliedbiosystems.com).

All the SNP assays were designed according to the standard PCR conditions set on the StepOne real time PCR and 7500 fast PCR (Applied Biosystems, UK).

figure 23 Taqman Probe-based assay chemistry



Reproduced from Applied Biosystems website- http://www.appliedbiosystems.com/absite/us/en/home/applications-technologies/real-time-pcr/taqman-and-sybr-green-chemistries.html (accessed on December 2009)

3.5.2.1 Genotyping of triallelic single nucleotide polymorphism, rs3091244 using Taqman PCR

Taqman SNP assays for the triallelic SNP rs3091244 was custom designed using bioinformatics tools. Figure 24 shows an overview of the steps involved in the custom designed taqman SNP assays for rs3091244.

figure 24 *Overview of the custom designed Taqman SNP assays for rs3091244*

Target sequence was obtained from the dbSNP database at

http://www.ncbi.nlm.nih.gov/SNP

dbSNP-rs3091244

GTCCATGGCTCTGGGAGGAGCATGTTTGTTTTCCTCATTTCCCAGTCTGTAAATAAGCA
AATTGAAAGGGGTTAGTGATAATGTCCATCTCCAGAAGCTGTCAGATTTCCTTTGTCAA
ACTCTATGATTTGGGCTGAAGTAGGTGTTGGAGAGGCAGCTACCACGTGCACCCAGAT
GGCCACT**[A/C/T]**GTTTAATATGTTACCATTTCCCATTATTTTCGCAGGATAGATAGCCAA
AGTGGAGCCCTGAGAGATTTCTTCATTTTTCCTGTCATAAAGAATTGGTAATTCAGTAG
TCATAGGAGTTTGTAATAAATAACTCACATTGATTTCTCTGTTCTGAAATAATTTTGCTT
CCCCTCTTCCCGAAGCTCTGACACCTGCCCCAA

↓

Assessing the quality of the sequence

1) The uniqueness of the sequences was established by BLAST (Basic Local Alignment Search Tool) at

http://www.ncbi.nlm.nih.gov/BLAST assessed on 27.10.2009

Sequence producing significant alignment;

Locus- NT 004487 -Homo sapiens chromosome 1 genomic contig, score -599, E-value =9.4e-169

2) There were no repetitive elements detected in this sequence after analysing the sequence through the program Repeat masker. (http://repeatmasker.genome.washington.edu)

3) Specialized BLAST was performed to identify SNPs not of interest and was masked with a N.

GTCCATGGCTCTGGGAGGAGCATGTTTGTTTTCCTCATTTCCCAGTCTGTAAATAAGCA
AATTGAAAGGGGTTAGTGATAATGTCCATCTCCAGAAGCTGTCAGATTTCCTTTGTCA
AACTCTATGATTTGGGCTGAAGTAGGTGTTGGAGAGGCAGCTACCACNTGCACCNAGA
TGGCCACT**[c/t]**GTTTAATATGTTACCATTTCCCATTATTTTCGCAGGATAGATAGCCAA
AGTGGAGCCCTNAGAGATTTCTTNATTTTTCCTGTCATAAAGAATTGGTAATTCAGTAG

4) The masked sequence with SNP of our interest was then submitted to Applied Biosytems for taqman SNP genotyping assay

Since the SNP was triallelic (A/C/T), two paired conventional Taqman SNP assays were designed to detect all three alleles of the triallelic SNP. One for C/A and one for C/T genotyping. The C allele was labelled with FAM, and the minor alleles A and T were labelled with VIC, both the genotyping assays were performed in separate tubes.

Table 29 show the details of the two biallelic assay used to detect rs3091244 and table 30 shows the Taqman primer and probe sequences used for the genotyping of rs3091244.

Genomic wet DNA was used for all genotyping experiments. Negative controls (sterile water) and two positive controls for the triallelic *CRP* SNPs were analysed on all plates to ensure there was no contamination. The reaction volume for each well was set to 20 µl (Table 31) and number of cycles to 50 (Table 32). The reaction volume and cycling stages for the PCR were similar for all taqman SNP assays.

Table 29 Taqman SNP assays to detect the triallelic SNP rs3091244

Assay name	Gene name	Gene symbol	rs ID	Alleles tested	Major allele to minor allele
AHO9VY8	C-reactive protein	CRP	rs3091244	C>T	C-T
AHN1ECS	C-reactive protein	CRP	rs3091244	C>A	C-A

Table 30Taqman primer and probe sequences used for the genotyping of rs3091244

Genotype	Primer (5'-3')		Allele	Allelic probe
	Forward	Reverse		
C/T	AGGTGTTGGAGAGGCAGCTA	TCCTGCGAAAATAATGGGAAATGGT	C	VIC* ATGGCCACTCGTTTAA MGBNFQ
			T	FAM* ATGGCCACTTGTTTAA MGBNFQ
C/A	GTTGGAGAGGCAGCTACCA	TCCTGCGAAAATAATGGGAAATGGT	C	VIC* AGATGGCCACTCGTTTA MGBNFQ
			A	FAM*AGATGGCCACTAGTTTA MGBNFQ

The positions of SNPs in the allelic probes are underlined.
**reporter dye used for allelic discrimination*

Table 31Reaction mix

Reagents	Volume (µl/ 96 well plate)
2 × Taqman SNP genotyping mastermix	1008.0
40 × Taqman SNP genotyping assay	50.40
DNAse-free water	756.0
Genomic DNA template	2.0 (per well)
Total volume	20.0 (per well)

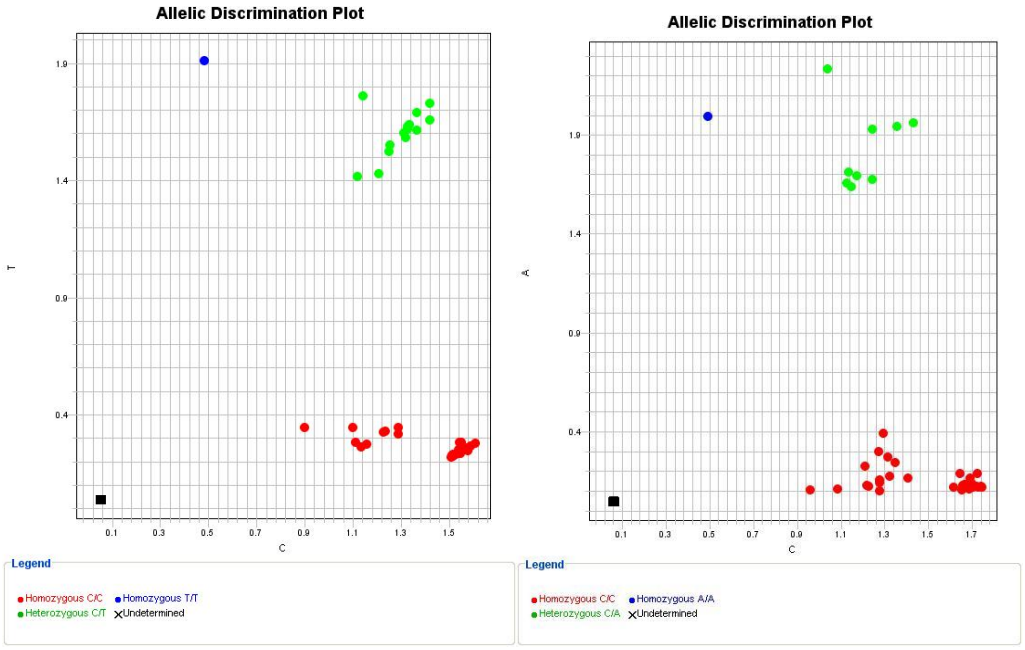
Table 32Cycling stage for the PCR

	Pre-PCR read	Thermal cycling			Post-PCR read
Stage	Holding stage	Holding stage	Cycling (50 cycles)		Holding stage
			Denature	Anneal/ extend	
Temperature	60^0C	95^0C	92^0C	60^0C	60^0C
Time (mm:ss)	00:30	10:00	00:15	01:00	00:30

In the experiment for C/T genotyping, three genotypes will be identified: CC, CT and TT. The CA genotype may be included in the samples identified as the CC genotype and the AT genotype may be included in the samples identified as the TT genotype. The

AA genotype will not be amplified. Similarly in the C/A genotyping experiment, three genotypes will be identified: CC, CA and AA. The CT genotype and the AT genotype may be included in the samples identified as the CC genotype and the AA genotype respectively. The TT genotype will not be amplified (Morita et al., 2007).

figure 25 Scatter diagram of the data for the genotyped single nucleotide polymorphism (rs3091244)



C allele labelled with FAM on the horizontal axis and the minor alleles A and T labelled with VIC on the vertical axis. Each genotype group is depicted in different colours and negative control is plotted as black squares

The genotyping results from 2 experiments, C/T and C/A Taqman assays will be combined to obtain all the results.

3.5.2.2 Genotyping of single nucleotide polymorphism rs1800795 using Taqman PCR

The -174G/C IL-6 promoter polymorphism is a biallelic SNP and figure 43 in appendix B 9.2.1.2 gives an overview of the custom designed taqman SNP assay for rs1800795. Table 33 show the details of the biallelic assay used to detect rs1800795 and table 34 shows the Taqman primer and probe sequences used for the genotyping of rs1800795.

Table 33Taqman SNP assays for the biallelic SNP rs1800795

Assay name	**Gene name**	**Gene symbol**	**rs ID**	**Alleles tested**	**Major allele to minor allele**
AHY9ICB	Interleukin-6	IL-6	rs1800795	G>C	G-C

Table 34Taqman primer and probe sequences used for the genotyping of rs1800795

Genotype	Primer (5'-3')		Allele	Allelic probe
	Forward	Reverse		
G/C	TGACGACCTAAGCTGCACTTTT	AGATTGTGCAATGTGACGTCCTT	G C	VIC* TTGTGTCTTGCGATGCTA MGBNFQ FAM* TTGTGTCTTGCCATGCTA MGBNFQ

The positions of SNPs in the allelic probes are underlined.
**reporter dye used for allelic discrimination*

3.5.2.3 Genotyping of single nucleotide polymorphism rs2854116 using Taqman PCR

The T455C (rs2854116) promoter polymorphism of *APOC3* gene is a biallelic SNP. An overview of the custom designed taqman SNP assay for rs2854116 is shown in figure 44 in appendix. B 9.2.1.2. Table 35 show the details of the biallelic assay used to detect rs2854116 and table 36 shows the Taqman primer and probe sequences used for the genotyping of rs2854116.

Table 35SNP assays for the biallelic SNP rs2854116

Assay name	**Gene name**	**Gene symbol**	**rs ID**	**Alleles tested**	**Major allele to minor allele**
AHBJG2W	Apolipoprotein C3	*APOC3*	rs2854116	T/C	T-C

Table 36 Taqman primer and probe sequences used for the genotyping of rs2854116

Genotype	Primer (5'-3')		Allele	Allelic probe
	Forward	Reverse		
T/C	CTGAACACAGCCTGGAGTAGAG	TGCCGGAGCCACTGATG	T C	VIC* ACTCCAAACA<u>T</u>CCCCC MGBNFQ FAM* CTCCAAACA<u>C</u>CCCCC MGBNFQ

The positions of SNPs in the allelic probes are underlined.
**reporter dye used for allelic discrimination*

3.5.2.4 Genotyping of single nucleotide polymorphism rs2854117 using Taqman PCR

The C482T (rs2854117) promoter polymorphism of APOC3 gene is a biallelic SNP. An overview of the custom designed taqman SNP assay for rs2854117 is shown in figure 45 in appendix B 9.2.1.2. Table 37 shows the details of the biallelic assay used to detect rs2854117 and table 38 shows the Taqman primer and probe sequences used for the genotyping of rs2854117.

Table 37Taqman SNP assays for the biallelic SNP rs2854117

Assay name	**Gene name**	**Gene symbol**	**rs ID**	**Alleles tested**	**Major allele to minor allele**
AHQJAN9	Apolipoprotein C3	*APOC3*	Rs	C/T	C-T

Table 38 Taqman primer and probe sequences used for the genotyping of rs2854117

Genotype	Primer (5'-3')		Allele	Allelic probe
	Forward	Reverse		
C/T	CTGAACACAGCCTGGAGTAGAG	AGAGCTCAGCCCTGTAACCA	C T	VIC* CAGAAGACCGGGCATC MGBNFQ FAM* CAGAAGACCAGGCATC MGBNFQ

The positions of SNPs in the allelic probes are underlined.
**reporter dye used for allelic discrimination*

3.5.2.5 Genotyping of single nucleotide polymorphism rs662799 using Taqman PCR

Taqman SNP genotyping assay for APOA5 polymorphism was a pre designed assay supplied by Applied Biosytem. Table 39 shows the sequence used for designing Taqman SNP genotyping for rs662799.

Table 39 Sequence for Taqman SNP genotyping assay for rs662799

Assay ID	Reference SNP number	Sequence used for Taqman SNP assay
C_2310403_10	rs662799	GAGCCCCAGGAACTGGAGCGAAAGT[A/G]AGATTTGCCCCA TGAGGAAAAGCTG

3.6 Zonal density gradient ultracentrifugation

Zonal ultracentrifugation is a technique used to separate lipid sub fractions in plasma based on their size, shape and density. Zonal separations are obtained by placing layer after layer of density gradient solutions based on the densities of different lipid sub fractions. During ultracentrifugation lipid sub fractions based on their size and density migrate to their respective gradient layer and form discreet bands (Wilcox and Heimberg, 1970). Detailed description is shown in appendix B 9.2.3.

3.7 Mass spectrometry for identification of haemoglobin variants by ESI-MS

Note: A total of 174 whole blood EDTA treated samples were analysed by electrospray ionization mass spectrometry (ESI-MS) for the identification of haemoglobin disorders. The analysis was

carried out by Charlotte Scarff under the supervision of Prof Jim Scrivens at Centre for biomedical mass spectrometry and proteomics, Department of Biological Sciences, University of Warwick. Detailed description is shown in appendix B 9.2.4.

3.8 Development of a novel enzyme linked immunoassay-Ferritin bound to Apolipoprotein B

3.8.1 Background

In the United Kingdom, the burden of CVD is more prominent amongst the country's ethnic minorities (Barnett et al., 2006; Gill, 2007), and this is mainly due to the preponderance of CVD risk factors, such as diabetes mellitus which are most evident amongst South Asians compared to the general population (Nicholl et al., 1986; Mckeigue, 1991; Patel, 2008a; Patel, 2012a).

Increased body iron stores have been implicated in the pathogenesis of diabetes mellitus (Jiang et al., 2004); moreover increased circulating levels of ferritin have been associated with ApoB concentrations (Fernandez-Real et al., 1998). Studies have identified ferritin heavy and light chains to directly bind ApoB (Rashid et al., 2002; Seki et al., 2008) and that ferritin regulates the secretion of ApoB (Hevi and Chuck, 2003). The physiological or the clinical implications of ferritin bound to ApoB have not been explored. In this thesis an ELISA method was developed to determine the levels of ferritin bound to ApoB. The study also hypothesised an ethnic difference in the levels of ferritin and ferritin bound to ApoB among non-diabetic South Asian and European subjects.

3.8.2 Research design and methods

Subjects consisted of healthy volunteers recruited for research at the Centre for Cardiovascular Sciences, City Hospital, Birmingham. Diabetes status was determined by evidence within hospital case notes or by measurement of fasting plasma glucose. All subjects provided written informed consent. Ethical approval for this study was obtained from the local ethics committee.

Serum cholesterol (CHOD-PAP method), HDL (direct method) and ApoB were determined using routine auto analyser assays (Cobas Integra 400, Roche Diagnostics, UK) Separated EDTA plasma were stored at -70^{0}C for batch analysis.

3.8.3 Enzyme linked immunoassay for ferritin and ferritin bound to apolipoprotein B

3.8.3.1 Principle of the test

An in-house sandwich ELISA was developed for the measurement of ferritin levels and ferritin bound to ApoB on EDTA plasma using commercially available antibodies (Rockland, Gilbertsville, PA USA and R& D systems, Abingdon, UK). The assay utilizes two capture antibodies IgG fraction of anti-human ferritin antibodies and anti-human apolipoprotein B antibody for solid phase i.e. microtiter wells. The assay was performed on 96-well micro titre plates, divided into two sections, the first half was coated with IgG fraction of anti-human ferritin antibodies that were highly specific for human ferritin and the second half was coated with anti-human apolipoprotein B antibody. Calibrators, controls and serum samples were incubated for 2 hours and allowed to react with the capture antibodies. The detection antibody, biotin conjugated IgG fraction of anti-ferritin was then added and was incubated for 2 hours. This resulted in

ferritin molecules and ferritin bound to ApoB molecules in the sample being sandwiched between the solid phase and the enzyme linked detection antibody.

After washing off excess secondary antibody, conjugate streptavidin-horseradish peroxidase (HRP) and chromogenic substrates were used to determine the concentration of ferritin and ferritin bound to ApoB. The lower limit of detection using this ELISA assay for ferritin was 78.12 pg/ml.

3.8.3.2 Ferritin bound to apolipoprotein B-ELISA assay reagents and buffers

3.8.3.2.1 Phosphate buffer

Phosphate buffer saline tablets (5 tablets) and 0.5 ml Tween were dissolved in one litre of water.

3.8.3.2.2 Antibodies

The capture antibodies for this assay were IgG fraction of anti-ferritin Ab [human spleen] [rabbit] (Rockland, USA) and anti-human apolipoprotein B (ApoB) (R&D systems, UK). The capture antibody, IgG fraction of anti-ferritin Ab was prepared by reconstituting the vial with 5.0ml of sterile water which gave a stock solution of 10.0 mg/ml. A working concentration of 250 ng/ml was obtained by adding 2.5µl stock to 10mls of PBS. The second capture antibody, anti-human ApoB antibody was prepared by reconstituting the vial 0.5ml PBS which gave a stock solution of 0.2 mg/ml. A working concentration of 1000 ng/ml was obtained by adding 25µl stock to 10ml PBS.

The detection antibody was biotin conjugated IgG fraction of anti-ferritin [human spleen] [rabbit] antibody and was prepared by reconstituting the vial with 1ml of sterile water which gave a stock

solution of 10.0mg/ml. A working concentration of 2000ng/ml was obtained by adding 1µl to 50ml of PBS.

3.8.3.2.3 Standards

The standards for this assay were the quality control for ferritin assay for COBAS Integra 400 plus (Roche Diagnostics, UK). The top standared of 5000pg/ml was obtained by adding 33.3µl of stock to 966.7µl of PBS). This was diluted 6 times to obtain a 7-point serial dilution standard curve as shown in table 24.

Table 40 Preparation of standards for ferritin bound to ApoB ELISA assay

Ferritin	**Volume from preceding standard**	**Volume of PBS**
5000pg/ml (1)	Stock	
2500pg/ml (2)	500µl from (1)	500µl
1250pg/ml (3)	500µl from (2)	500µl
625pg/ml (4)	500µl from (3)	500µl
312.5pg/ml (5)	500µl from (4)	500µl
156.25pg/ml (6)	500µl from (5)	500µl
78.12pg/ml	500µl from (6)	500µl

3.8.3.2.4 Preparation of samples and quality controls

Serum samples were used for this assay, 5µl of serum on anti-ferritin primary antibody coated side (1:20) and 25µl on anti-human ApoB coated side (1:4). Universal controls were used as quality controls for this assay.

3.8.3.2.5 Substrate solution

The conjugate, streptavidin-HRP was prepared by adding 10ml of PBS-T to 50µl. Substrate solution was prepared by mixing equal volumes of colour reagent A, hydrogen peroxide and colour reagent B, tetramethylbenzidine (R & D systems, UK) (5 ml of each is enough for one 96-well plate).

3.8.3.2.6 Stop solution

To obtain 1mol/L of hydrochloric acid, 913.8ml of deionised water was mixed with 86.2 ml of HCl (11.6(M)).

3.8.4 Statistical analysis

To detect a ½ a standard deviation in ferritin means with a power of 80% (P <0.05 using a 2-tailed test) 42 subjects in each group were needed. Data were analysed in SPSS v14 (SPSS Inc., Chicago, IL) using standard and non-parametric tests.

3.8.5 Results

3.8.5.1 Intra-assay and Inter-assay coefficients of variation for ferritin bound to apolipoprotein B assay

The intra-assay coefficient of variation (CV) for ferritin bound to ApoB assay was determined by replicates of 8 different serum samples. The intra-assay coefficient of variation for the assay is shown in table 25. The inter-assay coefficient of variation for the assay is shown in table 26.

Table 41 Intra-assay coefficient of variation for ferritin

Sample	OD 1	OD 2	Mean OD	Concentration of ferritin (pg/ml)
1	0.767	0.790	0.7785	7901.596
2	0.734	0.710	0.722	6431.198
3	0.739	0.730	0.7345	6744.144
4	0.760	0.724	0.742	6925.563
5	0.732	0.736	0.734	6722.107
6	0.725	0.759	0.742	6924.374
7	0.725	0.694	0.7095	6159.382
8	0.770	0.766	0.768	7602.516
Mean CV-2.0% (For a good assay CV should be <5%)				

OD- optical density, raw data from ELISA plate reader

Table 42 Inter-assay coefficient of variation for ferritin

Sample	Concentration of ferritin 1 (pg/ml)	Concentration of ferritin 2 (pg/ml)	Mean concentration of ferritin (pg/ml)
1	2051.563	2055.051	2053.307
2	2925.662	3273.29	3099.476
3	4507.394	4541.473	4524.434
4	2662.656	2299.913	2481.285
5	4265.547	4188.92	4227.234
6	970.372	949.61	959.991
7	5840.194	6046.832	5943.513
8	2662.656	2299.913	2481.285
Mean CV -5.0% (For a good assay CV should be <10%)			

Raw data from ELISA plate reader

3.8.5.2 Ethnic difference in the levels of ferritin and ferritin bound to ApoB

Age, gender and CVD risk were comparable for 41 South Asian and 41 Caucasian subjects. Table 27 shows the demographic and cardiovascular risk profiles. Ferritin levels for South Asians were median (IQR) 62.48 (34.56-90.26) ng/ml and for Caucasians were 75.52 (42.03-103.98) ng/ml (P=0.112). ApoB levels for South Asians were 0.63 (0.18) g/L and for Caucasians were 0.70 (0.16) g/L (P=0.680). The percentage of ferritin bound to ApoB for non-diabetic South Asians were 1.27 (0.69-2.52) % and for non-diabetic Caucasians were 0.68 (0.21-1.30) %

(P=0.004). Amongst all subjects, five South Asians and four Caucasians had a CRP level of >5mg/L.

Table 43 Ethnic differences in cardiovascular risk profiles amongst non-diabetic subjects

Variables	**South Asians (n=41)**	**Caucasians (n=41)**	**P value**
Age (years)	59.4 (13.14)	60.4 (12.62)	0.788
Gender (% male)	29 (70.7)	27 (65.9)	0.205
Body mass index (kg/m^2)	25.4 (4.28)	27.2 (3.90)	0.583
Systolic blood pressure (mmHg)	140.06 (22.97)	140.88 (23.37)	0.642
Diastolic blood pressure (mmHg)	84.17 (12.05)	83.39 (13.68)	0.610
Total cholesterol (mmol/L)	4.41 (1.08)	4.91 (0.93)	0.807
High density lipoprotein (mmol/L)	1.34 (0.32)	1.38 (0.41)	0.215
Apolipoprotein B (g/L)	0.63 (0.18)	0.70 (0.16)	0.680
Ferritin (ng/ml)	62.5 (34.6-90.3)	75.5 (42.0-104.0)	0.112
Ferritin bound to ApoB (%)	1.27 (0.69-2.52)	0.68 (0.21-1.30)	0.004

3.8.6 Discussion

The findings of this study suggest an ethnic difference in the ferritin bound to ApoB among non-diabetic subjects, with South Asians showing a higher percentage of ferritin-ApoB compared to Caucasians despite having comparable levels of ferritin and ApoB. The physiological role of ferritin binding proteins such as ApoB is not clear. ApoB forms a complex with circulating ferritin and is involved in its clearance from the circulation (Sakamoto et al., 2009; Orino and Watanabe, 2008).

One of the possible explanations for higher percentage of ferritin-ApoB in South Asians could be aberrant erythropoiesis and increased iron availability in the circulation (Patel et al., 2008b;

Camejo et al., 1998) due to haemolytic disease such as beta thalassemia. We showed in a previous study that in South Asian women with iron deficiency anemia, the prevalence of diabetes mellitus is exceptionally raised (Chackathayil et al., 2012), but this concept needs further investigation.

Sullivan (1981) proposed a link between body iron stores and heart disease, but the results from epidemiological studies to prove this hypothesis has been conflicting (Danesh and Appleby, 1999). Elevated body iron stores have been implicated in the pathogenesis of diabetes mellitus (Jiang, 2004), metabolic syndrome and insulin resistance (Tuomainen et al., 1997; Jehn et al., 2004). Elevated levels of ferritin have been associated with increased risk of diabetes mellitus in healthy subjects (Jehn et al., 2007). In our study, ferritin levels were within normal ranges and comparable in both non diabetic South Asian and Caucasian subjects.

In diabetes, in vitro studies have demonstrated that accumulation of amino acetone generates reactive oxygen species that could affect the ferroxidase and iron uptake ability of ferritin resulting in iron induced oxidative stress (Dutra et al., 2003). Elevated circulating levels of ferritin have been associated with plasma triglycerides, and ApoB concentrations (Fernandez-Real, 1998). However, whether high levels of ferritin bound to ApoB could be potentially toxic under normal physiological condition or pathophysiological condition such as diabetes mellitus requires further investigation.

Ferritin is a 450 kDa multimeric protein which consists of 24 units of two types - heavy and light chains. Ferritin has the capacity to store 4500mol of iron/mol of ferritin and is up regulated during oxidative stress. The ferroxidase activity of the heavy chain catalyses the oxidation of ferrous iron to ferric iron for intracellular iron storage (Balla et al., 2003). ApoB is a hydrophobic protein with molecular mass of 512 kDa and is the main constituent of chylomicrons, very low-

density lipoproteins (VLDL) and LDL. Seki (2008) demonstrated in vitro studies that ApoB binds ferritin via hemin (iron containing porphyrin) and suggested a link between iron metabolism and lipid metabolism. Proteomic studies have also identified ferritin heavy and light chains to directly bind ApoB (Rashid, 2002; Seki, 2008).

Camejo (1998) suggested a model for the binding of hemin to ApoB. There is a high affinity between the carboxylic groups of the hemin and positively charged segments of apoB rich in lysine and arginine on the surface of LDL monolayer whereby the porphyrin ring is interacting with fatty acid chains of phospholipids and free cholesterol and the tetrapyrrole ring is embedded in the hydrophobic core of LDL. The alteration in the charge and structure of the LDL particle after hemin oxidation leads to increase uptake and degradation of oxidised LDL by macrophages. The binding of ferritin bound to ApoB can be of pathological significance and further work is warranted as to whether high levels of ferritin bound to ApoB could be an indicative of onset of diabetes and increased risk of CVD in South Asians.

3.9 Statistical analysis

All statistical analysis performed for this thesis is detailed in appendix C 9.3.7 (9.3.7.1-9.3.7.4).

3.9.1 Statistical analysis

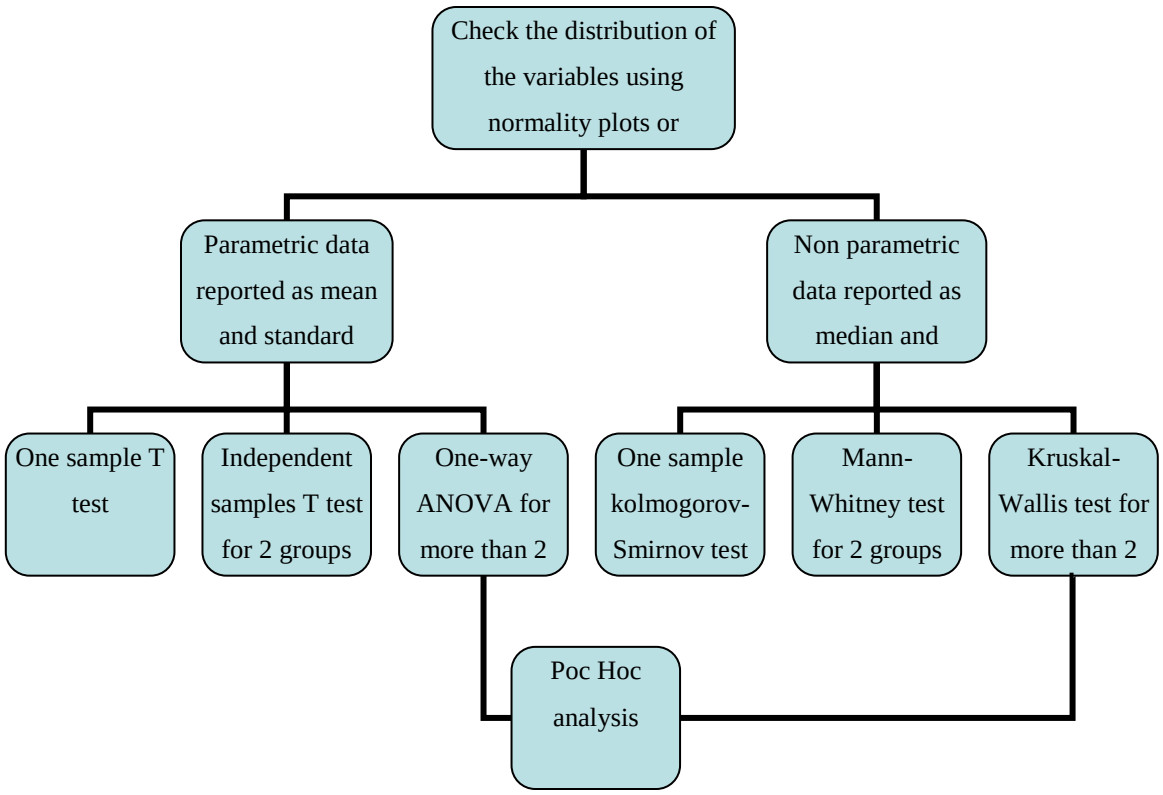
3.9.1.1 Descriptive statistics

All statistical analysis was performed using a computer based statistical package called 'SPSS' (version 18, SPSS Inc., USA) using standard and non-parametric tests and Kolmogorov-Smirnov normality plots. Central tendencies and variation for parametric data (normally distributed data) are presented as mean (standard deviation (SD)) or medians (interquartile range (IQR)) for non-

parametric data (skewed distribution). Comparisons were made by independent samples t-test or Mann-Whitney test, as appropriate.

Differences in genotype frequencies in the study population were assessed using 'chi-square' test. A probability level of $p<0.05$ was considered statistical significance. The significance of differences in characteristics such as demographic details were compared between the different genotypes using the One-Way ANOVA test for parametric test and Kruskal-Wallis test for non-parametric test.

figure 26 Flow chart for descriptive statistics



3.9.1.2 Correlation analysis

Bivariate correlation analysis was used to interpret the relationship between two variables assuming that such relationships were linear. The 'Pearson Rank Test' was used where variables

followed a normal distribution. The Pearson Test gave a correlation coefficient, r ranging from -1 to 1 based on the strength of the association and a probability level of $p<0.05$ was considered to be significant. The alternative to the Pearson Test is the 'Spearman Rank Test' and was used on non-parametric data. A similar score for correlation and probability was used.

Where a variable was thought to correlate with more than one variable, partial correlation analysis was used to calculate multiple correlation coefficients. For example the correlation between two variables could be influenced by a third variable.

3.9.1.3 Regression analysis

3.9.1.3.1 Linear Regression

Linear regression analysis was used to test relationships between normally distributed data (those assuming to have a linear relationship). It is the straight line that best describes the linear relationship between the two variables. Multivariable linear regression analysis was used to identify for instance clinical covariates associated with CRP levels and to predict the score of the dependent variable from a list of confounding factors or independent variables that were assumed to interact with the particular outcome.

For genotype phenotype association, multivariable linear regression model was used to test the null hypothesis that CRP levels did not differ by SNP genotype. The 'step' method on multiple regression analysis was used to determine correlation that was significant ($p<0.05$), the strength of which was determined using the beta coefficient. The beta coefficient represents how strongly the independent variable influences the dependent variable. For categorical variables, the regression coefficient indicates the average difference in the dependent variable between the two groups, adjusted for any difference between the groups with respect to other variables in the

model. This is equivalent to analysis of covariance. For quantitative independent variables, it is measured in standard deviation units. In a linear regression model to determine the independent predictors of CRP, a beta coefficient of 5 means that a one standard deviation change in the independent variable results in a 5 standard deviation change in CRP levels. R- square indicates percentage of variation in dependent variable explained by the independent variable.

3.9.1.3.2 Logistic Regression

To determine the probability of an event (reported as odds ratio) occurring in the presence of a particular genotype, multivariate logistic regression models were developed with various independent confounding factors included in all models.

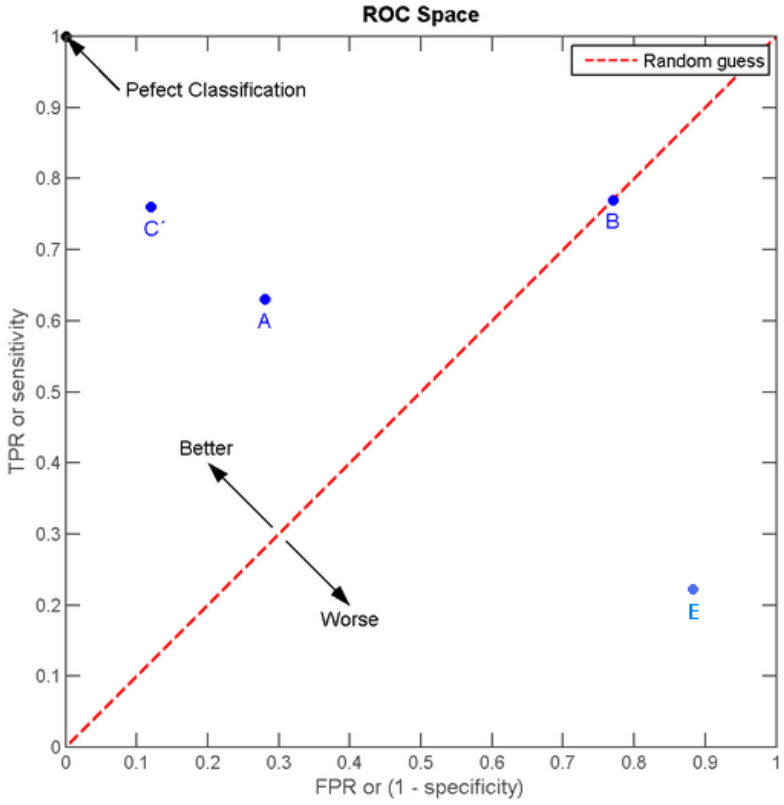
For instance from previous studies, body mass index, smoking status, total/HDL cholesterol ratio, hypertension, CVD, and triglycerides have been identified as predictors of CRP levels which were considered when the genotypes for the CRP triallelic SNP (wild type were coded as 0 and homozygous for mutant allele and heterozygous were coded as 1) were used as dependent variable in the model. A step-wise logististic regression analysis was performed to test the null hypothesis that the marker genotype is not associated with increased CVD risk or CVD event in this population. For all analyses, a $p<0.05$ will be considered significant.

3.9.1.4 Receiver operating characteristics curve analysis

The ability of each blood biomarkers to discriminate between the presence of CVD risk in South Asians were analysed on ROC graph, the area under the ROC curves (AUC) and cut off values were calculated. ROC is a graphical plot in which sensitivity or true positive rate is plotted on the Y-axis and false positive rate or 1-specificity is plotted on the X- axis.

Figure 27 shows a ROC graph with five different predictions. The perfect classification is represented by 0.1 point in the upper left corner, where there is 100% sensitivity and 100% specificity. A point on the diagonal line or otherwise called the line of no discrimination from the left bottom to the top right corners represents random guessing, the accuracy of B in the figure is 50%. Any points below the diagonal line represent poor classification while any points above the diagonal line represent good classification. Any classifiers on the left-hand side of an ROC graph closer to the X-axis make positive classification with strong evidence of few false positive errors but also low true positive rates. On the other hand, classifiers on the upper right-hand side of an ROC graph make positive classification with weak evidence of predicting all positive rates correctly but also have high false positive rates. In figure 48, C is a better predictor than A (Fawcett, 2006).

figure 27 *ROC graph showing four different prediction*



CHAPTER 4 RESULTS 1

4.1 The performance of inflammatory markers as discriminators of cardiovascular disease risk in South Asians in the UK

4.1.1 Aims and hypothesis

The aim of this section was to evaluate the diagnostic validity of blood biomarkers of inflammation, CRP and IL-6 in the prediction of CVD risk amongst South Asians asymptomatic for CVD in the UK. The hypothesis was that biomarkers of inflammation would be able to discriminate South Asians with CVD risk factors such as hypertension, obesity, diabetes mellitus, abnormal lipid profiles, metabolic syndrome and 10 year CHD risk score.

4.1.2 The validity of inflammatory markers as discriminators of cardiovascular disease risk in South Asian populations in the UK

4.1.2.1 Characteristics of the study cohort

The biomarker study cohort consisted of 505 South Asians asymptomatic for CVD and not on any therapies for modifiable CVD risk factors; of who 276 were men (mean age (SD), 55.0 years (8.3) and 229 were women (54.1 years (8.3). The characteristics of the study population are shown in Table 44. There were more male smokers than women, 14.1% versus 2%, $p<0.001$. The prevalence of hypertension was higher in South Asian men compared to South Asian women, $p=0.04$. The prevalence of diabetes mellitus was similar in South Asian men and women. The

prevalence of obesity was higher amongst South Asian women compared to South Asian men, 60.4% versus 42.4%, p<0.001. South Asian men showed a higher prevalence of metabolic syndrome and increased risk for CVD compared to South Asian women, prevalence rate for metabolic syndrome 53% versus 42.4%, 10 year CHD risk score 13% versus 6.5% respectively, all p<0.05.

There were no gender differences in mean systolic blood pressure, serum cholesterol and LDL-c. South Asian women showed higher levels of HDL-c and ApoAI compared to South Asian men (both p<0.001). South Asian men showed higher levels of ApoB and triglyceride compared to South Asian women, p=0.04 & p=0.005 respectively. There were significant gender differences in the prevalence of low HDL-c and raised triglycerides with South Asian men having higher rates compared to South Asian women, all p<0.05. There were significant gender differences in the levels of liver enzymes, with South Asian men having high levels of ALT, AST and GGT compared to South Asian women, all p<0.05. Similarly South Asian men had high levels of creatinine, a marker of renal function compared to South Asian women, p<0.05.

There were significant gender differences in the levels of inflammatory markers with South Asian women asymptomatic for CVD showing higher levels of CRP (p<0.001) and IL-6 (p=0.121, NS) compared to South Asian men. Distributions of all lipid measures, inflammatory markers and other biochemical measures for the study cohort are shown in Table 45.

Table 44Characteristics of the study population

Characteristics	Men (n=276)	Women (n = 229)	P value
Age (years)*	55.0 (8.3)	54.1 (8.3)	0.441
Current smokers, n (%)	39 (14.1)	2 (2.0)	<0.001
Hypertension (>130 mmHg or 85 mmHg), n (%)	190 (69.0)	138 (60.3)	0.044
Diabetes mellitus (HbA1c ≥ 6.5%), n (%)	32 (12.4)	28 (13.0)	0.870
Obesity (BMI≥27 kg/m^2), n (%)	117 (42.4)	137 (60.4)	<0.001
Increased waist Circumference (≥ 90 cm in men and ≥ 80 cm in women), n (%)	201 (73.0)	199 (87.3)	NS

Systolic blood pressure (mmHg)*	137.0 (16.1)	135.4 (20.1)	0.384
Diastolic blood pressure (mmHg)*	84.0 (10.0)	82.0 (11.0)	0.011
Waist circumference (cm)*	96.2 (11.1)	93.0 (12.3)	0.001
Body mass index (kg/m2)*	27.0 (4.0)	28.4 (5.0)	<0.001
Metabolic syndrome (%)	146 (53.0)	97 (42.4)	0.018
10 year coronary heart disease risk score (%)+	13.0 (8.3-18.0)	6.5 (3.9 -11.0)	<0.001

**Parametric data reported as mean (SD)-Independent sample T-test,+Non-parametric data reported as median (IQR)- Mann-Whitney test*

Metabolic syndrome –fasting glucose was not measured in E-ECHOES samples instead T2DM status was included in the ATP III clinical identification of the metabolic syndrome

Table 45Distribution of lipids, inflammatory markers and other biochemical measures in the study population

Characteristics	Men (n=276)	Women (n=229)	P value
Apolipoprotein AI (g/l)+	1.25 (1.11 -1.37)	1.37 (1.18 -1.53)	<0.001
Apolipoprotein B (g/l)*	0.80 (0.3)	0.72 (0.2)	0.041
Serum cholesterol (mmol/l)*	5.0 (1.3)	5.0 (1.3)	0.562
HDL cholesterol (mmol/l)*	1.00 (0.3)	1.20 (0.4)	<0.001
Low HDL cholesterol, n (%)	104 (39.2)	67 (31.0)	0.050
LDL cholesterol (mmol/l)*	3.0 (1.0)	3.0 (1.0)	0.950
Triglycerides (mmol/l)+	2.22 (1.51-3.05)	1.84 (1.30-2.64)	0.005
Raised triglyceride, n (%)	180 (67.2)	128 (57.4)	0.030
Calculated triglycerides+	2.07 (1.30-2.90)	1.85 (1.21-2.52)	0.042
Alkaline phosphatase (U/L)+	71.2 (59.0-88.0)	74.4(61.3-89.0)	0.299
Alanine transaminase (U/L)+	21.7 (17.0-30.0)	16.1 (13.0-21.1)	<0.001
Aspartate transaminase (U/L)+	20.0 (17.0-25.0)	19.0 (15.2-22.4)	0.006
Creatinine (µmol/l)+	69.0 (57.1-78.0)	52.0 (46.0-61.0)	<0.001
Gamma glutamyl transpeptidase (U/L)+	26.4 (18.0-40.0)	19.2 (13.0 -28.0)	<0.001
C-reactive protein (mg/L)+	1.67 (0.80-3.25)	2.80 (1.05-4.80)	<0.001
Interleukin 6+ (pg/ml)	11.0 (0.0-83.4)	20.0 (0.0-86.0)	0.121

**Parametric data reported as mean (SD)-Independent sample T-test*

,+Non-parametric data reported as median (IQR)- Mann-Whitney test

4.1.2.2 Levels of C-reactive protein across cardiovascular risk factor in South Asians asymptomatic for cardiovascular disease

The purpose of this section was to estimate CRP levels across each cardiovascular risk factor in South Asians asymptomatic for CVD. In the entire cohort, levels of CRP were higher in South Asians with greater obesity, diabetes mellitus, low HDL-c, metabolic syndrome and raised 10 year CHD risk score compared to healthy South Asian subjects. Differences in the levels of CRP

across CVD risk factors are shown in Table 46, figure 28-33. Levels of CRP were comparable in hypertensive and non-hypertensive subjects and in subjects with normal and elevated triglycerides in this cohort.

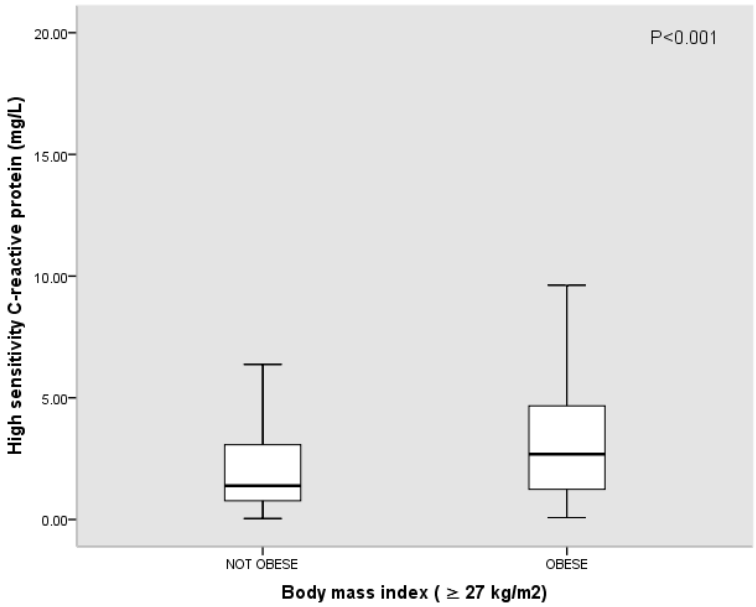
Table 46Differences in the levels of C-reactive protein across cardiovascular risk factors in South Asians asymptomatic for cardiovascular disease

Risk factors	CRP levels (mg/L)	P value (mann-whitney test)
Hypertensive (>130 mmHg or 85 mmHg) Not hypertensive	2.00 (0.90 – 4.43) 2.03 (0.92 – 4.04)	0.826
Obesity (BMI≥27 kg/m^2) Not obese	2.69 (1.23-4.68) 1.39 (0.77-3.08)	<0.001
Central obesity (≥ 90 cm in men and ≥ 80 cm in women) Not obese	2.26 (1.02-4.49) 1.10 (0.50-2.20)	<0.001
With diabetes mellitus (HbA1c ≥ 6.5%) Without diabetes mellitus	2.73 (1.45-4.66) 1.87 (0.83-3.98)	0.003
Low HDL cholesterol (<0.9 mmol/l in men & <1.0 mmol/l in women) Normal HDL Cholesterol	2.44 (1.02-5.66) 1.90 (0.85-3.63)	0.008
Elevated triglycerides (≥ 1.7 mmol/l) Normal triglycerides	2.13 (1.00 – 4.45) 1.82 (0.83 – 4.25)	0.136
With metabolic syndrome* Without metabolic syndrome	2.27 (1.06-4.67) 1.65 (0.80-3.63)	0.004
10 year CHD risk score (>20%) 10 year CHD risk score (<20%)	2.58 (1.26-5.90) 1.98 (0.89-4.02)	0.011

Reported as median and interquartile range

* *Metabolic syndrome –fasting glucose was not measured in E-ECHOES samples instead T2DM status was included in the ATP III clinical identification of the metabolic syndrome*

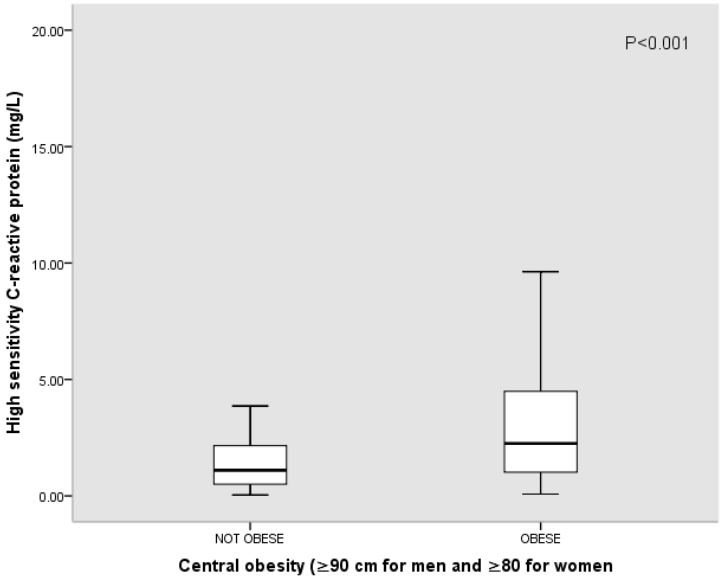
figure 28 *Levels of C-reactive proteins and obesity in South Asians asymptomatic for cardiovascular disease*



Mann-Whitney test

Boxes represent interquartile range and medians are shown as solid lines

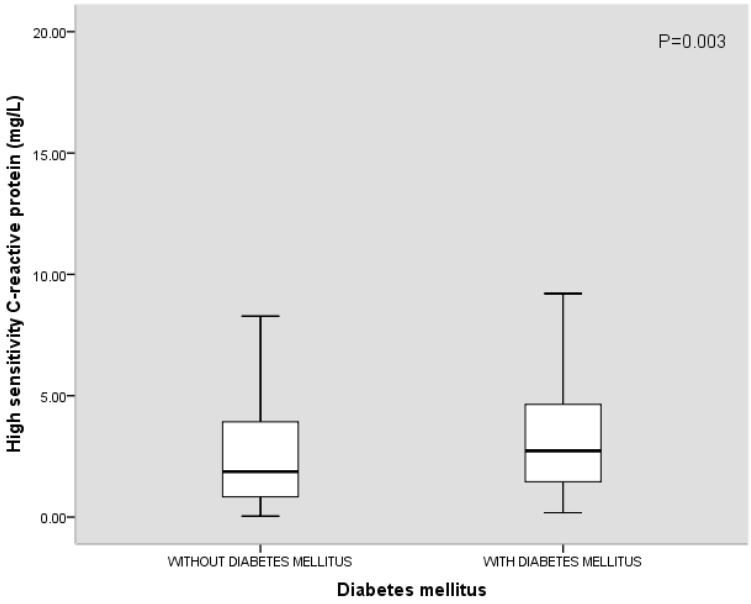
figure 29 *Levels of C-reactive protein and central obesity in South Asians asymptomatic for cardiovascular disease*



Mann-Whitney test

Boxes represent interquartile range and medians are shown as solid lines

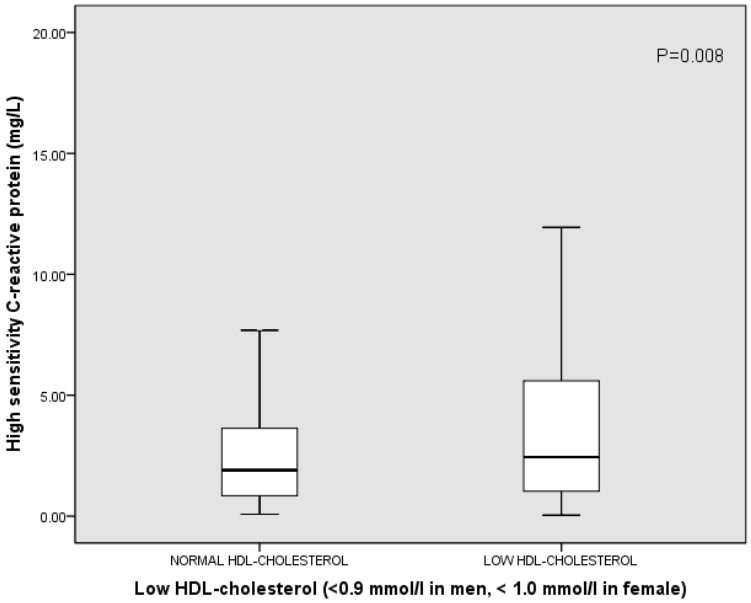
figure 30 *Levels of C-reactive protein and diabetes mellitus in South Asians asymptomatic for cardiovascular disease*



Mann-Whitney test

Boxes represent interquartile range and medians are shown as solid lines

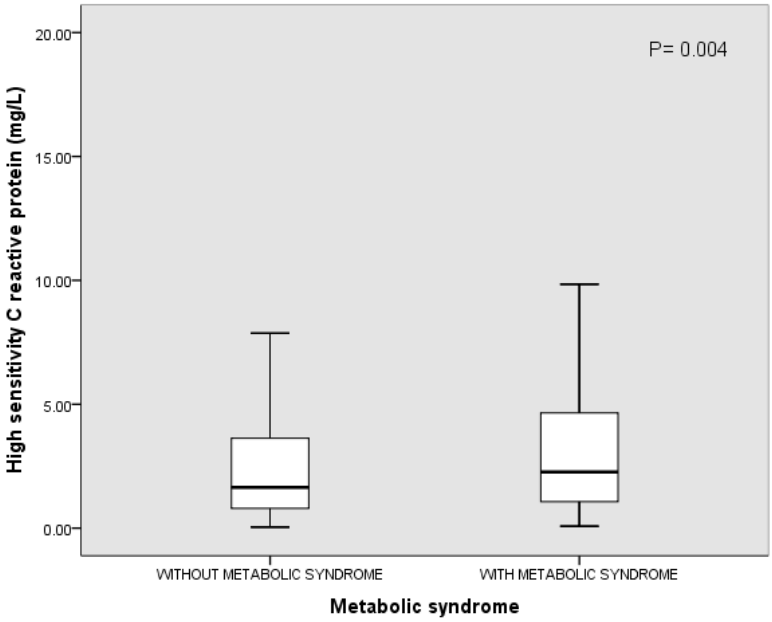
figure 31 *Levels of C-reactive protein and low HDL cholesterol in South Asians asymptomatic for cardiovascular disease*



Mann-Whitney test

Boxes represent interquartile range and medians are shown as solid lines

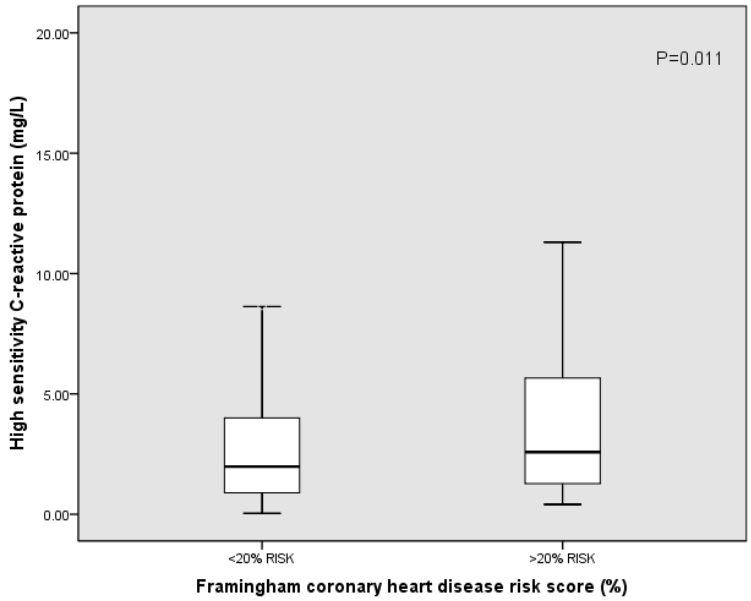
figure 32 Levels of C- reactive protein and metabolic syndrome in South Asians asymptomatic for cardiovascular disease



Mann-Whitney test

Boxes represent interquartile range and medians are shown as solid lines.

figure 33 Levels of C-reactive protein and Framingham 10 year coronary heart disease risk score in South Asians asymptomatic for cardiovascular disease



Mann-Whitney test

Boxes represent interquartile range and medians are shown as solid lines

4.1.2.3 The association between C - reactive protein and cardiovascular risk factors in South Asian men and women

Correlation analysis was performed to identify clinical variables that were associated with CRP levels in South Asian men and women.

CRP levels were positively associated with waist circumference, BMI, HbA1c, triglycerides, alkaline phosphatase, GGT and 10 year CHD risk score and negatively with HDL-c in South Asian men and women. Details are shown in Table 47.

Table 47Correlation analysis for CRP in South Asian men and women

Variables	South Asian men		South Asian women	
	Spearman's correlation coefficient, r	P value	Spearman's correlation coefficient, r	P value
Waist circumference (cm)	0.147	0.014	0.333	<0.001
Body mass index (kg/m^2)	0.147	0.014	0.369	<0.001
HbA1c (%)	0.170	0.006	0.198	0.003
Haemoglobin (g/dL)	0.241	0.003	NS	
White blood cell count	0.275	0.001	0.232	0.008
HDL cholesterol	-0.166	0.007	-0.186	0.006
Calculated fasting triglycerides	0.175	0.004	0.231	0.001
Triglycerides (mmol/l)	NS		0.199	0.003
Alkaline phosphatase (U/L)	0.356	<0.001	0.213	0.002
Creatinine (µmol/l)	NS		-0.140	0.040
Gamma glutamyl transpeptidase (U/L)	0.147	0.017	0.319	<0.001
CHD risk score	0.227	<0.001	0.232	0.001

Other variables that were included in the bivariate correlation analysis were age, mean systolic blood pressure, mean diastolic blood pressure, red blood cell count, ALT, AST, ApoA, ApoB, LDL-c, cholesterol, all p-NS.

Since CRP was positively associated with both waist circumference and BMI, partial correlation analysis was performed. On partial correlation analysis, BMI and waist circumference did not associate independently with CRP in South Asian men after controlling for each other. In South Asian women, BMI was positively and independently associated with CRP after controlling for waist circumference, correlation coefficient r = 0.179, p =0.00. Details are shown in Table 48.

Table 48Partial correlation for CRP and obesity in South Asian men and women

Control variables	South Asian men	South Asian women
Body mass index (kg/m^2)	Waist circumference, r =0.016, p=0.792	Waist circumference, r= 0.99, p=0.136
Waist circumference (cm)	Body mass index, r= 0.015, p=0.799	Body mass index, r=0.179, p=0.007

On simple linear regression, the strongest association was observed between CRP and BMI amongst South Asian women, adjusted R^2 = 0.125, beta coefficient =0.032, p<0.001 (figure 34) whilst in South Asian men, alkaline phosphatase showed the strongest association with CRP, adjusted R^2 = 0.119, beta coefficient (β) = 0.006, p<0.001 (figure 35).

figure 34 Association between log transformed C-reactive protein and body mass index amongst South Asian women

Simple linear regression

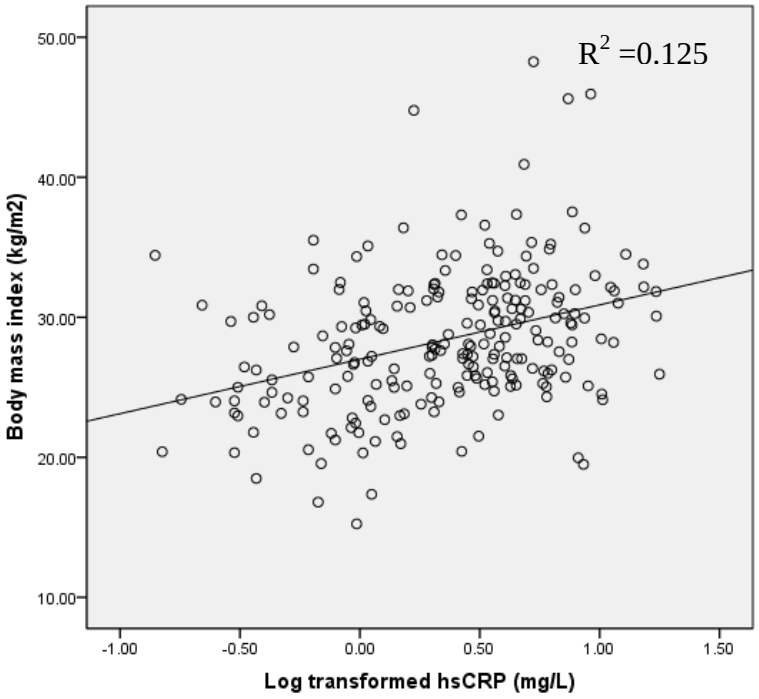
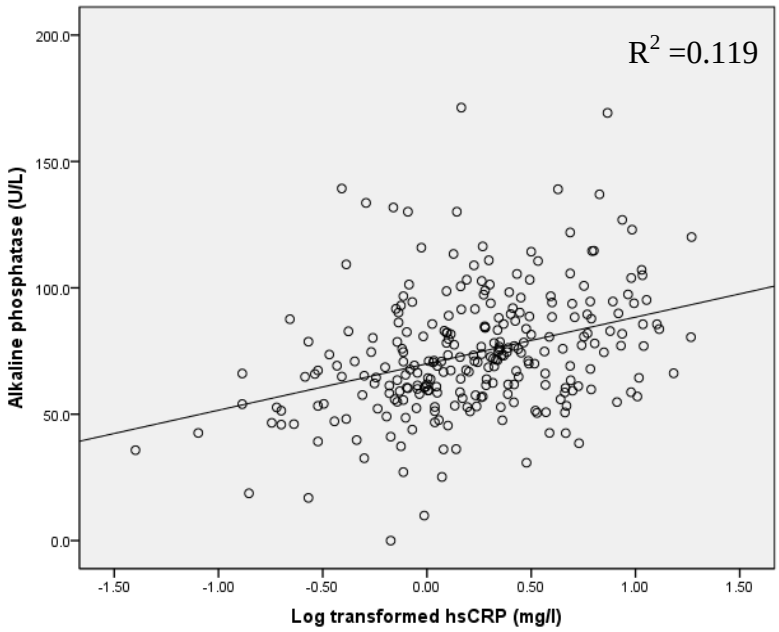


figure 35 Association between log transformed C-reactive protein and alkaline phosphatase amongst South Asian men

Simple linear regression



4.1.2.4 Clinical correlates of C- reactive protein in South Asian men and women: multiple regression models

The purpose of multiple regression models was to identify clinical correlates that independently explained variations in CRP levels in South Asian men and women. Multiple regression models were developed with variables associated with CRP levels on correlation analysis.

Using multiple linear regression, 10 year CHD risk score, alkaline phosphatase and BMI explained 16.0% of the variability in CRP levels amongst South Asian men asymptomatic for CVD, overall $R^2 = 0.157$, $p<0.001$(Table 49).

Table 49Clinical correlates of C-reactive protein in South Asian men: multiple linear regression model1

Predictor variables	Beta coefficients	95% Confidence Interval	P value	Adjusted R^2 (simple linear regression)
Alkaline phosphatase (U/L)	0.006	0.003 – 0.008	<0.001	0.116
10 year CHD risk score (%)	0.009	0.002 – .016	0.008	0.054
Body mass index (kg/m2)	0.018	0.004 – 0.032	0.013	0.024

Overall adjusted R^2 for the model 0.157, $p<0.001$

Variables included in the model- BMI, HbA1c, white blood cell count, calculated triglyceride levels, alkaline phosphatase and 10 year CHD risk score.
Please note, the overall adjusted R^2 for a similar model using non-fasted triglycerides instead of calculated triglycerides was 0.235, p<0.001

Amongst asymptomatic women, BMI, creatinine, alkaline phosphatase and calculated triglycerides explained 23.3% of the variations in CRP levels, overall $R^2 = 0.233$, p<0.001. BMI alone explained 12.1% of the variation in CRP levels in South Asian women.

Table 50Clinical correlates of C-reactive protein in South Asian women: multiple linear regression model 1

Predictor variables	Beta coefficients	95% Confidence Interval	P value	Adjusted R^2 (simple linear regression)
Body mass index (kg/m2)	0.033	0.021 - 0.044	<0.001	0.121
Creatinine (µmol/l)	-0.007	-0.011 - (-0.003)	<0.001	0.040
Alkaline phosphatase (U/L)	0.003	0.001 - 0.005	0.004	0.041
Calculated fasting triglyceride	0.040	0.007 - 0.073	0.018	0.040

Overall adjusted R^2 for the model 0.233, p<0.001
Variables included in the model- BMI, HbA1c, HDL cholesterol, calculated fasting triglycerides, alkaline phosphatase, creatinine and gamma glutamyl transpeptidase
Please note the overall adjusted R^2 for a similar model using non-fasted triglycerides instead of calculated triglycerides was 0.241, p<0.001

4.1.2.5 The performance of C-reactive protein as a discriminator of cardiovascular risk in South Asians asymptomatic for cardiovascular disease: ROC analysis

The purpose of this section was to evaluate the discriminative value for CRP in the identification of South Asians at an increased risk of CVD amongst South Asians asymptomatic for CVD. The discriminative measure was assessed by sensitivity and specificity and the area under the ROC curves.

On ROC analysis, CRP significantly discriminated those with greater obesity, diabetes mellitus, raised calculated fasting triglycerides, low HDL-c, raised 10 year CHD risk score and metabolic syndrome from healthy South Asians, all AUC- 0.573 - 0.658, p<0.05, (Table 51).

Validity of CRP was not evaluated in South Asians symptomatic for CVD risk factors (n=519) and CVD (n=198), this was due to the effect of lipid lowering therapy on CRP levels.

Table 51 C-reactive protein as a discriminator of metabolic and cardiovascular disease risk amongst South Asian men and women asymptomatic for cardiovascular disease

	Raised blood pressure (>130 mmHg or 85 mmHg) (328, 65.0% had hypertension)			Body mass index (> 27kg/m^2) (254, 50.1% were obese)		
	P value	AUC	95% CI	P value	AUC	95% CI
C-reactive protein (mg/L)	0.826	0.506	0.454-0.558	<0.001	0.630	0.581-0.679
Cut-off value for CRP (mg/L)	NS			1.985 mg/L		
	Central obesity (≥ 90 cm in men and ≥ 80 cm in women) (400, 79.3% were obese)			Diabetes mellitus (HbA1c ≥ 6.5%) (60, 13.0% were diabetic)		
	P value	AUC	95% CI	P value	AUC	95% CI
C-reactive protein (mg/L)	<0.001	0.658	0.596-0.720	0.003	0.618	0.549-0.688
Cut-off value for CRP (mg/L)	1.555 mg/L			2.255mg/L		
	Elevated measured triglycerides (≥ 1.7 mmol/l)			Elevated calculated triglycerides (≥ 1.7 mmol/l) (286, 61.0% had raised triglycerides)		
	P value	AUC	95% CI	P value	AUC	95% CI
C-reactive protein (mg/L	0.136	0.540	0.487-0.594	0.003	0.582	0.531 - 0.633
Cut-off value for CRP (mg/L)	NS			2.05 mg/L		
	Low HDL cholesterol (<0.9 mmol/l in men & <1.0 mmol/l in women, non-smokers) (171, 35.3% had low HDL-c)			10 years CHD risk score (>20%) (61, 13.0% had high CVD risk)		
	P value	AUC	95% CI	P value	AUC	95% CI
C-reactive protein (mg/L)	0.008	0.573	0.518-0.627	0.011	0.601	0.528-0.674
Cut-off value for CRP (mg/L)	2.065 mg/L			2.265 mg/L		
	Metabolic syndrome* (ATP III defined) (243, 48.1% had metabolic syndrome)					
	P value	AUC	95% CI			
C-reactive protein (mg/L)	0.004	0.575	0.525-0.625			
Cut-off value for CRP (mg/L)	1.995 mg/L					

**Metabolic syndrome –fasting glucose was not measured in E-ECHOES samples instead T2DM status was included in the ATP III clinical identification of the metabolic syndrome*

AUC- area under curve, 95% CI –confidence interval

4.1.3 The performance of interleukin-6 as a discriminator of cardiovascular disease risk in South Asians asymptomatic for cardiovascular disease

4.1.3.1 The association between interleukin-6 and cardiovascular risk factors in South Asian men and women

Correlation analysis was performed to identify clinical correlates that were associated with IL-6 levels but none of the clinical variables were associated with IL-6 in this cohort. All variables included in the spearman correlation analysis are shown in Table 52.

Table 52Correlation analysis for interleukin -6 in South Asian men and women

None of the following variables were associated with IL-6 levels on spearman correlation analysis; Age, gender, mean systolic blood pressure, mean diastolic blood pressure, waist measurement, body mass index, glycated haemoglobin, haemoglobin, red blood cell count, white blood cell count, ApoB, ApoAI, total cholesterol, LDL-c, HDL-c, triglycerides, ALT, AST, creatinine, gamma glutamyl transpeptidase and 10 years CHD risk score.

With regards to the relation between IL-6 levels and prevalence of CVD risk factors (hypertension, greater obesity, diabetes mellitus, elevated triglycerides, low HDL-c, metabolic syndrome and raised 10 year CHD risk score), only South Asian women with low HDL- c (<1.0 mmol/l in women) showed a significantly higher levels of IL-6 compared to women with normal HDL cholesterol, median (IQR) 42.5 (0.00 – 236.0) vs. 16.1 (0.00-61.0), p=0.05.

4.1.3.2 The performance of interleukin-6 as a discriminator of cardiovascular risk in South Asians asymptomatic for cardiovascular disease: ROC analysis

ROC analysis was done to evaluate the performance of IL-6 in the identification of South Asians at an increased risk of CVD amongst South Asians asymptomatic for CVD. On ROC analysis, IL-6 did not discriminate between South Asians with CVD risk factors (hypertension, greater

obesity, diabetes mellitus, elevated triglycerides, low HDL-c and metabolic syndrome) and raised 10 year CHD risk score from healthy South Asians (Table 53).

Table 53Interleukin 6 as discriminator of metabolic and cardiovascular disease risk amongst South Asian men and women asymptomatic for cardiovascular disease

Interleukin-6 (pg/ml)	Raised blood pressure (>130 mmHg or 85 mmHg)			Body mass index (> 27kg/m^2)		
	P value	AUC	95% CI	P value	AUC	95% CI
	0.332	0.527	0.473-0.580	0.569	0.485	0.434-0.536
	Central obesity (≥ 90 cm in men and ≥ 80 cm in women)			Diabetes mellitus (HbA1c ≥ 6.5%)		
	P value	AUC	95% CI	P value	AUC	95% CI
	0.591	0.517	0.453-0.581	0.425	0.467	0.387-0.547
	Elevated measured triglycerides (≥ 1.7 mmol/l)			Low HDL cholesterol (<0.9 mmol/l in men & <1.0 mmol/l in women, non-smokers)		
	P value	AUC	95% CI	P value	AUC	95% CI
	0.893	0.504	0.450-0.558	0.094	0.549	0.491-0.608
	Metabolic syndrome* (ATP III defined)			10 years CHD risk score (>20%)		
	P value	AUC	95% CI	P value	AUC	95% CI
	0.277	0.528	0.477-0.580	0.181	0.444	0.364-0.525

4.1.4 Discussion

Our aim was to evaluate the diagnostic validity of CRP and IL-6 in the discrimination of CVD risk in South Asians asymptomatic for CVD in the UK. Our findings suggest a lower discriminative range for CRP in South Asians than that determined by the current guidelines in the general population. The findings were replicated in two other South Asian cohorts i) a homogeneous Indian community in the UK ii) a homogeneous Indian community in India (Description of study population -Patel; 2006), results are shown in appendix C 9.3.1 table 145 & 146. Levels of CRP in the range of 1.43 –2.30 mg/L significantly discriminated South Asians with increased risk of CVD. IL-6 levels failed to discriminate South Asians with increased risk of CVD in our cohort. Prospective studies have shown that elevated levels of IL-6 predict incident CVD in Europeans (Ridker, 2000a; Ridker, 2000b; Danesh et al., 2008). In our cohort, the levels

of IL-6 were undetectable (<9.375 pg/ml) in 34.5% (174) of healthy subjects and hence maybe was unable to determine its association with CVD risk.

The performance of CRP was not evaluated in a White population but was compared to already established guidelines in the general population. CRP was measured in this cohort using commercially available Hs-CRP assay kits. The coefficient variation of this assay is generally <10%. The assay can detect very low levels of CRP in blood down to 0.3 mg/L in apparent healthy subjects and was introduced after a standardization program at the Centers for Disease Control and Prevention (Pearson, 2003; Fortmann, 2004; Myers, 2004). White blood cell count was available for only 279 South Asians in this cohort hence was unable to determine the effect of infection on CRP levels. Out of these 279 South Asians, only 6 subjects had mild leucocytosis which was reflected by high CRP levels. Another limitation for our study is the cross sectional study design and hence is unable to answer questions regarding the causal role of inflammatory markers in this population. The study was purely designed to determine the performance of inflammatory marker and its association with conventional CVD risk factors in South Asians.

There is a growing concern regarding surrogate markers such as CRP and its usefulness of to improve CVD risk prediction beyond those provided by classical risk factors (Danesh, 2004). Genetic studies so far argue against a causal relationship between CRP and CVD and show this marker as a mere bystander with no active role in the development of CVD (Kardys, 2006; Elliot et al., 2009;). Studies have shown that elevated levels of CRP in the general population are linked to the presence of conventional CVD risk factors. Current guidelines do not recommend CRP as an independent predictor of CVD but it is essential to define patient groups where it could be applicable (Miller, 2005; Emerging risk factor collaboration, 2010).

Consistent with previous studies, CRP levels were higher in women compared to men in our cohort. In the study by Forouhi et al., (2001) elevated CRP levels were correlated with central obesity after adjustment for BMI in South Asian women which reflected the higher prevalence of metabolic syndrome. This study suggested visceral adipose tissue as a key promoter of low-grade chronic inflammation in South Asian women. The main source for interleukin-6 (IL6), an inducer of hepatic CRP synthesis is the visceral adipose tissue. However in our cohort, central obesity was significantly lower in South Asian women compared to South Asian men while BMI was higher amongst South Asian women. The likely explanation is ectopic fat being stored in sites other than the visceral adipose tissue like in liver, skeletal and cardiac muscle cells which could contribute to pro inflammatory state and stimulate the production of CRP in liver (Smith, 2008).

The performance of obesity and central obesity as discriminators of CVD risk factors and increased risk of CVD was evaluated in this population (results are shown in appendix C 9.3.2 table 147). These findings are consistent with previous prospective studies which have highlighted measures of central obesity as cost effective, accurate in predicting CVD risk and should be incorporated in established CVD risk assessment tools (de Koning et al., 2007; Czernichow et al., 2011).

The study also examined the clinical correlates of CRP, which included traditional cardiovascular risk factors which have been demonstrated in prior studies (Kathiresan, 2006). In this cohort of South Asians asymptomatic for CVD, liver function test, alkaline phosphatase and kidney function test, creatinine levels were found to be correlates of CRP. In South Asian men, alkaline phosphatase alone explained 11.6% of the total variance in CRP levels while in South Asian women, BMI alone explained 12.1% of the total variance in CRP levels.

4.1.4.1 Conclusion

Our findings suggest that CRP levels in the range of 1.43-2.30 mg/L discriminates South Asians at increased risk of CVD. This level is lower than that seen in white populations. Our findings were replicated in two cohorts i) a homogeneous Indian community in the UK ii) a homogeneous Indian community in India. IL-6 levels did not discriminate South Asians at increased risk of CVD.

4.2 Genotype- phenotype and genotype-disease association of inflammatory markers

4.2.1 Aims and hypothesis

There is increasing evidence supporting the use of CRP as a biomarker of CVD. South Asians have an increased risk of CVD, and this is reflected by higher circulating levels of CRP. Genetic association studies in Europeans have identified -390C>T>A (rs3091244) within the promoter region of *C-reactive protein gene* on chromosome 1q21-q23 and -174G>C (rs1800795) within the promoter region of *Interleukin-6 gene* on chromosome 7 to be associated with levels of C-reactive protein and CVD, yet genetic association studies amongst South Asians are scarce. The aim of this section was to determine the allelic frequency of rs3091244 and rs1800795 and the contribution of these SNPs to inter-individual variability in CRP & IL-6 levels and CVD in South Asians.

4.2.2 Genotype-phenotype and genotype-disease association of inflammatory markers in South Asians

4.2.2.1 Characteristics of the study cohort

The genetic study cohort consisted of 1049 South Asians, of whom 600 (57.2%) were men mean age (SD) 59.0 years (11.0) and 449 (43.0%) were women, 57.3 years (9.3), p=0.047. There were more male smokers than females, 34.4% versus 2%, p<0.001. There were significant gender differences in the prevalence of hypertension, obesity, central obesity and existing CVD, all p<0.05. There were no significant gender differences in the prevalence of diabetes mellitus and mean levels of systolic blood pressure and diastolic blood pressure (Table 54).

Table 54 Characteristics of the study population

Characteristics	Male (n=602)	Female (n=449)	P value
Age (years)	59.0 (11.0)	57.3 (9.3)	0.047
Current smokers, n (%)	180 (34.4)	8 (2.0)	<0.001
Hypertension, n (%)	479 (80.0)	347 (77.3)	0.018
Diabetes mellitus, n (%)	212 (35.3)	134 (30.0)	0.116
Obesity, n (%)	287 (50.0)	292 (67.3)	<0.001
Increased waist circumference, n (%)	484 (84.5)	402 (92.2)	<0.001
Systolic blood pressure (mmHg)*	139.4 (19.0)	142.0 (21.0)	0.60
Diastolic blood pressure (mmHg)*	82.2 (11.0)	83.0 (11.2)	0.876
Waist circumference (cm)*	94.2 (25.4)	93.0 (20.4)	0.312
Body mass index (kg/m2)*	27.4 (4.1)	30.0 (5.3)	<0.001
Prevalent cardiovascular disease, n (%)	168 (28.0)	79 (17.6)	<0.001

** Parametric data reported as mean (SD)-Independent sample T-test,*
+ Non-parametric data reported as median (IQR)- Mann-Whitney test

In the case of lipids, lipoproteins and other biochemical measures, South Asian women showed higher ApoAI/ApoB ratio, higher levels of HDL-c compared to South Asian men (both p<0.001). Levels of triglyceride were higher in South Asian men compared to women, p=0.005. There was a significant gender difference in the levels of inflammatory markers with South Asian women showing high levels of CRP (p<0.001) and IL-6 (p=NS) compared to South Asian men. There were significant gender differences in the levels of liver enzymes, with South Asian men having high levels of ALT, AST and GGT compared to South Asian women, all p<0.001. Similarly South Asian men had high levels of creatinine compared to South Asian women, p<0.001.

Distributions of all lipid measures and other biochemical measures in the study population are shown in Table 55.

Table 55 Distribution of lipid measures and other biochemical measures in the study population

Characteristics	Male (n=600)	Female (n=449)	P value
Apolipoprotein A (g/l)+	1.25 (1.10-1.44)	1.34 (1.14-1.54)	0.001
Apolipoprotein B (g/l)+	0.70 (0.53 -0.86)	0.66 (0.50 -0.81)	0.005
ApoA/ApoBratio+	1.83 (1.43-2.37)	2.04 (1.63-2.54)	<0.001
Cholesterol (mmol/l)*	4.07 (1.6)	4.20 (1.5)	0.313
HDL cholesterol (mmol/l)*	0.97 (0.33)	1.10 (0.37)	<0.001
LDL cholesterol (mmol/l)*	2.25 (0.93)	2.25 (0.90)	0.934
Triglycerides (mmol/l)+	2.00 (1.40-2.93)	1.84 (1.33-2.66)	0.047
Calculated triglycerides+	1.87 (1.03-2.77)	1.77 (1.10-2.51)	0.194

Alkaline phosphatase (U/L)[+]	70.0 (57.0-86.0)	74.0 (59.0-91.3)	0.030
Alanine transaminase (U/L)[+]	21.2 (16.1-30.2)	16.0 (12.2-21.0)	<0.001
Aspartate transaminase (U/L)[+]	19.7 (17.0-25.0)	18.0 (14.4-22.0)	<0.001
Creatinine (µmol/l)[+]	70.0 (60.0-81.0)	54.0 (45.2-64.0)	<0.001
Gamma glutamyl transpeptidase (U/L)[+]	27.4 (18.0-46.0)	19.4 (13.0-30.0)	<0.001
C-reactive protein (mg/L)[+]	1.45 (0.73-3.33)	2.44 (1.10-5.00)	<0.001
Interleukin 6 (pg/ml)[+$]	10.2 (0.00-59.0)	19.2 (0.00-81.0)	0.155

[*] *Parametric data reported as mean (SD)-Independent sample T-test*
,[+] *Non-parametric data reported as median (IQR)- Mann-Whitney test*
[$] IL-6 levels were available only in EECHOES samples, 327 South men and 287 women, total=614)

4.2.2.2 Genotype frequency and allelic frequency of rs3091244 and rs1800795 in South Asians

The allelic and genotype frequencies for rs3091244 and rs1800795 are shown in Table 56 & 57. For rs3091244, the minor allele frequency for T allele was 0.23 and A allele was 0.11. The carrier frequency for this triallelic SNP in our cohort was 55%. The minor allelic frequency for rs1800795 was 0.18 and the carrier frequency in our cohort was 32%.

Table 56Genotype and minor allelic frequencies of rs3091244 in South Asians in the UK

rs3091244	CC (wild type)	CT	CA	TA	TT	AA	Total	Minor allelic frequency (95% CI) (T allele)	Minor allelic frequency (95% CI) (A allele)
Number of subjects (%)	447 (45)	302 (30.3)	124 (12.4)	37 (4)	56 (6)	30 (3)	996	23.0 (21.2-25.0)	11.0 (9.6-12.4)

Table 57 Genotype and minor allelic frequencies of rs1800795 in South Asians in the UK

rs1800795	GG (wild type)	GC	CC	Total	Minor allelic frequency (95% CI)
Number of subjects (%)	690 (68%)	284 (28%)	44 (4.3%)	1018	18.0 (16.2-20.0)

4.2.2.3 Differences in cardiovascular risk factors and serum C-reactive protein levels across C-reactive SNP rs3091244 genotypes

The purpose of this section was to determine whether any significant difference in demographics, CRP levels and CVD were evident amongst South Asians with carriers of minor allele of SNP rs3091244 compared to those with wild type.

There was no significant difference in terms of demographics, lipid levels and prevalent CVD among the two groups divided as rs3091244 genotype CC, wild type and carriers of minor allele, T & A (Table 58 and Table 59). Except for the prevalence of increased waist circumference, where carriers of minor allele showed a higher percentage of subjects with increased central obesity compared to those with wild type genotype, p=0.012. A significant difference was also observed in the levels of CRP among the two genotype groups, with carriers of minor allele showing higher levels of CRP compared to wild type, p =0.003 (Table 59 & figure 36).

Table 58 Characteristics of the study population across rs3091244 genotype

	CC (wild type) n = 447	Carriers of minor alleles (alleles T & A) n = 549	P value
Age (years)	59.0 (10.0)	58.0 (11.0)	0.101
Male gender, n (%)	255 (57.4)	314 (58.0)	0.659
Current smokers, n (%)	88 (21.8)	90 (18.6)	0.230
Hypertension, n (%)	350 (78.3)	436 (79.4)	0.667
Diabetes mellitus, n (%)	144 (33.0)	179 (33.0)	0.958
Obesity, n (%)	245 (57.2)	300 (58.0)	0.835
Increased waist circumference, n (%)	363 (85)	468 (90.2)	0.012
Systolic blood pressure (mmHg)*	140.1 (18.3)	141.0 (21.0)	0.703
Diastolic blood pressure (mmHg)*	82.1 (11.1)	82.0 (11.3)	0.920
Waist circumference (cm)*	94.0 (24.0)	93.0 (24.0)	0.463
Body mass index (kg/m2)*	28.1 (5.0)	28.4 (5.0)	0.393
Prevalent cardiovascular disease, n (%)	104 (23.3)	135 (24.6)	0.627

*Parametric data reported as mean (SD)- Independent T-test & n(%) –chi-square test.

Table 59 Distribution of lipids, lipoproteins and inflammatory markers across rs3091244 genotype

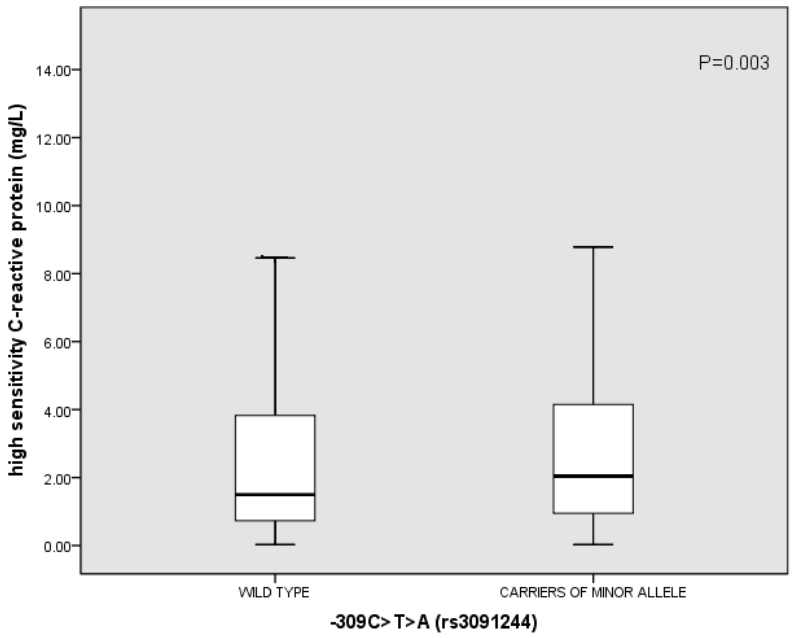
	CC (wild type) n = 447	Carriers of minor alleles (alleles T & A) n = 549	P value
Apolipoprotein A (g/l)[+]	1.27 (1.09-1.49)	1.28 (1.13-1.51)	0.350
Apolipoprotein B (g/l)[+]	0.67 (0.53-0.84)	0.69 (0.52-0.84)	0.615
ApoA/ApoBratio[+]	1.91 (1.50-2.41)	1.96 (1.53-2.45)	0.549
Cholesterol (mmol/l)[*]	4.05 (1.6)	4.11 (1.7)	0.582
HDL cholesterol (mmol/l)[*]	1.02 (0.4)	1.01 (0.32)	0.616
LDL cholesterol (mmol/l)[*]	2.21 (0.93)	2.30 (0.91)	0.477
Triglycerides (mmol/l)[+]	1.94 (1.35-2.69)	1.91 (1.32-2.90)	0.771
Calculated triglycerides[+]	1.78 (0.95-2.62)	1.87 (1.05-2.62)	0.334
C-reactive protein (mg/L)[+]	1.50 (0.73-3.83)	2.04 (0.95-4.20)	0.003
Interleukin 6 (pg/ml)[+$]	13.0 (0.00-79.1)	14.0 (0.00-63.2)	0.992

[*]Parametric data reported as mean (SD)- Independent T-test. [+] Non-parametric data reported as median (IQR)- Mann-Whitney test

[$]IL-6 levels were available only in EECHOES samples, 327 South men and 287 women, total=614)

figure 36 Levels of C-reactive protein across the genotypes

Mann-Whitney test



Boxes represent interquartile range and medians are shown as solid lines
Wild type (CC), n = 447 Carriers of minor alleles (alleles T & A), n =n = 549

Simple linear regression was performed to determine whether SNP rs3091244 was associated with its phenotype, CRP levels. rs3091244 was associated with CRP levels when comparing wild type with carriers of minor allele (CC vs carriers of A & T), $\beta = 0.171$ (95% CI, 0.033-0.308), adjusted $R^2 = 0.005$, $p = 0.015$.

There was no significant difference in the frequency of genotypes in South Asians with prevalent CVD compared to South Asians without CVD (Table 60).

Table 60Genotype and allelic distribution in the study population

rs3091244	South Asians without cardiovascular disease (n=757)	South Asians with cardiovascular disease (n=239)	P value
Wild type (CC) (%) Heterozygous (CT/CA) (%) Homozygous for minor allele (TA/TT/AA) (%)	343 (45.3) 321 (42.4) 93 (12.3)	104 (43.5) 105 (43.9) 30 (12.6)	NS
Allelic distribution C T A	 0.67 0.23 0.11	 0.65 0.23 0.12	 NS

4.2.2.4 Clinical correlates of C- reactive protein in South Asian men and women: multiple regression models

Correlation analysis was performed to identify clinical correlates of CRP in South Asian men and women. CRP was positively associated with waist, BMI, ApoB, alkaline phosphatase, GGT and negatively wth HDL-c in South Asian men. Amongst South Asian women, CRP was positively associated with waist, BMI, diastolic blood pressure, white blood cell count, calculated triglycerides, triglycerides, alkaline phosphatase, GGT and negatively with HDL-c and creatinine. Details are shown in Table 61.

Table 61Correlation analysis for CRP in South Asian men and women in the UK

Variables	South Asian men		South Asian women	
	Spearman's correlation coefficient, r	P value	Spearman's correlation coefficient, r	P value
Waist circumference (cm)	0.203	<0.001	0.241	<0.001
Body mass index (kg/m^2)	0.210	<0.001	0.353	<0.001
Diastolic blood pressure (mmHg)	NS		0.141	0.004
White blood cell count	NS		0.146	0.012
Apolipoprotein B (g/l)	0.149	<0.001	NS	
HDL cholesterol	-0.101	0.018	-0.130	0.008
Calculated fasting triglycerides	NS		0.104	0.020
Triglycerides (mmol/l)	NS		0.114	0.012
Alkaline phosphatase (U/L)	0.345	<0.001	0.208	<0.001
Creatnine (µmol/l)	NS		-0.137	0.007
Gamma glutamyl transpeptidase (U/L)	0.197	<0.001	0.271	<0.001

Other variables included in the analysis were age, systolic blood pressure, glycated haemoglobin, glucose levels, haemoglobin, red blood cell count, ALT, AST, ApoAI, cholesterol and LDL-c.

Since CRP was positively associated with both waist circumference and BMI in South Asian women, partial correlation analysis was performed. On partial correlation analysis, waist circumference was positively and independently associated with CRP after controlling for BMI in South Asian men, r = 0.114, p=0.007. In South Asian women, BMI was positively and independently associated with CRP after controlling for waist circumference, correlation coefficient r = 0.243, p = <0.001. Details are shown in Table 62.

Table 62Partial correlation for CRP and obesity in South Asian men and women

Control variables	South Asian men	South Asian women
Body mass index (kg/m^2)	Waist circumference, r =0.114, p=0.007	Waist circumference, r= 0.000, p=0.993
Waist circumference (cm)	Body mass index, r= 0.075, p=0.079	Body mass index, r=0.243, p=<0.001

On multiple linear regression model 1 (developed with variables associated with CRP), alkaline phosphatase, BMI and -309C>T>A (CC vs. carriers of minor allele) explained 14% of the

interindividual variability in CRP levels in South Asian men with alkaline phosphatase explaining 10% of the total variance (Table 63).

Table 63 Clinical correlates of C-reactive protein in South Asian men: multiple linear regression model 1

Predictor variables	Beta coefficients	95% Confidence Interval	P value	Adjusted R^2 (simple linear regression)
Alkaline phosphatase (U/L)	0.013	0.010 - 0.017	<0.001	0.100
Body mass index (kg/m2)	0.047	0.026 - 0.069	<0.001	0.041
-309C>T>A (CC vs. carriers of minor allele)	0.235	0.055 – 0.415	0.011	0.005

Overall adjusted R^2 for the model 0.139, p<0.001
Variables included in the model- BMI, HDL-c, alkaline phosphatase, GGT and -309C>T>A (CC vs. carriers of minor allele

On multiple regression model 2, alkaline phosphatase, BMI, GGT and HDL cholesterol explained 14.3% of the interindividual variability in CRP levels in South Asian men again with alkaline phosphatase explaining 10% of the total variance (Table 64).

Table 64 Clinical correlates of C-reactive protein in South Asian men: multiple linear regression model 2

Predictor variables	Beta coefficients	95% Confidence Interval	P value	Adjusted R^2 (simple linear regression)
Alkaline phosphatase (U/L)	0.013	0.010 - 0.017	<0.001	0.100
Body mass index (kg/m2)	0.0471	0.019 - 0.0693	<0.001	0.041
Gamma glutamyl transpeptidase (U/L)	0.004	0.001 – 0.007	0.015	0.022
HDL cholesterol (mmol/l)	-0.283	-0.566 – 0.000	0.050	0.006

Overall adjusted R^2 for the model 0.143, p<0.001
Variables included in the model- BMI, HDL-c, alkaline phosphatase and GGT

In South Asian women, BMI, alkaline phosphatase, creatinine and diastolic blood pressure explained 17.1% of the interindividual variability in CRP levels with BMI explaining 10% of the total variance (Table 65).

Table 65 Clinical correlates of C-reactive protein in South Asian women: multiple linear regression model

Predictor variables	Beta coefficients	95% Confidence Interval	P value	Adjusted R^2 (simple linear regression)
Body mass index (kg/m2)	0.066	0.047 - 0.085	<0.001	0.104
Alkaline phosphatase (U/L)	0.007	0.003 - 0.011	0.001	0.030
Creatnine (µmol/l)	-0.008	-0.014 – (-0.001)	0.016	0.014
Diastolic blood pressure (mmHg)	0.010	0.002 – 0.019	0.021	0.021

Overall adjusted R^2 for the model 0.171, p<0.001

Variables included in the model- BMI, diastolic blood pressure, HDL-c, calculated fasting triglyceride, alkaline phosphatase, GGT and creatnine

In the entire cohort, BMI, alkaline phosphatase, lipid lowering therapy (yes/no), rs3091244, gender, HDL cholesterol and GGT explained 24% of the interindividual variability in CRP levels with BMI explaining 8% of the total variance. The triallelic SNP located in the promoter sequence explained only 0.5% of the variability in CRP levels in a model that included all clinical covariates (Table 66).

Table 66 Clinical correlates of C-reactive protein in South Asians in the UK: multiple linear regression model

Predictor variables	Beta coefficients	95% Confidence Interval	P value	Adjusted R^2 (simple linear regression)
Body mass index (kg/m2)	0.063	0.045 - 0.080	<0.001	0.081
Alkaline phosphatase (U/L)	0.010	0.007 - 0.014	<0.001	0.068
Lipid lowering therapy (no/yes)	-0.265	-0.433- (-0.098)	0.002	0.006
-309C>T>A (CC vs. carriers of minor allele)	0.256	0.092 - 0.420	0.002	0.005
Gender (male/female)	0.330	0.154 - 0.505	<0.001	0.010
HDL cholesterol (mmol/l)	-0.274	-0.505 – (-0.043)	0.020	0.003
Gamma glutamyl transpeptidase (U/L)	0.003	0.000 - 0.005	0.050	0.012

Overall adjusted R^2 for the model 0.235, p<0.001
Variables included in the model- gender, BMI, diastolic blood pressure, haemoglobin, HDL-c, alkaline phosphatase, GGT, creatinine, lipid lowering therapy (yes/no) and -309C>T>A (CC vs. carriers of minor allele)

4.2.2.5 The association between Interleukin-6 SNP rs1800795, cardiovascular risk factors and inflammatory markers

There was no significant difference in terms of demographics, lipid levels, inflammatory markers and prevalent CVD among the two groups divided as rs1800795 genotype GG, wild type and carriers of minor allele, C. Characteristics of the study population and distribution of lipid and inflammatory markers across rs1800795 genotype are shown in Table 67 & 68.

Table 67Characteristics of the study population across rs1800795 genotype

Characteristics	GG (wild type)	Carriers of minor alleles (GC & CC)	P value
Age (years)	57.4 (11.0)	58.0 (10.0)	0.743
Male gender, n (%)	324 (58.0)	143 (52.0)	0.106

Current smokers, n (%)	101 (21.0)	36 (15.0)	0.053
Hypertension, n (%)	442 (78.1)	226 (80.1)	0.492
Diabetes mellitus, n (%)	173 (31.0)	82 (29.4)	0.703
Obesity, n (%)	304 (57.0)	163 (61.0)	0.305
Increased waist circumference, n (%)	469 (88.0)	235 (88.0)	0.993
Systolic blood pressure (mmHg)*	140.0 (22.0)	141.0 (20.0)	0.479
Diastolic blood pressure (mmHg)*	82.0 (12.0)	83.3 (11.3)	0.071
Waist circumference (cm)*	92.0 (26.4)	94.0 (25.0)	0.310
Body mass index (kg/m2)*	28.3 (5.0)	29.0 (5.0)	0.319
Prevalent cardiovascular disease, n (%)	138 (24.4)	68 (24.1)	0.932

**Parametric data reported as mean (SD)- Independent T-test & n(%) -chi-square test.*

Table 68 Distribution of lipids, lipoproteins and inflammatory markers across rs1800795 genotype

Characteristics	GG (wild type)	Carriers of minor alleles (GC & CC)	P value
Apolipoprotein A (g/l)+	1.27 (1.12-1.51)	1.27 (1.09-1.48)	0.792
Apolipoprotein B (g/l)+	0.68 (0.52-0.84)	0.70 (0.51-0.84)	0.894
ApoAI/ApoBratio+	1.93 (1.51-2.41)	1.91 (1.50-2.44)	0.785
Cholesterol (mmol/l)*	4.00 (1.7)	4.10 (1.7)	0.469
HDL cholesterol (mmol/l)*	1.01 (0.34)	1.01 (0.40)	0.986
LDL cholesterol (mmol/l)*	2.20 (0.92)	2.25 (0.91)	0.428
Triglycerides (mmol/l)+	1.92 (1.31-2.90)	1.84 (1.35-2.66)	0.509
Calculated triglycerides+	1.78 (0.91-2.62)	1.78 (0.88-2.62)	0.934
High sensitivity C-reactive protein (mg/L)+	1.88 (0.82-4.10)	1.83 (0.90-4.20)	0.602
Interleukin 6 (pg/ml)	12.0 (0.00-59.0)	15.0 (0.00-105.0)	0.383

**Parametric data reported as mean (SD)- Independent T-test*
+ Non-parametric data reported as median (IQR)-Mann-Whitney test.
$IL-6 levels were available only in EECHOES samples, 327 South men and 287 women, total=614)

4.2.3 Discussion

The *CRP* promoter polymorphism rs3091244 contributed minimally to variation in CRP levels in South Asians and was not associated with CVD. The triallelic SNP located in the promoter sequence explained only 0.5% of the variability in CRP levels. Carriers of minor alleles (CT, CA, TT or AA) had higher levels of CRP compared to subjects with major alleles (CC). The allelic frequency for CRP-390 T and A were 23% and 11% respectively similar to those reported in non-Asian populations (Kathiresan, 2006). The IL-6 SNP, rs1800795 did not contribute to inter-individual variability in CRP and IL-6 levels in South Asians.

Several studies have demonstrated the association between rs3091244 and CRP levels in non-Asian populations. In the Framingham Heart Study, the contribution of rs3091244 was modest and explained only 1.4% of the variation in CRP levels. Subjects homozygous for CC genotype had the lowest level of CRP compared to heterozygotes (CT or CA genotypes) who had intermediate level of CRP and minor allele homozygotes (TT or AA) who showed the highest level of CRP. However, the triallelic SNP rs3091244 was not associated with prevalent CVD (Kathiresan, 2006). Suk Danik (2006) demonstrated that the triallelic -390C/T/A SNP was associated with the highest level of CRP in patients after ACS whilst Kovacs (2005) associated the triallelic SNP with baseline CRP levels in patients with CHD.

With regards to association between rs3091244 and CVD, the genotypes were not associated with increased risk of ischemic heart disease (Zacho, 2008). Similarly, studies by Zee and Ridker (2002), Kardys (2006) and a meta-analysis by C Reactive Protein Coronary Heart Disease Genetics Collaboration (CCGC) (2011) also failed to associate CRP genotypes with the risk of CHD respectively, despite showing a substantial genetic influence on CRP levels. In the third National Health and Nutrition Examination Survey, the functional -390C/T/A SNP was associated with CRP levels and with prevalent CHD in the non-Hispanic population, thus suggesting a true causal relationship between CRP and CHD (Crawford, 2006). This was not the case in our cohort, this could be due to insufficient statistical power or there might be other multiple rare variants that could be associated with CVD in South Asians.

The functional significance of this triallelic SNP in the promoter region of CRP gene has been demonstrated in vitro. Hage (2007) identified two SNPs at the promoter region of the CRP gene which are functional, -409G/A (rs3093062) and -390C/T/A (rs3091244). These polymorphisms directly contributed to the interindividual variation of blood CRP levels. Higher CRP levels were

observed in carriers of -409GG and -390TT. The two SNPs were reported to reside within the E box elements E-box 1 and E-box -2, which are functional since transcription factors bind to them and is altered in the presence of -409GG and -390TT SNPs. Szalai (2005) and Carlson (2005) demonstrated that the rs3091244 triallelic SNP in the promoter region alters the affinity for transcription factors and affects the transcription of the gene which results in variation of blood CRP levels. In vitro study using CRP promoter-firefly luciferase reporters showed that individuals with 409G/-390T haplotype had a highest promoter activity, with high transcription factor binding version of E-box 1 and E-box 2 and high levels of CRP when compared to -409A/-390T individuals who showed weak promoter activity and hence lowest CRP levels (Szalai, 2005).

The study also examined the clinical correlates of CRP, which included gender, cardiovascular risk factors such as BMI, lipid lowering therapy (yes/no), HDL cholesterol which have been demonstrated in prior studies (Kathiresan, 2006). Apart from these clinical variables, liver function tests, alkaline phosphatase and gamma glutamyl transpeptidase levels were found to be correlates of CRP. In the entire cohort, BMI, alkaline phosphatase, lipid lowering therapy (yes/no), rs3091244 (CC vs. carriers of minor allele), gender, HDL cholesterol and GGT explained 24% of the interindividual variability in CRP levels with BMI explaining 8% of the total variance. The triallelic SNP located in the promoter sequence was independently associated with CRP levels and explained only 0.5% of the variability in CRP levels in a model that included all clinical covariates. However in South Asian men, alkaline phosphatase explained 10% of the total variance in CRP levels while in South Asian women, BMI explained 10% of the total variance in CRP levels.

4.2.3.1 Conclusion

The *CRP* promoter polymorphism rs3091244 contributed minimally to variation in CRP levels in South Asians and were not associated with clinical CVD.

CHAPTER 5 RESULTS 2

5.1 Lipids and lipoproteins in South Asian populations in the UK

5.1.1 Aims and hypothesis

Fasting triglycerides are a better discriminator of CVD risk in South Asian populations compared to other lipids and lipoprotein measures. Current guidelines recommend measures of triglycerides to be obtained in a fasting state. Due to logistical issues associated with obtaining fasted bloods, there are often not measured in the primary prevention setting. In this cross sectional study, the aim was to look at the performance of non-fasting triglycerides and calculated levels of triglycerides estimated by the Friedewald formula as discriminators of CVD risk in South Asians asymptomatic for CVD in the UK. The analysis also looked at the ethnic differences in lipids and lipoproteins amongst South Asians in the UK.

5.1.2 The validity of calculated triglycerides and lipoproteins as a discriminator of cardiovascular disease in South Asians asymptomatic for cardiovascular disease

5.1.2.1 Characteristics of the study cohort

The study population were recruited through the E-ECHOES study; the characteristics of the study population and distribution of all lipid profiles and biochemical measures are shown in Chapter 4 Result section 1, Table 44 & 45.

With regards to the characteristics amongst men by ethnic groups, more Bangladeshi men smoked, p<0.001. The prevalence of diabetes mellitus was highest amongst Pakistani men 22% compared to 7% and 9.1% in Indian and Bangladeshi men respectively, p=0.004. The prevalence of obesity and central obesity were also highest amongst Pakistani men p=0.010 & <0.001 respectively. Characteristics of South Asian men by ethnic groups are shown in Table 69. Characteristics of South Asian women by ethnic groups are shown in Table 70. There were no significant differences in demographic details amongst women belonging to different ethnic groups.

Table 69Characteristics of men by ethnic groups

Characteristics	Men by ethnic groups^ (Total n=250)			P value
	Indians (n= 129)	Pakistanis (n= 99)	Bangladeshi (n= 22)	
Age (years)*	54.4 (7.3)	56.1 (9.5)	54.1 (10.1)	0.285
Current smokers, n (%)	7 (5.4)	18 (18.2)	11 (50.0)	<0.001
Hypertension (>130 mmHg or 85 mmHg), n (%)	90 (70.0)	65 (66.0)	12 (55.0)	0.357
Diabetes mellitus (HbA1c ≥ 6.5%), n (%)	8 (7.0)	20 (22.0)	2 (9.1)	0.004
Obesity (BMI≥27 kg/m^2), n (%)	51 (40.0)	48 (49.0)	3 (14.0)	0.010
Increased waist Circumference (≥ 90 cm in men and ≥ 80 cm in women), n (%)	96 (74.4)	81 (82.0)	9 (41.0)	<0.001
Systolic blood pressure (mmHg)*	138.0 (16.5)	135.4 (16.2)	130.0 (12.4)	0.074
Diastolic blood pressure (mmHg)*	85.0 (10.1)	82.1 (9.4)	82.5 (9.5)	0.110
Waist circumference (cm)*	96.1 (12.0)	98.2 (9.3)	90.0 (7.5)	0.003
Body mass index (kg/m2)*	27.0 (3.5)	27.1 (3.7)	24.1 (3.0)	0.002
Metabolic syndrome (%)#	71 (55.0)	54 (55.0)	7 (32.0)	0.118
10 year coronary heart Disease risk score (%)+	18 (15.3)	25 (25.3)	4 (18.2)	0.179

**Parametric data reported as mean (SD)- One way anova, n(%) -chi-square test.*

^ Included only subjects who reported their ethnicity as Indian, Pakistani or Bangladeshi, excluded ones other Asian background, n = 4, East African Asian, n=22

#Metabolic syndrome –fasting glucose was not measured in E-ECHOES samples instead T2DM status was included in the ATP III clinical identification of the metabolic syndrome

Table 70Characteristics of women by ethnic groups

Characteristics	Women by ethnic groups^ (Total n=211)			P value
	Indians (n= 119)	Pakistanis (n= 77)	Bangladeshi (n= 15)	
Age (years)*	55.0 (8.1)	53.0 (9.0)	57.0 (8.2)	0.179
Current smokers, n (%)	1 (1.0)	2 (2.6)	1 (7.0)	0.256
Hypertension (>130 mmHg or 85 mmHg), n (%)	71 (60.0)	46 (60.0)	9 (60.0)	1.00
Diabetes mellitus (HbA1c ≥ 6.5%), n (%)	18 (16.2)	9 (12.3)	0 (0)	0.211
Obesity (BMI≥27 kg/m^2), n (%)	72 (61.0)	51 (68.0)	6 (40.0)	0.115
Increased waist Circumference (≥ 90 cm in men and ≥ 80 cm in women), n (%)	104 (87.4)	69 (91.0)	13 (87.0)	0.746
Systolic blood pressure (mmHg)*	136.0 (19.4)	135.1 (20.1)	137.0 (31.0)	0.957
Diastolic blood pressure (mmHg)*	81.0 (10.4)	82.0 (11.0)	83.0 (16.0)	0.729
Waist circumference (cm)*	92.0 (13.0)	95.1 (12.0)	94.0 (10.2)	0.245
Body mass index (kg/m2)*	28.3 (5.0)	29.0 (5.1)	27.0 (4.0)	0.288
Metabolic syndrome (%)	49 (41.2)	37 (48.1)	6 (40.0)	0.611
Framingham coronary heart Disease risk score (%) +	4 (3.5)	3 (4.1)	2 (13.3)	0.220

** Parametric data reported as mean (SD)- One way anova, n(%) -chi-square test.*
^ Included only subjects who reported their ethnicity as Indian, Pakistani or Bangladeshi, excluded ones East African Asian, n=18.

Triglyceride levels were lowest amongst Bangladeshi men, p=0.027. Levels of alkaline phosphatase and creatinine were lowest in Indian men, p=0.001. Lipids and biochemical measures in South Asian men by ethnic groups are shown in Table 71. Distribution of lipids and biochemical measures in South Asian women by ethnic groups are shown in Table 72. There were no significant differences in the levels of lipids and biochemical measures in South Asian women belonging to different ethnic groups.

Table 71Distribution of lipids and biochemical measures in South Asian men by ethnic groups

Characteristics	Men by ethnic groups^ (Total n=250)			P value
	Indians (n= 129)	Pakistanis (n= 99)	Bangladeshi (n= 22)	
Apolipoprotein AI (g/l)+	1.25 (1.09 – 1.39)	1.22 (1.12 – 1.35)	1.25 (1.11 – 1.39)	0.985
Apolipoprotein B (g/l)*	0.79 (0.26)	0.74 (0.23)	0.75 (0.24)	0.412
Serum cholesterol (mmol/l)*	4.70 (1.40)	4.64 (1.31)	4.70 (1.15)	0.946
HDL cholesterol (mmol/l)*	0.99 (0.35)	0.98 (0.26)	1.03 (0.32)	0.759
LDL cholesterol (mmol/l)*	2.67 (0.96)	2.60 (0.81)	2.71 (0.82)	0.786
Triglycerides (mmol/l)+	2.33 (1.68 – 3.18)	2.13 (1.50 – 2.90)	1.54 (1.20 – 2.30)	0.027

Calculated fasting triglycerides[+]	1.95 (1.22 – 3.02)	2.10 (1.41 – 2.80)	1.90 (1.31 – 2.50)	0.658
Alkaline phosphatase (U/L)[+]	66.1 (54.1 – 81.0)	77.0 (62.0 – 94.0)	75.0 (59.0 – 88.4)	0.001
Alanine transaminase (U/L)[+]	22.1 (17.0 – 31.0)	22.0 (17.0 – 31.4)	19.5 (15.3 – 27.0)	0.370
Aspartate transaminase (U/L)[+]	19.1 (16.2 – 24.4)	20.7 (18.0 – 25.0)	20.0 (15.4 – 23.0)	0.162
Creatinine (µmol/l)[+]	66.0 (55.0 – 75.0)	73.0 (61.0 – 83.0)	74.0 (62.0 – 91.2)	0.005
Gamma glutamyl transpeptidase (U/L)[+]	23.8 (17.4 – 37.5)	30.0 (18.2 – 42.0)	24.3 (17.4 – 30.2)	0.111

[*] *Parametric data reported as mean (SD)- One way anova,* [+] *Non-parametric data reported as median (IQR) Kruskal-Wallis test*

[^] *Included only subjects who reported their ethnicity as Indian, Pakistani or Bangladeshi, excluded ones other Asian background, n = 4, East African Asian, n=22.*

Table 72Distribution of lipids and biochemical measures in South Asian women by ethnic groups

Characteristics	Women by ethnic groups[^] (Total n=211)			P value
	Indians (n= 119)	Pakistani (n= 77)	Bangladeshis (n= 15)	
Apolipoprotein AI (g/l)[+]	1.37 (1.20 – 1.54)	1.40 (1.17 – 1.60)	1.32 (1.14 – 1.52)	0.936
Apolipoprotein B (g/l)[*]	0.72 (0.20)	0.72 (0.21)	0.75 (0.30)	0.817
Serum cholesterol (mmol/l)[*]	4.75 (1.24)	4.80 (1.40)	4.81 (1.20)	0.973
HDL cholesterol (mmol/l)[*]	1.20 (0.40)	1.15 (0.40)	1.15 (0.41)	0.630
LDL cholesterol (mmol/l)[*]	2.70 (0.82)	2.60 (0.90)	2.70 (0.90)	0.691
Triglycerides (mmol/l)[+]	1.83 (1.45 – 2.60)	2.01 (1.30 – 2.84)	2.04 (1.23 – 2.40)	0.849
Calculated fasting triglycerides[+]	1.76 (1.21 – 2.40)	2.10 (1.20 – 2.80)	1.91 (1.50 – 2.50)	0.347
Alkaline phosphatase (U/L)[+]	72.0 (59.4 – 87.0)	76.0 (63.2 – 89.2)	74.0 (64.1 – 95.0)	0.716
Alanine transaminase (U/L)[+]	17.0 (13.0 – 20.4)	15.4 (12.1 – 21.1)	14.0 (11.3 – 21.4)	0.648
Aspartate transaminase (U/L)[+]	18.0 (15.0 – 22.0)	19.0 (15.4 – 25.1)	19.2 (14.0 – 23.1)	0.525
Creatinine (µmol/l)[+]	53.0 (47.0 – 61.0)	51.0 (45.3 – 63.0)	51.0 (48.0 – 58.0)	0.931
Gamma glutamyl transpeptidase (U/L)[+]	17.1 (12.1 – 28.0)	19.4 (13.0 – 30.0)	17.0 (13.0 – 27.4)	0.392

[*] *Parametric data reported as mean (SD)- One way anova,* [+] *Non-parametric data reported as median (IQR)-* Kruskal-Wallis test.[^] Included only subjects who reported their ethnicity as Indian, Pakistani or Bangladeshi, excluded ones East
African Asian, n=18.

5.1.2.2 Dyslipidaemia characteristics of South Asian men and women asymptomatic for cardiovascular disease in the UK

The section gives an overview of the prevalence of dyslipidaemia in South Asian men and women asymptomatic for CVD in the UK.

In South Asians asymptomatic for CVD, the prevalence of raised triglyceride, low HDL-c and combined raised triglyceride and low HDL-c were significantly more amongst South Asian men

compared to South Asian women, all p<0.05. Table 73 shows the dyslipidaemia characteristics of South Asian men and women in the UK.

Table 73 Dyslipidaemia characteristics of South Asian men and women in the UK

Dyslipidaemic category	South Asian men	South Asian women	P value
Raised serum cholesterol (>5.18 mmol/l), n (%)	89 (34.0)	80 (37.0)	0.480
Raised serum triglyceride (>1.7 mmol/l), n (%)	178 (66.4)	127 (57.0)	0.031
Low HDL cholesterol (<0.9 mmol/l in men & <1.0 mmol/l in women), n (%)	104 (39.2)	67 (31.0)	0.050
Isolated HDL cholesterol (<0.9 mmol/l in men & <1.0 mmol/l in women in the absence of other lipid abnormalities), n (%)	18 (26.1)	20 (28.2)	0.782
Raised serum triglyceride and low HDL cholesterol, n (%)	81 (31.0)	45 (20.4)	0.011

Chi-square test

Amongst South Asian men, the prevalence of raised serum triglyceride (≥ 1.7 mmol/l) was higher in Indian men compared to Pakistani men and Bangladeshi men, p=0.010. Dyslipidaemia characteristics of South Asian men by ethnic groups in the UK are shown in Table 74. Dyslipidaemic characteristics of South Asian women by ethnic groups in the UK are shown in Table 75. There were no significant differences in the prevalence of dyslipidaemia characteristics amongst South Asian women belonging to different ethnic groups.

Table 74 Dyslipidaemic characteristics of South Asian men by ethnic groups in the UK

Dyslipidaemic category	South Asian men by ethnic groups (Total n =250)			P value
	Indians (n= 129)	Pakistani (n= 99)	Bangladeshi (n= 22)	
Raised serum cholesterol (>5.18 mmol/l), n (%)	45 (37.2)	25 (25.3)	8 (36.4)	0.154
Raised serum triglyceride (>1.7 mmol/l), n (%)	93 (73.2)	63 (64.3)	8 (40.0)	0.010
Low HDL cholesterol (<0.9 mmol/l in men & <1.0 mmol/l in women), n (%)	44 (37.0)	41 (41.4)	10 (46.0)	0.644
Isolated HDL cholesterol (<0.9 mmol/l in men & <1.0 mmol/l in women in the absence of other lipid abnormalities), n (%)	7 (5.4)	6 (6.1)	3 (13.6)	0.342
Raised serum triglyceride and low HDL cholesterol, n (%)	37 (30.3)	32 (32.7)	6 (27.3)	0.863

Chi-square test

Table 75Dyslipidaemic characteristics of South Asian women by ethnic groups in the UK

Dyslipidaemic category	South Asian women by ethnic groups (Total n =250)			P value
	Indians (n= 119)	Pakistanis (n= 77)	Bangladeshi (n= 15)	
Raised serum cholesterol (>5.18 mmol/l), n (%)	47 (42.0)	22 (30.0)	5 (33.3)	0.202
Raised serum triglyceride (>1.7 mmol/l), n (%)	69 (59.0)	43 (58.1)	9 (64.3)	0.908
Low HDL cholesterol (<0.9 mmol/l in men & <1.0 mmol/l in women), n (%)	30 (27.0)	26 (34.2)	5 (33.3)	0.506
Isolated HDL cholesterol (<0.9 mmol/l in men & <1.0 mmol/l in women in the absence of other lipid abnormalities), n (%)	9 (8.0)	7 (9.1)	0 (0)	0.477
Raised serum triglyceride and low HDL cholesterol, n (%)	20 (17.4)	17 (23.0)	5 (33.3)	0.291

Chi-square test

5.1.2.3 The association between lipids, lipoproteins and cardiovascular risk factors in South Asian men and women asymptomatic for cardiovascular disease in the UK

Correlation analysis was performed to determine the clinical correlates of total cholesterol, LDL-c, HDL-c, triglycerides and apolipoproteins.

Total cholesterol and LDL-c levels were comparable in South Asian men and South Asian women; hence correlation analysis was performed for the entire cohort. Total cholesterol was positively associated with ApoB, HDL-c, LDL-c, diastolic blood pressure, HbA1c, ApoA, triglycerides measured & calculated, alkaline phosphatase, ALT, AST, creatinine, GGT and 10 year CVD risk score, all $p<0.05$. LDL-c levels were positively associated with ApoB, total cholesterol, HDL-c, HbA1c, ApoAI, triglycerides measured & calculated, alkaline phosphatase, ALT, AST, creatinine, GGT and 10 year CVD risk score, all $p<0.05$. Correlation analysis for total cholesterol and LDL-c in South Asians are shown in table 76.

Table 76Correlation analysis for total cholesterol and LDL-cholesterol in South Asians asymptomatic for cardiovascular disease in the UK

Variables	Cholesterol (mmol/l)	LDL cholesterol (mmol/l)
Pearson correlation coefficient, r		
Apolipoprotein B (g/l)	0.772**	0.810**
Cholesterol (mmol/l)	-	0.822**
HDL cholesterol (mmol/l)	0.352**	0.348**
LDL cholesterol (mmol/l)	0.822**	-
Diastolic blood pressure (mmHg)	0.106*	NS
Spearman correlation coefficient, r		
Glycated haemoglobin (HbA1c) (%)	0.101*	0.120*
Apolipoprtoein A (g/l)	0.491**	0.388**
Calculated triglyceride	0.505**	0.201**
Triglyceride (mmol/l)	0.293**	0.097*
Alkaline phosphatase (U/L)	0.224**	0.160**
Alanine transaminase (U/L)+	0.192**	0.179**
Aspartate transaminase (U/L)+	0.218**	0.195**
Creatnine (µmol/l)+	0.160**	0.163**
Gamma glutamyl transpeptidase (U/L)+	0.234**	0.183**
10 year CHD risk score (%)	0.257**	0.225**

**p<0.05, **p<0.001, NS- non significant, Other variables included in the analysis were age, gender, BMI, waist measurement, systolic blood pressure, haemoglobin, red blood cell count and white blood cell count.*

Correlation analysis for HDL-c, triglycerides and calculated fasting triglycerides in South Asian men and women are shown in table 77 and 78. In South Asian men, HDL-c was positively associated with total cholesterol, LDL-c, ApoAI and AST and negatively associated with waist measurement, BMI, red blood cell count, triglyceride –non-fasting & calculated, and 10 year CHD risk score, all $p<0.05$. Triglyceride levels and calculated fasting triglycerides were positively associated with ApoB, total cholesterol, waist measurement, BMI, systolic blood pressure, diastolic blood pressure, alkaline phosphatase, ALT, GGT and 10 year CVD risk score and were negatively associated with HDL-c in South Asian men. Furthermore, calculated fasting triglycerides alone were positively associated red blood cell count, haemoglobin, AST and creatinine while triglyceride levels were negatively associated with age.

In South Asian women, HDL-c was positively associated with ApoB, total cholesterol, LDL-c, ApoA, ALT, AST and creatinine, and negatively with BMI, white blood cell, triglycerides- non-fasting & calculated and 10 year CVD risk score. Triglyceride was positively associated with ApoB, total cholesterol, waist circumference, alkaline phosphatase, GGT and 10 year CVD risk score and negatively with HDL-c, ApoA and AST. Calculated fasting triglyceride levels were positively associated with ApoB, total cholesterol, LDL-c, waist measurement, alkaline phosphatase, GGT and 10 year CHD risk score and negatively with HDL-c.

Table 77Correlation analysis for lipids in South Asian men in the UK

Variables	HDL cholesterol (mmol/l)	Triglycerides (mmol/l)	Calculated fasting triglycerides
Pearson correlation coefficient, r		Spearman's correlation coefficient, r	
Age (years)	NS	-0.139*	NS
Apolipoprotein B (g/l)	NS	0.302**	0.431**
Cholesterol (mmol/l)	0.298**	0.320**	0.548**
HDL cholesterol (mmol/l)	-	-0.396**	-0.217**
LDL cholesterol (mmol/l)	0.319**	NS	0.208*
Waist circumference (cm)	-0.233**	0.188*	0.140*
Body mass index (kg/m^2)	-0.197*	0.212**	0.237**
Systolic blood pressure (mmHg)	NS	0.194*	0.165*
Diastolic blood pressure (mmHg)	NS	0.232**	0.198*
Red blood cell count	-0.191*	NS	0.208*
Haemoglobin (g/l)	NS	NS	0.207*
Spearman's correlation coefficient, r			
Apolipoprotein A (g/l)	0.800**	NS	NS
Calculated triglyceride	-0.217**	0.704**	-
Triglyceride (mmol/l)	-0.396**	-	0.704**
Alkaline phosphatase (U/L)	NS	0.135*	0.310**
Alanine transaminase (U/L)$^+$	NS	0.191*	0.247**
Aspartate transaminase (U/L)$^+$	0.193*	NS	0.162*
Creatnine (µmol/l)$^+$	NS	NS	0.130*
Gamma glutamyl transpeptidase (U/L)$^+$	NS	0.211*	0.274**
10 year CHD risk score (%)	-0.296**	0.371**	0.462**

**p<0.05, **p<0.001, NS- non significant*

Other variables included in the analysis were age systolic blood pressure and white blood cell count.

Table 78Correlation analysis for lipids in South Asian women in the UK

Variables	HDL cholesterol (mmol/l)	Triglycerides (mmol/l)	Calculated fasting triglycerides
Pearson correlation coefficient, r		Spearman's correlation coefficient, r	
Apolipoprotein B (g/l)	0.190*	0.285**	0.367**
Cholesterol (mmol/l)	0.423**	0.280**	0.461**
HDL cholesterol (mmol/l)	-	-0.400**	-0.223*
LDL cholesterol (mmol/l)	0.406**	NS	0.190*
Waist circumference (cm)	NS	0.210*	0.230*
Body mass index (kg/m^2)	-0.141*	NS	NS
Systolic blood pressue (mmHg)	NS	NS	NS
Diastolic blood pressure (mmHg)	NS	NS	NS
White blood cell count	-0.235*	NS	NS
Haemoglobin (g/l)	NS	NS	NS
Spearman's correlation coefficient, r			
Apolipoprotein A (g/l)	0.888**	-0.136*	NS
Calculated fasting triglyceride	-0.223*	0.725**	-
Triglyceride (mmol/l)	-0.400**	-	0.725**
Alkaline phosphatase (U/L)	NS	0.185*	0.204*
Alanine transaminase $(U/L)^+$	0.188*	NS	NS
Aspartate transaminase $(U/L)^+$	0.401**	-0.145*	NS
Creatinine $(\mu mol/l)^+$	0.271**	NS	NS
Gamma glutamyl transpeptidase $(U/L)^+$	NS	0.220*	0.250**
10 year CHD risk score (%)	-0.416**	0.501**	0.425**

**p<0.05, **p<0.001, NS- non significant*
Other variables included in the analysis were age and red blood cell count.

Correlation analysis for lipoproteins in South Asian men and women are shown in table 79. ApoAI was positively associated with ApoB, total cholesterol, HDL-c, LDL-c, alkaline phosphatase, ALT, AST, creatinine and negatively with 10 year CHD risk score in South Asian men and women. In South Asian men only, ApoAI was positively associated with systolic blood pressure and GGT.

In South Asian men and South Asian women, ApoB was positively associated with total cholesterol, LDL-c, ApoAI, calculated fasting triglycerides, triglycerides, alkaline phosphatase, AST, creatinine, and 10 year CVD risk score, all $p<0.05$. In South Asian men alone, ApoB was negatively associated with age and positively with waist circumference, BMI, diastolic blood

pressure, red blood cell count, haemoglobin, ALT and gamma glutamyl transpeptidase. In South Asian women alone, ApoB was positively associated with HDL-c and HbA1c, $p<0.05$.

Table 79 Correlation analysis for lipoproteins in South Asian men and women in the UK

Variables	South Asian men	South Asian women	South Asian Men	South Asian women
	Apolipoprtein AI (g/l)		Apolipoprotein B (g/l)	
Correlation coefficient, r	Spearman's correlation coefficient, r		Pearson's correlation coefficient, r	
Age (years)	NS	NS	-0.169*	NS
Apolipoprotein B (g/l)	0.290**	0.322**	-	-
Cholesterol (mmol/l)	0.463**	0.537**	0.755**	0.814**
HDL cholesterol (mmol/l)	0.800**	0.888**	NS	0.190*
LDL cholesterol (mmol/l)	0.350**	0.445**	0.778**	0.868**
Waist circumference (cm)	NS	NS	0.166*	NS
Body mass index (kg/m^2)	NS	NS	0.142*	NS
Systolic blood pressue (mmHg)	0.140*	NS	NS	NS
Diastolic blood pressure (mmHg)	NS	NS	0.139*	NS
Glycated hemoglobin (%)	NS	NS	NS	0.151*
Red blood cell count	NS	NS	0.170*	NS
Hemoglobin (g/dL)	NS	NS	0.273*	NS
			Spearman's correlation coefficient, r	
Apolipoprtoein A (g/l)	-	NS	0.290**	0.322**
Calculated triglyceride	NS	NS	0.431**	0.367**
Triglyceride (mmol/l)	NS	NS	0.302**	0.285**
Alkaline phosphatase (U/L)	0.222**	0.167*	0.183*	0.190*
Alanine transaminase (U/L)	0.186*	0.238**	0.258**	NS
Aspartate transaminase (U/L)	0.276**	0.409**	0.138*	0.153*
Creatnine (µmol/l)	0.172*	0.301**	0.166*	0.190*
Gamma glutamyl transpeptidase (U/L)	0.200*	NS	0.313**	NS
10 year CHD risk score (%)	-0.127*	-0.221**	0.302**	0.344**

5.1.2.4 The performance of non-fasting triglycerides, calculated triglycerides and lipoproteins in the prediction of cardiovascular risk in South Asians asymptomatic for cardiovascular disease: ROC analysis

ROC analysis was done to evaluate the performance of non-fasting triglycerides and calculated triglycerides in the identification of South Asians at an increased risk of CVD.

On ROC analysis, measured non-fasting triglycerides significantly discriminated South Asians with greater obesity (those with increased BMI and waist circumference), metabolic syndrome and raised 10 year CHD risk score from healthy South Asians, all $p<0.05$ whilst calculated triglycerides significantly discriminated South Asians with obesity, metabolic syndrome and raised 10 year CHD risk score from healthy South Asians, all $p<0.05$ (Table 80).

Amongst apolipoproteins, ApoAI significantly discriminated South Asians with hypertension, metabolic syndrome and raised 10 year CHD risk score from healthy South Asians, all $p<0.05$. ApoB significantly discriminated those with metabolic syndrome and raised 10 year CHD risk score from healthy South Asians, $p<0.001$ (Table 80).

Validity of lipids and lipoproteins were not evaluated in South Asians symptomatic for CVD risk factors (n=519) and CVD (n=198), this was due to the effect of lipid lowering therapy and anti-diabetic therapy on lipids.

Table 80 Calculated triglycerides and lipoprotein measures as a discriminator of cardiovascular risk in South Asians asymptomatic for cardiovascular disease

Triglyceride & lipoproteins	Raised blood pressure (>130 mmHg or 85 mmHg)			Diabetes mellitus (HbA1c ≥ 6.5%)		
	P value	AUC	95% CI	P value	AUC	95% CI
Calculated triglycerides	0.170	0.538	0.484-0.591	0.958	0.502	0.420-0.585
Triglycerides (mmol/l)	0.080	0.548	0.494-0.602	0.790	0.489	0.408-0.570
Apolipoprotein AI (g/l)	0.041	0.556	0.503-0.609	0.350	0.462	0.383-0.541
Apolipoprotein B (g/l)	0.939	0.498	0.444-0.552	0.604	0.521	0.434-0.608
Triglyceride & lipoproteins	Body mass index (> 27kg/m^2)			Central obesity (≥ 90 cm in men and ≥ 80 cm in women)		
	P value	AUC	95% CI	P value	AUC	95% CI
Calculated triglycerides	0.010	0.568	0.516-0.619	0.095	0.554	0.489-0.620
Triglycerides (mmol/l)	0.040	0.555	0.504-0.606	0.052	0.563	0.494-0.631
Apolipoprotein A1 (g/l)	0.817	0.494	0.442-0.545	0.496	0.900	0.431-0.561
Apolipoprotein B (g/l)	0.523	0.517	0.465-0.569	0.056	0.562	0.497-0.627

Triglyceride & lipoproteins	Metabolic syndrome* (ATP III defined)			10 years CHD risk score (>20% high risk)		
	P value	AUC	95% CI	P value	AUC	95% CI
Calculated triglycerides	<0.001	0.727	0.682-0.771	<0.001	0.709	0.630 – 0.788
Triglycerides (mmol/l)	<0.001	0.822	0.784-0.860	<0.001	0.665	0.588-0.743
Apolipoprotein A1 (g/l)	<0.001	0.356	0.307-0.405	<0.001	0.349	0.288-0.410
Apolipoprotein B (g/l)	0.005	0.574	0.523-0.625	0.005	0.611	0.533-0.690

Metabolic syndrome –fasting glucose was not measured in E-ECHOES samples instead T2DM status was included in the ATP III clinical identification of the metabolic syndrome
ROC anal;ysis in 505 South Asians aymptomatic for CVD

5.1.3 Discussion

The aim was to evaluate the diagnostic validity of calculated triglycerides and non-fasting triglycerides in the discrimination of CVD risk in South Asians asymptomatic for CVD in the UK. Both measures of triglycerides discriminated those South Asians with obesity or metabolic syndrome or raised 10 year CHD risk score from healthy South Asians.

The direct pathophysiogical role of triglycerides in the development of CVD is not clear. Recent evidence support the hypothesis that atherosclerosis is a postprandial phenomenon. After food consumption, hydrolysis of triglycerides within chylomicrons generates atherogenic triglyceride-rich remnant lipoprotein, hence greater accumulation of atherogenic particles with elevated levels of postprandial triglyceride. Non-fasting triglycerides have shown to be strongly correlated with atherogenic triglyceride-rich remnant lipoprotein and are a strong independent predictor of incident CVD after adjustment for traditional CVD risk factors compared to fasting levels of triglyceride (Jeppesen et al., 1995; Bansal et al., 2007; Nordestgaard et al., 2007).

Amongst lipoproteins, ApoAI significantly discriminated South Asians with hypertension, metabolic syndrome and raised 10 year CHD risk score from healthy South Asians, all AUC

(0.349 – 0.556), p<0.05. ApoB significantly discriminated those with metabolic syndrome and raised 10 year CHD risk score from healthy South Asians, all AUC (0.574 – 0.611), p<0.001.

The study also characterised lipid and lipoprotein measures in South Asians belonging to various backgrounds living in the UK. With regards to lipid and lipoprotein measures, a significant difference was observed only in triglyceride levels amongst South Asian men, with Indian men and Pakistani men having a higher triglyceride levels [2.33 mmol/l (1.68 – 3.18)] and [2.13 mmol/l (1.50 – 2.90)] respectively compared to Bangladeshi men [1.54 mmol/l (1.20 – 2.30)], p=0.027.

The prevalence of raised triglycerides was higher amongst South Asian men compared to South Asian women. Further analysis showed the prevalence of raised triglycerides was higher in Indian men compared to Pakistani and Bangladeshi men. The prevalence of low HDL-c was also higher amongst South Asian men but was comparable across different ethnic groups. Similarly, the prevalence of combined raised triglycerides and low HDL-C was significantly higher amongst South Asian men compared to South Asian women but was comparable across ethnic groups.

5.1.3.1 Conclusion

Calculated triglycerides and non-fasting triglyceride levels discriminated South Asians with increased risk of CVD and hence can be used as an alternative for fasting triglyceride measurements in routine clinical practice.

5.2 Genotype- phenotype and genotype-disease association of lipids in South Asians

5.2.1 Aims and hypothesis

Genetic association studies in non-Asian populations have identified -455T>C (rs2854116) and -482C>T (rs2854117) within the promoter region of *Apolipoprotein C3 gene* on chromosome 11q23-q24 and -1131T>C (rs662799) on *Apolioprotein A5 gene* on chromosome 11q23 to be associated with levels of triglyceride and CVD, yet genetic association studies amongst South Asians are scarce. The aim of this section was to determine the allelic frequency of rs2854116, rs2854117 and rs662799 and the contribution of these SNPs to inter-individual variability in blood lipids, metabolic syndrome and CVD in South Asians.

5.2.2 Genotype-phenotype and genotype-disease association of lipids in South Asians

5.2.2.1 Characteristics of the study cohort

The study population were recruited through the E-ECHOES and NIASTAR study, the characteristics of the genetic study cohort and distribution of lipid profiles and biochemical measures are shown in Chapter 4 Result section 2, Table 54 & 55.

With regards to the characteristics amongst men by ethnic groups, as seen previously, there were more smokers amongst Bangladeshi men. The prevalence of hypertension was higher amongst Indian men, and the prevalence of T2DM was highest in Bangladeshi men. The prevalence of obesity and central obesity were highest amongst Pakistani men. Characteristics of South Asian men by ethnic groups are shown in Table 81. Characteristics of South Asian women by ethnic groups are shown in Table 82. Amongst women belonging to different ethnic groups, Indian

women were much older compared to Pakistani and Bangladeshi women, p=0.015. The prevalence of obesity was higher in Pakistani women.

Table 81 Characteristics of men by ethnic groups

Characteristics	Men by ethnic groups^ (Total n=558)			P value
	Indians (n= 296)	Pakistanis (n= 207)	Bangladeshis (n= 55)	
Age (years)*	59.5 (10.2)	59.0 (11.0)	57.2 (12.0)	0.339
Current smokers, n (%)	49 (20.0)	83 (44.0)	38 (73.1)	<0.001
Hypertension (>130 mmHg or 85 mmHg), n (%)	246 (83.1)	154 (74.4)	39 (71.0)	0.021
Diabetes mellitus (HbA1c ≥ 6.5%), n (%)	102 (35.0)	93 (45.4)	26 (47.3)	0.024
Obesity (BMI≥27 kg/m^2), n (%)	134 (48.0)	111 (55.0)	19 (36.0)	0.030
Increased waist Circumference (≥ 90 cm in men and ≥ 80 cm in women), n (%)	236 (85.0)	178 (88.0)	40 (74.1)	0.050
Systolic blood pressure (mmHg)*	141.5 (18.2)	137.0 (19.5)	134.4 (18.0)	0.004
Diastolic blood pressure (mmHg)*	83.0 (10.2)	82.0 (12.2)	80.2 (10.3)	0.267
Waist circumference (cm)*	93.0 (28.0)	97.4 (19.4)	94.4 (8.1)	0.119
Body mass index (kg/m2)*	27.4 (4.0)	28.0 (4.0)	26.0 (3.6)	0.002
Metabolic syndrome, n (%)#	169 (59.0)	119 (58.3)	26 (50.0)	0.505
Prevalent cardiovascular disease, n (%)	88 (30.0)	59 (29.0)	11 (20.0)	0.338

** Parametric data reported as mean (SD)- One way anova, n(%)-Chi-square test.*

^ Included only subjects who reported their ethnicity as Indian, Pakistani or Bangladeshi, excluded ones other Asian background, n = 4, East African Asian, n=32.

 # #Metabolic syndrome –fasting glucose was not measured in E-ECHOES samples instead T2DM status was included in the ATP III clinical identification of the metabolic syndrome

Table 82Characteristics of women by ethnic groups

Characteristics	Women by ethnic groups^ (Total n= 419)			P value
	Indians (n= 230)	Pakistanis (n= 148)	Bangladeshis (n= 41)	
Age (years)*	58.4 (10.0)	56.0 (9.5)	57.1 (7.1)	0.015
Current smokers, n (%)	3 (1.4)	4 (3.0)	1 (3.0)	0.629
Hypertension (>130 mmHg or 85 mmHg), n (%)	182 (79.1)	108 (73.0)	33 (81.0)	0.328
Diabetes mellitus (HbA1c ≥ 6.5%), n (%)	74 (33.0)	53 (36.0)	13 (32.0)	0.770
Obesity (BMI≥27 kg/m^2), n (%)	139 (62.1)	115 (79.3)	21 (53.0)	<0.001
Increased waist Circumference (≥ 90 cm in men and ≥ 80 cm in women), n (%)	203 (91.0)	140 (95.2)	38 (95.0)	0.208
Systolic blood pressure (mmHg)*	143.1 (20.2)	140.4 (21.1)	139.0 (23.0)	0.277
Diastolic blood pressure (mmHg)*	82.0 (11.0)	83.0 (12.0)	83.0 (12.2)	0.479
Waist circumference (cm)*	90.4 (20.3)	98.1 (13.0)	95.4 (21.2)	<0.001
Body mass index (kg/m2)*	29.0 (5.0)	31.0 (5.3)	29.0 (6.0)	0.008
Metabolic syndrome, n (%) #	136 60.0)	90 (62.1)	26 (65.0)	0.799
Prevalent cardiovascular disease, n (%)	43 (19.0)	20 (14.0)	6 (15.0)	0.393

** Parametric data reported as mean (SD) - One way anova, n(%)-Chi-square test.*

^ Included only subjects who reported their ethnicity as Indian, Pakistani or Bangladeshi, excluded ones East African Asian, n=21. Sri Lankan =1, Other Asian background=2.

 # # Metabolic syndrome –fasting glucose was not measured in E-ECHOES samples instead T2DM status was included in the ATP III clinical identification of the metabolic syndrome

With regards to lipids and biochemical measures in South Asian men by ethnic groups, levels of ApoAI were higher in Indian men [1.28 g/l (1.10 – 1.53)] compared to Pakistani men [1.21 g/l (1.10 – 1.34)] and Bangladeshi men [1.19 g/l (1.10 – 1.32)], p=0.005. Levels of total cholesterol were lowest in Indian men at 3.90 mmol/l (1.8), p=0.051 and levels of HDL-c were highest in Indian men at 1.01 mmol/l (0.4), p=0.015. Levels of alkaline phosphatase, and creatinine were highest amongst Bangladeshi men, p≤0.015. Distribution of lipids and biochemical measures in South Asian men by ethnic groups are shown in Table 83. Distribution of lipids and biochemical measures in South Asian women by ethnic groups are shown in Table 84. There were no significant differences in the levels of lipid measures in South Asian women belonging to different ethnic groups.

Table 83Distribution of lipids and biochemical measures in South Asian men by ethnic groups

Characteristics	Men by ethnic groups^ (Total n=558)			P value
	Indians (n= 296)	Pakistanis (n= 207)	Bangladeshi (n= 55)	
Apolipoprotein AI (g/l)+	1.28 (1.10 – 1.53)	1.21 (1.10 – 1.34)	1.19 (1.10 – 1.32)	0.005
Apolipoprotein B (g/l)+	0.71 (0.53 – 0.86)	0.69 (0.54 – 0.84)	0.65 (0.52 – 0.89)	0.735
Serum cholesterol (mmol/l)*	3.90 (1.8)	4.24 (1.4)	4.24 (1.4)	0.051
HDL cholesterol (mmol/l)*	1.01 (0.40)	0.94 (0.30)	0.90 (0.22)	0.015
LDL cholesterol (mmol/l)*	2.21 (0.95)	2.24 (0.90)	2.34 (0.94)	0.627
Triglycerides (mmol/l)+	1.89 (1.31 – 2.76)	2.22 (1.50 – 2.97)	1.93 (1.18 – 3.01)	0.132
Alkaline phosphatase (U/L)+	68.1 (53.1 – 82.4)	71.4 (61.0 – 90.0)	73.0 (62.0 – 88.1)	0.015
Alanine transaminase (U/L)+	21.2 (16.0 – 30.0)	21.2 (16.2 – 31.2)	21.0 (17.3 – 31.0)	0.823
Aspartate transaminase (U/L)+	19.1 (16.3 – 24.3)	20.4 (17.0 – 25.0)	20.4 (17.0 – 26.2)	0.277
Creatinine (µmol/l)+	67.0 (56.0 – 77.0)	74.0 (64.0 – 84.0)	76.0 (65.0 – 90.0)	<0.001
Gamma glutamyl transpeptidase (U/L)+	26.0 (17.0 – 46.0)	30.0 (19.0 – 44.0)	30.0 (19.1 – 45.1)	0.369

*Parametric data reported as mean (SD) – One way anova, + Non-parametric data reported as median (IQR) – Kruskal –Wallis test.

^ Included only subjects who reported their ethnicity as Indian, Pakistani or Bangladeshi, excluded ones other Asian background, n = 4, East African Asian, n=32.

Table 84Distribution of lipids and biochemical measures in South Asian women by ethnic groups

Characteristics	Women by ethnic groups^ (Total n = 419)			P value
	Indians (n= 230)	Pakistanis (n= 148)	Bangladeshis (n= 41)	
Apolipoprotein AI (g/l)+	1.35(1.17 – 1.55)	1.32 (1.16 – 1.51)	1.27(1.01 – 1.54)	0.485
Apolipoprotein B (g/l)*	0.66 (0.52 – 0.81)	0.66 (0.51 – 0.82)	0.58 (0.43 – 0.80)	0.186
Serum cholesterol (mmol/l)*	4.20 (1.7)	4.33 (1.4)	4.00 (1.4)	0.280
HDL cholesterol (mmol/l)*	1.12 (0.4)	1.10 (0.3)	1.10 (0.4)	0.588
LDL cholesterol (mmol/l)*	2.28 (0.9)	2.28 (0.9)	2.04 (0.9)	0.272
Triglycerides (mmol/l)+	1.81 (1.34 – 2.62)	1.92 (1.31 – 2.81)	2.03 (1.47 – 2.82)	0.531
Alkaline phosphatase (U/L)+	74.0 (60.0 – 92.2)	75.0 (61.1 – 91.0)	66.1 (48.0 – 82.20	0.132
Alanine transaminase (U/L)+	16.0 (12.0 – 20.2)	16.1 (13.0 – 22.2)	16.0 (13.0 – 21.2)	0.386
Aspartate transaminase (U/L)+	17.4 (14.2 – 21.3)	18.3 (14.3 – 22.3)	19.1 (15.2 – 22.0)	0.306
Creatinine (µmol/l)+	55.0 (46.0 – 64.0)	50.5 (45.0 – 65.0)	55.0 (48.0 – 60.3)	0.836
Gamma glutamyl transpeptidase (U/L)+	17.7 (12.3 – 26.4)	21.1 (15.0 – 33.0)	21.2 (16.0 – 32.2)	0.002

**Parametric data reported as mean (SD)-* One way anova, *+ Non-parametric data reported as median (IQR)- Kruskal-Wallis test.*

^ Included only subjects who reported their ethnicity as Indian, Pakistani or Bangladeshi, excluded ones East African Asian, n=21. Sri Lankan =1, Other Asian background=2.

5.2.2.2 Genotype frequency and allelic frequency of rs2854116, rs2854117 and rs662799 in South Asians in the UK

For rs2854116 and rs2854117, the minor allele frequency for C allele was 0.52 and T allele was 0.41 respectively. The carrier frequency for rs2854116 and rs2854117 in our cohort was 76% and 64.2% respectively. The minor allelic frequency for rs662799 was 0.17 and the carrier frequency in our cohort was 32%. The minor allele frequency is higher than those reported in Whites, 0.17 vs. 0.04. All the SNPs met the assumption of the Hardy Weinberg Theory.

The genotype frequencies for rs2854116, rs2854117 and rs662799 were comparable by ethnic groups. Table 85 shows the genotype frequencies and allelic frequencies of rs2854116, rs2854117 and rs662799 in South Asians by ethnic groups in the UK.

Table 85 Genotype and allelic frequencies of rs2854116, rs2854117 and rs662799 in South Asians by ethnic groups in the UK

Genotypes		Indians n = 527	Pakistanis n = 357	Bangladeshis n = 97	Total number of subjects (%)*	Minor allele frequency, (95% CI)	P value
rs2854116	TT (wild type)	106 (21.0)	23 (25.3)	95 (28.0)	245 (24.0)	52.0 (51.0-53.4)	0.135
	TC	257 (51.0)	48 (53.0)	153 (45.0)	495 (48.5)		
	CC	142 (28.1)	20 (22.0)	96 (28.0)	279 (27.4)		
rs2854117	CC (wild type)	178 (36.0)	27 (31.0)	120 (37.0)	353 (36.0)	41.3 (40.0-43.0)	0.726
	TC	226 (46.0)	46 (53.0)	146 (44.4)	453 (46.0)		
	TT	90 (18.2)	14 (16.1)	63 (19.1)	182 (18.4)		
rs662799	TT (wild type)	362 (27.0)	58 (60.4)	246 (70.0)	715 (68.4)	17.4 (16.1-19.0)	0.407
	TC	137 (27.0)	34 (35.4)	96 (27.3)	294 (28.2)		
	CC	18 (3.5)	4 (4.2)	10 (2.8)	35 (3.4)		

**This included East African Asians, Sri Lankan, subjects from other Asian background*

5.2.2.3 The association between APOC3 promoter polymorphisms (rs2854116 & rs2854117), cardiovascular risk factors and lipid measures in South Asians in the UK

There was no significant difference in terms of demographics, lipid levels and prevalent CVD among the two groups divided as rs2854116 genotype, wild type (TT) and carriers of minor allele (TC & CC) (Table 86 & 87).

Table 86Distribution of cardiovascular disease risk factors across rs2854116 genotypes

	TT (wild type) (n = 245)	Carriers of minor alleles (TC & CC) (n = 774)	P value
Age (years)	58.0 (10.3)	58.0 (11.0)	0.701
Male gender, n (%)	136 (56.0)	442 (58.0)	0.640
Current smokers, n (%)	39 (18.0)	137 (20.3)	0.449
Hypertension, n (%)	191 (78.0)	605 (78.2)	0.946
Diabetes mellitus, n (%)	31 (13.0)	95 (12.3)	0.843
Obesity, n (%)	139 (60.0)	418 (57.0)	0.508
Increased waist circumference, n (%)	213 (91.0)	640 (87.1)	0.105
Systolic blood pressure (mmHg)*	140.0 (23.0)	140.4 (20.0)	0.728
Diastolic blood pressure (mmHg)*	82.4 (13.0)	82.0 (11.3)	0.609
Waist circumference (cm)*	95.4 (23.0)	93.0 (24.2)	0.127
Body mass index (kg/m2)*	29.0 (5.0)	28.2 (5.0)	0.297
Metabolic syndrome, n (%)#	146 (61.0)	434 (60.0)	0.432
Prevalent cardiovascular disease, n (%)	54 (22.0)	191 (25.0)	0.400

**Parametric data reported as mean (SD) – Independent T-test, n(%) –chi-square test.*

 # #Metabolic syndrome –fasting glucose was not measured in E-ECHOES samples instead T2DM status was included in the ATP III clinical identification of the metabolic syndrome

Table 87Lipid levels across rs2854116 genotypes

	TT (wild type) (n = 245)	Carriers of minor alleles (TC & CC) (n = 774)	P value
Apolipoprotein A1(g/l)+	1.25 (1.12-1.41)	1.29 (1.11-1.51)	0.153
Apolipoprotein B (g/l)+	0.67 (0.50-0.83)	0.69 (0.53-0.84)	0.138
ApoA/ApoBratio+	1.90 (1.48-2.39)	1.95 (1.52-2.44)	0.807
Cholesterol (mmol/l)*	4.06 (1.4)	4.10 (1.6)	0.732
HDL cholesterol (mmol/l)*	0.99 (0.3)	1.03 (0.4)	0.108
LDL cholesterol (mmol/l)*	2.20 (0.9)	2.25 (0.9)	0.339
Triglycerides (mmol/l)+	1.94 (1.38-2.66)	1.92 (1.32-2.82)	0.654
Calculated triglycerides+	1.78 (1.10-2.54)	1.85 (1.00-2.67)	0.717

**Parametric data reported as mean (SD)- Independent T-test, + Non-parametric data reported as median (IQR)- Mann-Whitney test.*

There was a significant difference in the prevalence of diabetes mellitus across rs2854117 genotype, wild type (CC) and carriers of minor allele (CT & TT), p=0.05 (Table 88). There was no difference in other demographic, lipid levels and prevalent CVD among the two rs2854117 genotype group (Table 89).

Table 88Distribution of cardiovascular disease risk factors across rs2854117 genotypes

	CC (wild type) (n = 307)	Carriers of minor alleles (CT & TT) (n = 516)	P value
Age (years)	58.0 (9.3)	58.0 (11.0)	0.741
Male gender, n (%)	164 (54.0)	291 (57.0)	0.479
Current smokers, n (%)	40 (15.4)	90 (20.3)	0.109
Hypertension, n (%)	243 (79.2)	411 (80.0)	0.864
Diabetes mellitus, n (%)	80 (26.4)	169 (33.0)	0.054
Obesity, n (%)	176 (60.1)	278 (57.3)	0.451
Increased waist circumference, n (%)	265 (90.4)	421 (90.0)	0.128
Systolic blood pressure (mmHg)*	141.0 (21.0)	140.4 (20.0)	0.915
Diastolic blood pressure (mmHg)*	83.0 (11.2)	82.0 (11.4)	0.233
Waist circumference (cm)*	94.0 (25.0)	92.0 (26.0)	0.331
Body mass index (kg/m2)*	29.0 (5.0)	28.2 (5.0)	0.219
Metabolic syndrome (%) #	181 (60.0)	301 (60.0)	0.926
Prevalent cardiovascular disease, n (%)	74 (24.1)	124 (24.0)	0.981

**Parametric data reported as mean (SD)- Independent T-test, n(%)- chi-square test.*

 # #Metabolic syndrome –fasting glucose was not measured in E-ECHOES samples instead T2DM status was included in the ATP III clinical identification of the metabolic syndrome

Table 89Lipid levels across rs2854117 genotypes

	CC (wild type) (n = 307)	Carriers of minor alleles (CT & TT) (n = 516)	P value
Apolipoprotein A (g/l)+	1.25 (1.11-1.50)	1.28 (1.11-1.52)	0.354
Apolipoprotein B (g/l)+	0.67 (0.52-0.82)	0.70 (0.52-0.84)	0.299
ApoA/ApoB ratio+	1.92 (1.50 -2.50)	1.94 (1.52-2.40)	0.597
Cholesterol (mmol/l)*	4.00 (1.6)	4.05 (1.7)	0.371
HDL cholesterol (mmol/l)*	1.00 (0.3)	1.01 (0.4)	0.258
LDL cholesterol (mmol/l)*	2.20 (0.9)	2.22 (0.94)	0.692
Triglycerides (mmol/l)+	1.90 (1.39-2.67)	1.91 (1.30-2.90)	0.601
Calculated triglycerides+	1.74 (0.94-2.46)	1.83 (0.86-2.71)	0.325

**Parametric data reported as mean (SD)- Independent T-test, + Non-parametric data reported as median (IQR)- Mann-Whitney test*

5.2.2.4 The association between APOA5 promoter polymorphism rs662799, cardiovascular risk factors and lipid measures in South Asians in the UK

The section shows the significant difference in demographics and lipid levels in South Asians with carriers of minor allele of rs662799 compared to those with wild type.

The prevalence of metabolic syndrome was significantly higher in carriers of minor allele, C allele of rs662799 compared to those with wild type, p = 0.030 (Table 90).

Table 90 Distribution of CVD risk factors across rs662799 genotypes

	TT (wild type) (n = 715)	Carriers of minor alleles (TC & CC) (n =329)	P value
Age (years)	58.0 (10.1)	59.0 (11.0)	0.168
Current smokers, n (%)	121 (19.1)	66 (23.0)	0.168
Hypertension, n (%)	551 (77.1)	262 (80.0)	0.352
T2DM, n (%)	236 (33.2)	102 (31.1)	0.503
Obesity, n (%)	403 (59.0)	171 (55.2)	0.267
Increased waist circumference, n (%)	607 (88.4)	267 (86.4)	0.386
Systolic blood pressure (mmHg)*	140.0 (20.3)	141.0 (19.3)	0.344
Diastolic blood pressure (mmHg)*	82.3 (12.0)	82.0 (11.0)	0.452
Waist circumference (cm)*	95.0 (22.3)	92.0 (25.1)	0.060
Body mass index (kg/m2)*	28.4 (5.0)	28.2 (5.0)	0.616
High sensitivity C-reactive protein (mg/L)+	1.86 (0.80-4.12)	1.68 (1.00-4.00)	0.949
Metabolic syndrome (%) #	391 (56.3)	204 (64.0)	0.030
Prevalent cardiovascular disease, n (%)	162 (23.0)	81 (25.0)	0.486

** Parametric data reported as mean (SD)- Independent T-test, n(%)- chi-square test.*

 # Metabolic syndrome –fasting glucose was not measured in E-ECHOES samples instead T2DM status was included in the ATP III clinical identification of the metabolic syndrome

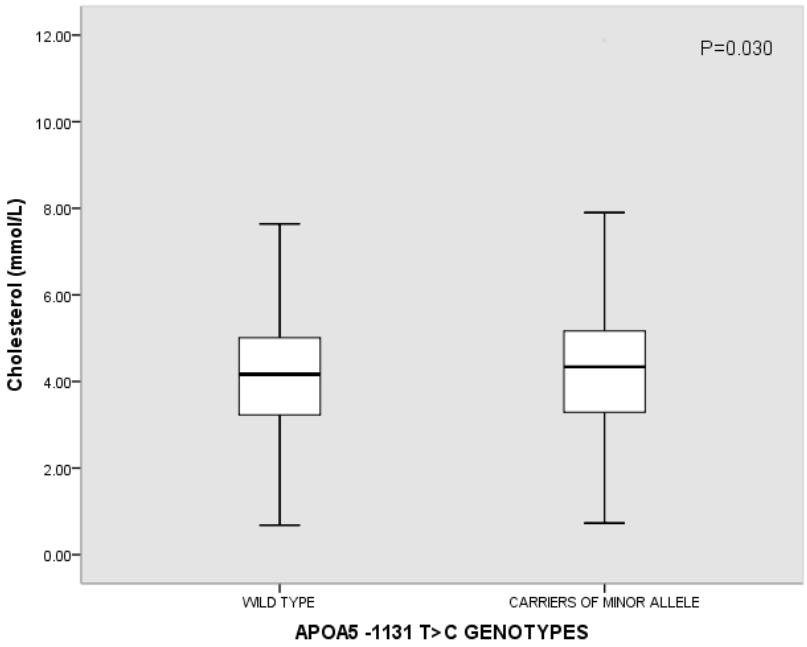
With regards to lipid measures, carriers of minor allele, C allele of rs662799 had significantly higher levels of ApoB, total cholesterol and triglyceride compared to those with wild type, all p <0.05 (Table 91, figure 37 & 38).

Table 91 Lipid levels across rs662799 genotypes

	TT (wild type) (n = 715)	Carriers of minor alleles (TC & CC) (n =329)	P value
Apolipoprotein A (g/l)[+]	1.30 (1.11-1.48)	1.30 (1.11-1.52)	0.243
Apolipoprotein B (g/l)[+]	0.67 (0.52-0.83)	0.71 (0.54-0.90)	0.004
ApoA/ApoBratio[+]	1.94 (1.53-2.45)	1.92 (1.50-2.40)	0.102
Cholesterol (mmol/l)[*]	4.04 (1.62)	4.30 (1.53)	0.030
HDL cholesterol (mmol/l)[*]	1.03 (0.40)	1.02 (0.40)	0.736
LDL cholesterol (mmol/l)[*]	2.22 (0.88)	2.33 (1.00)	0.066
Triglycerides (mmol/l)[+]	1.84 (1.30-2.66)	2.20 (1.50-3.10)	<0.001
Calculated triglycerides[+]	1.83 (1.00 -3.00)	1.90 (1.21-2.90)	0.114

[*]*Parametric data reported as mean (SD)- Independent T-test,* [+] *Non-parametric data reported as median (IQR)- Mann-Whitney test*

figure 37 Levels of total cholesterol across rs662799genotypes

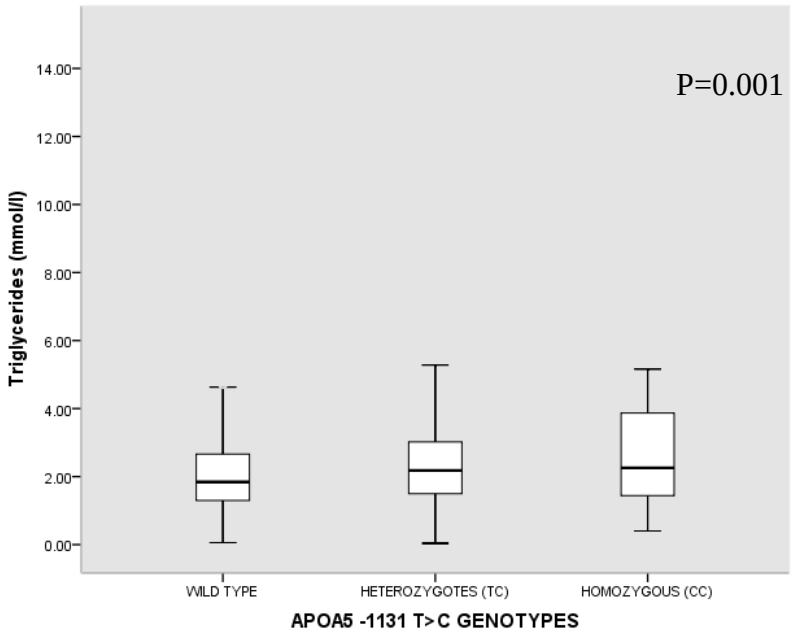


Independent T-test

Boxes represent interquartile range and medians are shown as solid lines

figure 38 Levels of triglyceride across rs662799 genotype

Kruskal-Wallis test



Boxes represent interquartile range and medians are shown as solid lines

Linear regression was performed to determine whether lipid measures were associated with SNP rs662799 (for categorical variables, the regression coefficient indicates the average difference in the dependent variable between the two groups). SNP rs662799 was associated with levels of triglyceride (β= 0.341 (95% CI 0.144-0.537), r^2=0.011, P=0.001), total cholesterol (β= 0.241 (95% CI 0.028-0.454), r^2=0.004, P=0.030) and Apo B (β = 0.090 (95% CI 0.024-0.156), r^2=0.006, P=0.08) when comparing wild type (TT) with carriers of minor allele (TC & CC).

5.2.2.5 The association between APOA5 promoter polymorphism rs662799 with triglyceride levels and metabolic syndrome in South Asians in the UK

Logistic regression was performed to determine the odds of having raised triglycerides and metabolic syndrome in carriers of -1131 C allele compared to non-carriers.

The odds ratio for raised plasma triglyceride in carriers of -1131 C allele compared to non-carriers was 1.21, 95% CI 1.10 -1.35, P>0.001. The odds ratio for metabolic syndrome in carriers of -1131 C allele compared to non-carriers was 0.738, (95% CI, 0.562 – 0.968).

5.2.2.6 Clinical correlates of triglycerides in South Asian men and women: multiple regression models

Multiple regression models were developed with variables associated with triglycerides in correlation analysis and was performed to determine clinical correlates that independently explained variation in triglyceride levels in South Asians.

On correlation analysis, triglyceride was positively associated with waist circumference, BMI, red blood cell count, white blood cell count, ApoB, cholesterol, ALT and GGT and was negatively associated with ApoAI, ApoAI/Bratio and HDL-c in South Asian men and women, all $p<0.05$. In South Asian men alone, triglyceride was positively associated with diastolic blood pressure, haemoglobin and LDL-c and negatively with age, all $p<0.05$. In South Asian women alone, triglyceride was positively associated with HbA1c, $p<0.05$. Correlation analysis for triglycerides in South Asians is shown in table 92.

Table 92Correlation analysis for triglycerides in South Asian men and women in the UK

Variables	South Asian men		South Asian women	
	Spearman's correlation coefficient, r	P value	Spearman's correlation coefficient, r	P value
Age (years)	-0.187	<0.001	NS	
Waist circumference (cm)	0.130	0.002	0.187	<0.001
Body mass index (kg/m^2)	0.133	0.002	0.127	0.010
Diastolic blood pressure (mmHg)	0.156	<0.001	NS	
Glycated haemoglobin (%)	NS	NS	0.152	0.003
Haemoglobin (g/l)	0.268	<0.001	NS	
Red blood cells	0.247	<0.001	0.135	0.022
White blood cells	0.156	0.002	0.300	<0.001

Apolipoprotein AI (g/l)	-0.153	<0.001	-0.117	0.020
Apolipoprotein B (g/l)	0.285	<0.001	0.197	<0.001
ApoAIBratio	-0.409	<0.001	-0.296	<0.001
Cholesterol (mmol/l)	0.359	<0.001	0.203	<0.001
HDL cholesterol (mmol/l)	-0.315	<0.001	-0.336	<0.001
LDL cholesterol (mmol/l)	0.167	<0.001	NS	
Alanine transaminase (U/L)[+]	0.205	<0.001	0.130	0.012
Gamma glutamyl transpeptidase (U/L)[+]	0.263	<0.001	0.251	<0.001

[+]*Measured only in E-ECHOES samples*

On multiple linear regression models, total cholesterol, HDL-c, ApoB, GGT and rs662799 explained 40% of the variability in triglyceride levels in South Asian men with total cholesterol explaining 12% of the total variance (Table 93).

Table 93Clinical correlates of triglyceride in South Asian men: multiple linear regression model

Predictor variables	Beta coefficients	95% Confidence Interval	P value	Adjusted R^2 (simple linear regression)
Total cholesterol (mmol/l)	1.038	0.781 – 1.296	<0.001	0.115
HDL cholesterol (mmol/l)	-2.775	-3.275 – (-2.276)	<0.001	0.080
Apolipoprotein B (g/l)	-2.814	-4.140 – (-1.488)	<0.001	0.019
Gamma glutamyl transpeptidase (U/L)	0.010	0.004 – 0.015	0.001	0.014
rs662799 (TT vs. TC & CC)	0.392	0.076 – 0.707	0.015	0.006

Overall adjusted R^2 for the model 0.389, p<0.001
Variables included in the model- Age, waist, diastolic blood pressure, HbA1c, haemoglobin, ApoB, total cholesterol, HDL cholesterol,ALT,GGT and rs662799 genotype (TT vs. TC & CC)

In South Asian women, HDL-c, total cholesterol, GGT, rs662799 and HbA1c explained 31% of the variability in triglyceride levels with HDL cholesterol explaining 10% of the total variance (Table 94).

Table 94Clinical correlates of triglyceride in South Asian women: multiple linear regression model

Predictor variables	Beta coefficients	95% Confidence Interval	P value	Adjusted R^2 (simple linear regression)
HDL cholesterol (mmol/l)	-1.647	-1.971 – (-1.323)	<0.001	0.090
Total cholesterol (mmol/l)	0.398	0.306 – 0.490	<0.001	0.052
Gamma glutamyl transpeptidase (U/L)	0.005	0.002 – 0.009	0.001	0.036
rs662799 (TT vs. TC & CC)	0.302	0.068 – 0.536	0.012	0.017
Glycated haemoglobin (%)	0.125	0.027 – 0.224	0.013	0.030

Overall adjusted R^2 for the model 0.307, p<0.001
Variables included in the model- Age, waist, diastolic blood pressure, HbA1c, haemoglobin, ApoB, total cholesterol, HDL cholesterol, ALT, GGT and rs662799 genotype (TT vs. TC & CC)

5.2.3 Discussion

The *APOA5* -1131C>T (rs662799) is a determinant of plasma triglyceride levels in the South Asian population. The odds ratio for raised plasma triglyceride in carriers of -1131 C allele was 1.21 compared to non-carriers, p>0.001. Carriers of the minor allele -1131 C had higher levels of triglycerides, total cholesterol and more metabolic syndrome compared to wild type homozygotes. *APOC3* promoter polymorphisms rs2854116 and rs2854117 did not contribute to any variations in blood lipid levels and metabolic syndrome. None of the polymorphisms were associated with CVD in this cohort.

The minor allele frequency of rs662799 observed in our cohort was similar to those reported in Asian Indians and was higher in South Asians compared to those in Whites, 17% versus 4% (Chandak, 2006). The minor allele frequencies of rs2854116 and rs2854117 were higher than those reported in Whites but similar to those in South Asian populations (Pollex, 2007). There was no significant difference in the genotype frequencies for *APOC3* promoter polymorphisms and *APOA5* -1131T>C in subjects belonging to Indian, Pakistani and Bangladeshi origin.

The *APOA5* gene is located 27kb distal to apoA-IV in apoA1/C3/A4/A5 gene cluster on chromosome 11q23. The functionality of *APOA5* gene was demonstrated in gene knockout studies, where the human APOA5 transgenic mice had low levels of plasma triglyceride (0.32 ± (SD) 0.11 mg/ml) compared to APOA5 knockout mice (1.53 ± 0.77 mg/ml). Our findings were consistent with prior studies that have associated *APOA5* -1131C allele with elevated levels of triglycerides in Europeans (Aouizerat, 2003; Dorfmeister, 2007; De Caterina, 2011; Evans et al., 2011) and Asian Indians (Chandak, 2006). The APOA5 -1131T>C polymorphism has also been associated with increased risk of CVD (De Caterina, 2011; Evans, 2011).

APOC3 codes for a 79-residue glycoprotein and is synthesised in the liver and is a protein component of triglyceride rich lipoprotein particles. Over expression of ApoCIII delays the catabolism of triglycerides by down regulating LPL and impairs the apoE mediated clearance of triglyceride-rich lipoprotein by the liver, resulting in hypertriglyceridemia (Ito et al., 1990; Li, 1995). The expression of ApoCIII is down regulated by insulin, transcriptional activity of promoter constructs containing the mutant alleles of the *APOC3* promoter polymorphisms located within an insulin-responsive element (-490 to -449) is compromised and fails to respond effectively to insulin-mediated down-regulation hence remains active constantly (Li, 1995; Waterworth, 2003).

APOC3 promoter polymorphisms have been associated with variations in triglyceride levels (Hegele et al., 1997; Waterworth, 2000), increased risk of metabolic syndrome (Miller et al., 2007; Pollex, 2007) and CHD in multi ethnic groups (Olivieri et al., 2002). In our cohort, levels of triglyceride were comparable in South Asians with minor alleles of *APOC3* promoter polymorphisms and in those with wild type homozygotes. The prevalence of metabolic syndrome was also comparable in those with minor allele of *APOC3* promoter polymorphisms and wild type homozygotes. *APOC3* promoter polymorphisms are the most studied SNPs in lipid metabolism and have mostly generated positive associations with lipids in multi ethnic groups. However, along with the present study, there are studies that have failed to confirm this association (Dallongeville et al., 2006; Sentinelli, 2011).

Finally, the strongest evidence for the association of triglycerides and CVD has been observed in subjects with low HDL-C (Criqui et al., 1993) and diabetes mellitus (Laakso et al., 1993); such an effect could blank out a modest effect (Emerging Risk Factors Collaboration, 2009). The

increased risk of CVD attributed to hypertriglyceridemia could be due to dysfunctional HDL and small dense LDL particles that are more prone to oxidative modification (Miller et al, 2011).

5.2.3.1 Conclusion

APOA5 -1131T>C is a determinant of plasma triglyceride levels in the South Asian population; however this polymorphism was not associated with CVD. Prior studies have confirmed the role of *APOC3* promoter polymorphisms on serum triglyceride levels and CVD, but these findings were not replicated in our South Asian cohort.

CHAPTER 6 RESULTS 3

6.1 The validity of biomarkers of thrombosis, haemostasis, and endothelial dysfunction as discriminators of cardiovascular disease risk in South Asians in the UK

6.1.1 Aims and hypothesis

South Asians have an increased risk of CVD compared to the general population in the UK. However, there is no substantial population based evidence for the performance of D-dimer, P-selectin and vWF as discriminators of CVD risk in South Asians. The aim of this section was to evaluate the performance of these biomarkers in the identification of South Asians at an increased risk of CVD initially in South Asians asymptomatic for CVD and then to validate the findings in a group of South Asians with CVD risk factors (on medication for anti-hypertensive, anti – diabetic or lipid lowering therapy) and existing CVD events.

6.1.2 The validity of biomarkers of thrombosis, haemostasis and endothelial dysfunction as discriminators of cardiovascular disease in South Asians asymptomatic for cardiovascular disease

6.1.2.1 Characteristics of the study cohort

The characteristics of the study population (E-ECHOES) and distribution of all lipid profiles and biochemical measures are shown in Chapter 4 Result section 1, Table 44 & 45. There was no significant gender difference in the levels of P-selectin in South Asians. The levels of D-dimer

were higher in South Asian women compared to South Asian men, p <0.001. The levels of vWF were higher amongst South Asian men compared to South Asian women, p=0.04. Table 95 shows the distribution of plasma biomarkers in the study population.

Table 95Distribution of plasma biomarkers in the study population

Characteristics	Men (n=276)	Women (n=229)	P value
D-dimer (µg/ml)[+]	0.21 (0.12-0.33)	0.29 (0.19-0.44)	<0.001
P-selectin (ng/ml)[+]	32.0 (24.4-40.0)	30.5 (23.3-39.3)	0.442
Von willebrand factor (IU/dL)*	79.4 (15.4)	77.0 (15.0)	0.04

[+]*Reported as median and interquartile range -Mann-Whitney test*
**Reported as mean (SD) –Independent T-test*

6.1.2.2 Levels of D-dimer, P selectin, von willebrand factor and cardiovascular risk factors in South Asians asymptomatic for cardiovascular disease

The section shows the levels of D-dimer, P selectin and vWF across each CVD risk factor in South Asians asymptomatic for CVD.

In the entire cohort of South Asians asymptomatic for CVD, levels of D-dimer were higher in those with greater central obesity compared to non-obese subjects, p =0.002. Levels of D-dimer were comparable in those with other CVD risk factors such as hypertension, obesity, diabetes mellitus, low HDL-c, raised triglycerides, metabolic syndrome, 10 year CVD risk score and healthy South Asians (table 96).

Table 96Difference in the levels of D-dimer across cardiovascular risk factors in South Asians asymptomatic for cardiovascular disease

Risk factors	D-dimer (µg/ml)	P value (Mann-Whitney test)
Hypertensive (>130 mmHg or 85 mmHg) Not hypertensive	0.23 (0.13 – 0.39) 0.24 (0.15 – 0.40)	0.397
Obesity (BMI≥27 kg/m^2) Not obese	0.24 (0.15 – 0.41) 0.23 (0.14 - 0.38)	0.323
Central obesity (≥ 90 cm in men and ≥ 80 cm in women) Not obese	0.25 (0.15 – 0.40) 0.20 (0.12 – 0.31)	0.002

With diabetes mellitus (HbA1c ≥ 6.5%) Without diabetes mellitus	0.27 (0.18– .39) 0.23 (0.14 – 0.39)	0.252
Low HDL cholesterol (<0.9 mmol/l in men & <1.0 mmol/l in women) Normal HDL Cholesterol	0.23 (0.12 – 0.38) 0.24 (0.15 – 0.40)	0.419
Elevated triglycerides (≥ 1.7 mmol/l) Normal triglycerides	0.23 (0.14 – 0.40) 0.24 (0.20 – 0.40)	0.349
With metabolic syndrome* Without metabolic syndrome	0.24 (0.14–0.38) 0.23 (0.14 – 0.40)	0.617
10 year CHD risk score (>20%) 10 year CHD risk score (<20%)	0.31 (0.12 – 0.48) 0.23 (0.14 – 0.38)	0.187

Reported as median and interquartile range

**Metabolic syndrome –fasting glucose was not measured in E-ECHOES samples instead T2DM status was included in the ATP III clinical identification of the metabolic syndrome*

Levels of P-selectin were higher in those with elevated triglycerides compared to those with normal triglycerides, p=0.050. Levels of P-selectin were comparable in those with CVD risk factors such as hypertension, greater obesity, diabetes mellitus, low HDL-c, metabolic syndrome and 10 year CVD risk score and healthy South Asians (Table 97).

Table 97 Difference in the levels of P selectin across cardiovascular risk factors in South Asians asymptomatic for cardiovascular disease

Risk factors	P selectin (ng/ml)	P value (Mann-Whitney test)
Hypertensive (>130 mmHg or 85 mmHg) Not hypertensive	31.3 (24.0 – 40.0) 31.0 (24.0 – 38.3)	0.705
Obesity (BMI≥27 kg/m^2) Not obese	31.0 (24.0–39.0) 32.0 (24.0 – 40.0)	0.605
Central obesity (≥ 90 cm in men and ≥ 80 cm in women) Not obese	31.4 (24.0 – 40.0) 31.0 (24.0 – 39.2)	0.696
With diabetes mellitus (HbA1c ≥ 6.5%) Without diabetes mellitus	31.1 (24.4–41.0) 31.2 (24.0 – 39.0)	0.646
Low HDL cholesterol (<0.9 mmol/l in men & <1.0 mmol/l in women) Normal HDL Cholesterol	32.1 (24.5 – 40.1) 31.0 (24.0 – 39.1)	0.342
Elevated triglycerides (≥ 1.7 mmol/l) Normal triglycerides	32.0 (25.0 – 40.0) 31.0 (23.0 – 37.4)	0.050
With metabolic syndrome* Without metabolic syndrome	32.1 (25.0 – 40.1) 31.0 (23.0 – 38.0)	0.071
10 year CHD risk score (>20%) 10 year CHD risk score (<20%)	34.0 (24.0 – 40.2) 31.0 (24.2 – 39.5)	0.413

Reported as median and interquartile range

**Metabolic syndrome –fasting glucose was not measured in E-ECHOES samples instead T2DM status was included in the ATP III clinical identification of the metabolic syndrome*

Levels of vWF were higher in South Asians with obesity compared to non-obese subjects, p =0.022 and in those with higher 10 year CVD risk score compared to those with lower 10 year CVD risk score, p=0.002 (Table 98).

Table 98 Difference in the levels of von willebrand factor across cardiovascular risk factors in South Asians asymptomatic for cardiovascular disease

Risk factors	Von willebrand factor (IU/dL)	P value (Independent sample T test)
Hypertensive (>130 mmHg or 85 mmHg) Not hypertensive	77.0 (14.3) 79.0 (16.0)	0.174
Obesity (BMI≥27 kg/m^2) Not obese	80.0 (15.2) 77.0 (15.0)	0.022
Central obesity (≥ 90 cm in men and ≥ 80 cm in women) Not obese	79.0 (15.0) 76.2 (16.0)	0.146
With diabetes mellitus (HbA1c ≥ 6.5%) Without diabetes mellitus	80.5 (14.3) 78.0 (16.1)	0.516
Low HDL cholesterol (<0.9 mmol/l in men & <1.0 mmol/l in women) Normal HDL Cholesterol	79.0 (14.0) 78.0 (16.1)	0.516
Elevated triglycerides (≥ 1.7 mmol/l) Normal triglycerides	79.0 (16.0) 78.0 (15.0)	0.540
With metabolic syndrome* Without metabolic syndrome	79.3 (14.0) 77.0 (16.1)	0.074
10 year CHD risk score (>20%) 10 year CHD risk score (<20%)	84.0 (16.3) 77.2 (15.0)	0.002

Reported as mean and standard deviation

**Metabolic syndrome –fasting glucose was not measured in E-ECHOES samples instead T2DM status was included in the ATP III clinical identification of the metabolic syndrome*

6.1.2.3 The association between D-dimer, P-selectin, von willebrand factor and cardiovascular risk factors in South Asian men and women

Correlation analysis were performed to identify clinical correlates of D-dimer, P selectin and Vwf in South Asians.

D-dimer was positively associated with age, HbA1c, and CRP, $p<0.05$ in South Asian men. D-dimer levels were negatively associated with triglycerides only (correlation coefficient, r=-0.151, p=0.030) in South Asian women. In the entire cohort of South Asians asymptomatic for CVD, D-dimer was positively associated with age, gender, CRP and was negatively associated with red blood cell count, haemoglobin, ALT and triglyceride, $p<0.05$. Correlation analyses for D-dimer are shown in table 99 & 100.

Table 99 Correlation analysis for D-dimer in South Asian men and women asymptomatic for cardiovascular disease

Variables	South Asian men		South Asian women	
	Speraman's correlation coefficient, r	P value	Speraman's correlation coefficient, r	P value
Age (years)	0.179	0.003	NS	
Glycosylated hemoglobin (%)	0.123	0.050	NS	
High sensitivity C-reactive protein (mg/L)	0.204	0.001	NS	
Triglycerides (mmol/l)	NS		-0.151	0.025

Variables included in the analysis were age, waist circumference, BMI, systolic blood pressure, diastolic blood pressure, white blood cell count, red blood cell count, hemoglobin, HbA1c, alkaline phosphatase, ALT, AST, GGT, creatnine, ApoAI, ApoB, total cholesterol, LDL-c, HDL-c, triglycerides, 10 year CHD risk score, vWF , CRP and P selectin.

Table 100 Correlation analysis for D-dimer in the entire cohort

Variables	South Asians asymptomatic for CVD	
	Speraman's correlation coefficient, r	P value
Age (years)	0.096	0.032
Gender	0.227	<0.001
High sensitivity C-reactive protein (mg/L)	0.161	<0.001
Red blood cell count	-0.166	0.006
Haemoglobin (g/dL)	-0.155	0.010
Alanine transaminase (U/L)	-0.119	0.010
Triglycerides (mmol/l)	-0.109	0.017

Variables included in the analysis were waist circumference, BMI, systolic blood pressure, diastolic blood pressure, white blood cell count, HbA1c, alkaline phosphatase, AST, GGT, creatnine, ApoAI, ApoB, total cholesterol, LDL-c, HDL-c, triglycerides, 10 year CHD risk score, vWF , CRP and P selectin.

In the entire cohort, levels of P-selectin were positively associated with white blood cell count, alkaline phosphatase, ApoB, total cholesterol, GGT, LDL-c, triglycerides and 10 year CHD risk score, all $p<0.05$. Correlation analyses for P selectin are shown in table 101.

Table 101 Correlation analysis for P-selectin in South Asians asymptomatic for cardiovascular disease

Variables	Speraman's correlation coefficient, r	P value
White blood cell count	0.199	0.001
Alkaline phosphatase (U/L)	0.135	0.003
Apolipoprotein B (g/l)	0.135	0.003
Total cholesterol (mmol/l)	0.140	0.002
Gamma glutamyl transpeptidase (U/L)	0.125	0.007
LDL cholesterol (mmol/l)	0.107	0.020
Triglycerides (mmol/l)	0.117	0.010
10 year CHD risk score (%)	0.115	0.012

Variables included in the analysis were age, waist circumference, BMI, systolic blood pressure, diastolic blood pressure, red blood cell count, hemoglobin, HbA1c, ALT, AST, GGT, creatnine, ApoAI, ApoB, total cholesterol, HDL-c,10 year CHD risk score vWF , CRP and P selectin.

Levels of vWF were positively associated with age, waist measurement, BMI, systolic blood pressure, CRP, white blood cell count, alkaline phosphatase, AST, creatinine, GGT and 10 year CHD risk score, all $p<0.05$ in South Asian men. In South Asian women, levels of vWF were

negatively correlated with ApoB and ApoAI, $p<0.05$ and positively with red blood cell count. Correlation analyses for vWF are shown in table 102.

Table 102 Correlation analysis for von willebrand factor in South Asian men and women asymptomatic for cardiovascular disease

Variables	South Asian men		South Asian women	
	Pearson's correlation coefficient, r	P value	Pearson's correlation coefficient, r	P value
Age (years)	0.153	0.011	NS	
Waist measurement (cm)	0.181	0.003	NS	
Body mass index (kg/m^2)	0.154	0.011	NS	
Systolic blood pressure (mmHg)	0.156	0.010	NS	
Apolipoprotein B (g/L)	NS		-0.148	0.031
	Speraman's correlation coefficient, r	P value	Speraman's correlation coefficient, r	P value
Red blood cell count	NS		0.185	0.040
White blood cell count	0.192	0.020	NS	
Apolipoprotein AI (g/L)	NS		-0.135	0.050
High sensitivity C-reactive protein (mg./L)	0.207	0.001	NS	
Alkaline phosphatase (U/L)	0.150	0.015	NS	
Aspartate transaminase (U/L)	0.126	0.042	NS	
Creatinine (µmol/l)	0.147	0.017	NS	
Gamma glutamyl transpeptidase (U/L)	0.169	0.006	NS	
10 year coronary heart Disease risk score (%)	0.227	<0.001	NS	

Variables included in the analysis were diastolic blood pressure, white blood cell count, hemoglobin, HbA1c, ApoAI, ApoB, total cholesterol, LDL-c,HDL-c, triglycerides, vWF , CRP and P selectin.

6.1.2.4 Clinical correlates of D-dimer, P selectin and von willebrand factor in South Asian men and women: multiple regression models

Multiple regression models were developed with variables associated with D-dimer, P selectin and vWF and was performed to identify clinical correlates that independently explained variation in the above mentioned plasma biomarkers in South Asians.

On multiple regression models, red blood cell count alone explained 7% of the variation in D-dimer levels. GGT explained 2% of the variation in P selectin levels. Table 103 & 104 shows the clinical correlates of D-dimer and P selectin in South Asians asymptomatic for CVD.

Table 103 Clinical correlates of D-dimer in South Asians: multiple linear regression model

Predictor variables	Beta coefficients	95% Confidence Interval	P value	Adjusted R^2 (simple linear regression)
Red blood cell count	-0.109	-0.169 - -0.050	<0.001	0.068

Variables included in the model- age, gender, CRP, haemoglobin, ALT, BNP and triglycerides

Table 104 Clinical correlates of P-selectin in South Asians: multiple linear regression model

Predictor variables	Beta coefficients	95% Confidence Interval	P value	Adjusted R^2 (simple linear regression)
Gamma glutamyl transpeptidase (U/L)	0.156	0.044 – 0.268	0.007	0.024

Variables included in the model- white blood cell count, alkaline phosphatase, GGT, triglycerides and 10 year CVD risk score

Table 105 & 106 shows the clinical correlates of vWF in South Asians, model 1 & 2. In model 1, creatinine, white blood cell count, age and BMI explained 7% of the variation in vWF levels. In model 2, creatinine, white blood cell count and waist circumference explained 6% of the variation in vWF levels.

Table 105 Clinical correlates of von willebrand factor in South Asians: multiple linear regression model 1

Predictor variables	Beta coefficients	95% Confidence Interval	P value	Adjusted R^2 (simple linear regression)

Creatinine (µmol/l)	0.157	0.051 – 0.263	0.004	0.020
White blood cell count	1.104	0.180 – 2.028	0.019	0.020
Age (years)	0.285	0.061 – 0.509	0.013	0.013
Body mass index (kg/m^2)	0.430	0.029 – 0.831	0.040	0.006

Overall adjusted R^2 for the model- 0.07
Variables included in the model- age, gender, red blood cell count, white blood cell count, ApoAI, CRP, alkaline phosphatase, ALT, creatinine, GGT

Table 106 Clinical correlates of von Will brand factor in South Asians: multiple linear regression model 2

Predictor variables	Beta coefficients	95% Confidence Interval	P value	Adjusted R^2 (simple linear regression)
Creatinine (µmol/l)	0.159	0.053 – 0.266	0.003	0.020
White blood cell count	1.019	0.093 – 1.946	0.031	0.020
Waist circumference (cm)	0.174	0.004 – 0.343	0.050	0.007

Overall adjusted R^2 for the model- 0.060
Variables included in the model- waist circumference, red blood cell count, white blood cell count, CRP, alkaline phosphatase, ALT, creatinine, GGT and 10 year CVD risk score

6.1.2.5 The performance of D-dimer, P selectin and von willebrand factor in the prediction of cardiovascular risk in South Asians asymptomatic for cardiovascular disease: ROC analysis

The performance of D-dimer, P selectin and vWF as discriminators of CVD risk in South Asians was evaluated using ROC analysis. On ROC analysis, D-dimer significantly discriminated those with greater central obesity from non-obese South Asians, AUC- 0.601 (95% CI 0.542 – 0.659), p=0.002. P selectin significantly discriminated South Asians with elevated triglycerides from South Asians with normal triglycerides, AUC-0.553 (95% CI 0.501 – 0.606), p=0.050. Levels of vWF significantly discriminated subjects with greater obesity, metabolic syndrome and higher 10 year CVD risk score from healthy South Asians, all AUC 0.551 -0.642, $p<0.05$. Plasma biomarkers as discriminators of metabolic and cardiovascular disease risk in South Asians asymptomatic for cardiovascular disease are shown in Table 107 -109.

Table 107 D-dimer as a discriminator of metabolic and cardiovascular disease risk amongst South Asian men and women asymptomatic for cardiovascular disease

D-dimer (mg/L)	Raised blood pressure (>130 mmHg or 85 mmHg)			Body mass index (> 27kg/m^2)		
	P value	AUC	95% CI	P value	AUC	95% CI
	0.397	0.477	0.424 - 0.530	0.323	0.526	0.475 - 0.577
	Central obesity (≥ 90 cm in men and ≥ 80 cm in women)			Diabetes mellitus (HbA1c ≥ 6.5%)		
	P value	AUC	95% CI	P value	AUC	95% CI
	0.002	0.601	0.542 – 0.659	0.253	0.547	0.469 – 0.625
	Elevated measured triglycerides (≥ 1.7 mmol/l)			Low HDL cholesterol (<0.9 mmol/l in men & <1.0 mmol/l in women, non-smokers)		
	P value	AUC	95% CI	P value	AUC	95% CI
	0.346	0.474	0.422 – 0.527	0.419	0.478	0.422 – 0.533
	Metabolic syndrome* (ATP III defined)			10 years CHD risk score (>20%)		
	P value	AUC	95% CI	P value	AUC	95% CI
	0.617	0.487	0.436 – 0.538	0.187	0.553	0.467 – 0.639

**Metabolic syndrome –fasting glucose was not measured in E-ECHOES samples instead T2DM status was included in the ATP III clinical identification of the metabolic syndrome*

Table 108 P–selectin as a discriminator of metabolic and cardiovascular disease risk amongst South Asian men and women asymptomatic for cardiovascular disease

P selectin (ng/ml)	Raised blood pressure (>130 mmHg or 85 mmHg)			Body mass index (> 27kg/m^2)		
	P value	AUC	95% CI	P value	AUC	95% CI
	0.705	0.510	0.457 – 0.564	0.605	0.487	0.435 – 0.538
	Central obesity (≥ 90 cm in men and ≥ 80 cm in women)			Diabetes mellitus (HbA1c ≥ 6.5%)		
	P value	AUC	95% CI	P value	AUC	95% CI
	0.696	0.512	0.451 – 0.573	0.646	0.519	0.441 – 0.596
	Elevated measured triglycerides (≥ 1.7 mmol/l)			Low HDL cholesterol (<0.9 mmol/l in men & <1.0 mmol/l in women, non-smokers)		
	P value	AUC	95% CI	P value	AUC	95% CI
	0..050	0.553	0.501 – 0.606	0.342	0.526	0.472 – 0.581
	Metabolic syndrome* (ATP III defined)			10 years CHD risk score (>20%)		
	P value	AUC	95% CI	P value	AUC	95% CI
	0.071	0.547	0.496 – 0.598	0.413	0.533	0.454 – 0.612

**Metabolic syndrome –fasting glucose was not measured in E-ECHOES samples instead T2DM status was included in the ATP III clinical identification of the metabolic syndrome*

Table 109 Von willebrand factor as a discriminator of metabolic and cardiovascular disease risk amongst South Asian men and women asymptomatic for cardiovascular disease

vWF (IU/dL)	Raised blood pressure (>130 mmHg or 85 mmHg)			Body mass index (> 27kg/m^2)		
	P value	AUC	95% CI	P value	AUC	95% CI
	0.174	0.537	0.484 – 0.590	0.040	0.555	0.504 – 0.605
	Central obesity (≥ 90 cm in men and ≥ 80 cm in women)			Diabetes mellitus (HbA1c ≥ 6.5%)		
	P value	AUC	95% CI	P value	AUC	95% CI
	0.335	0.531	0.467 – 0.594	0.165	0.557	0.481 – 0.632
	Elevated measured triglycerides (≥ 1.7 mmol/l)			Low HDL cholesterol (<0.9 mmol/l in men & <1.0 mmol/l in women, non-smokers)		
	P value	AUC	95% CI	P value	AUC	95% CI
	0.807	0.493	0.440 – 0.547	0.389	0.524	0.471 – 0.577
	Metabolic syndrome* (ATP III defined)			10 years CHD risk score (>20%)		
	P value	AUC	95% CI	P value	AUC	95% CI
	0.050	0.551	0.500 – 0.602	<0.001	0.642	0.567 – 0.716

Asian men and women asymptomatic for cardiovascular disease

**Metabolic syndrome –fasting glucose was not measured in E-ECHOES samples instead T2DM status was included in the ATP III clinical identification of the metabolic syndrome*

6.1.3 The validity of biomarkers of thrombosis, haemostasis and endothelial dysfunction as discriminators of cardiovascular disease in South Asians with cardiovascular disease risk factors and existing cardiovascular disease

The performance of plasma biomarkers were then analysed in a larger cohort which consisted of South Asians with CVD risk factors and existing CVD along with South Asians asymptomatic for CVD. The characteristic of South Asian men and women are shown separately in Table 110 & Table 111.

A significant age difference was observed in South Asian men and women with subjects with CVD being older compared to subjects with CVD risk factors and subjects asymptomatic for CVD, both p<0.001. The prevalence of hypertension, diabetes mellitus, obesity and central obesity were higher amongst South Asian men and women with CVD risk factors and lowest

amongst subject with CVD compared to those asymptomatic for CVD, all p<0.05. The use of CVD medications were higher amongst South Asian men and women with CVD compared to those with CVD risk factors, all p<0.001.

Table 110 Characteristics of South Asian men

Characteristics	South Asians asymptomatic for CVD, n=276	South Asians with CVD risk factors, n = 294	Subjects with CVD, n = 132	P value
Age (years)*	55.0 (8.3)	59.0 (10.0)	65.0 (10.1)	<0.001
Current smokers, n (%)	39 (14.1)	52 (17.7)	25 (18.9)	0.369
Hypertension (>130 mmHg or 85 mmHg), n (%)	190 (69.0)	265 (90.1)	126 (96.0)	<0.001
Diabetes mellitus (HbA1c ≥ 6.5%), n (%)	33 (12.0)	181 (62.0)	64 (49.0)	<0.001
Obesity (BMI≥27 kg/m^2), n (%)	117(42.4)	169 (58.0)	67(51.0)	0.002
Increased waist Circumference (≥ 90 cm in men and ≥ 80 cm in women), n (%)	201 (73.0)	256 (87.1)	109 (83.0)	<0.001
Systolic blood pressure (mmHg)*	137.0 (16.1)	143.0 (19.0)	139.1 (20.3)	0.001
Diastolic blood pressure (mmHg)*	85.0 (21.0)	84.2 (21.0)	78.0 (11.3)	0.001
Waist circumference (cm)*	96.2 (11.1)	100.1 (12.0)	101.0 (13.0)	<0.001
Body mass index (kg/m2)*	27.0 (4.0)	28.1 (4.3)	28.0 (4.4)	<0.001
Past medical history				
Previous HF (%)	0	0	6 (4.5)	
Previous MI (%)	0	0	67 (51.0)	
Angina (%)	0	0	79 (60.0)	
Drug therapy (%)				
ACE inhibitors	0	118 (40.1)	62 (47.0)	<0.001
Beta-blockers	0	38 (13.0)	57 (43.2)	<0.001
Calcium blockers	0	82 (28.0)	37 (28.0)	<0.001
Diuretics	0	62 (21.1)	32 (24.2)	<0.001
Anti-platelet therapy	0	64 (22.0)	54 (41.0)	<0.001

*Parametric data reported as mean (SD), Statistical tests - One-way anova & n (%)- chi square test.

Table 111 Characteristics of South Asian women

Characteristics	South Asians asymptomatic for CVD, n =229	South Asians with CVD risk factors, n ==225	Subjects with CVD, n=66	P value
Age (years)*	54.3 (7.5)	58.2 (8.8)	62.0 (9.0)	<0.001
Current smokers, n (%)	4 (1.7)	3 (1.3)	1 (1.5)	0.938
Hypertension (>130 mmHg or 85 mmHg), n (%)	139 (61.0)	201 (89.3)	61 (92.4)	<0.001
Diabetes mellitus (HbA1c ≥ 6.5%), n (%)	30 (13.1)	103 (46.0)	37 (56.1)	<0.001
Obesity (BMI≥27 kg/m^2), n (%)	139 (61.0)	159 (71.0)	49 (74.2)	0.040
Increased waist Circumference (≥ 90 cm in men and ≥ 80 cm in women), n (%)	199 (87.3)	213 (95.0)	64 (99.0)	0.002
Systolic blood pressure (mmHg)*	135.4 (20.1)	144.0 (19.0)	140.1 (23.0)	<0.001
Diastolic blood pressure (mmHg)*	83.0 (23.0)	83.0 (11.0)	77.3 (10.2)	0.061
Waist circumference (cm)*	93.0 (12.3)	97.0 (14.0)	100.2 (14.1)	<0.001
Body mass index (kg/m2)*	29.0 (6.5)	30.1 (5.4)	30.3 (5.3)	0.042
Past medical history				
Previous HF (%)	0	0	6 (9.1)	
Previous MI (%)	0	0	24 (36.4)	
Angina (%)	0	0	36 (56.1)	
Drug therapy (%)				
ACE inhibitors	0	61 (27.1)	24 (36.4)	<0.001
Beta-blockers	0	35 (16.0)	25 (38.0)	<0.001
Calcium blockers	0	45 (20.0)	19 (29.0)	<0.001
Diuretics	0	67 (30.0)	18 (27.3)	<0.001
Anti-platelet therapy	0	28 (12.4)	25 (38.0)	<0.001

*Parametric data reported as mean (SD), Statistical tests - One-way anova & n (%)- chi square test.

The distribution of lipids, biochemical measures and plasma biomarkers are shown in Table 112 & 113. Levels of ApoB, total cholesterol and LDL-c were higher in South Asian men and women asymptomatic for CVD compared to those with modifiable CVD risk factors and existing CVD, all p<0.05. In women alone, HDL-c was significantly higher in subjects asymptomatic for CVD compared to those with CVD risk factors and existing CVD, p =0.012. Levels of liver enzymes, ALT and GGT were significantly higher in South Asian men with CVD risk factors compared to those asymptomatic for CVD and existing CVD, both p<0.05. Creatinine levels were higher in

South Asian men with CVD compared to those asymptomatic for CVD and with CVD risk factors, p<0.001.

With regards to plasma biomarkers, levels of D-dimer were significantly higher in South Asian men with existing CVD compared to those asymptomatic for CVD and those with CVD risk factors, both p<0.05. In South Asian women, all plasma biomarkers were comparable in subjects asymptomatic for CVD, those with CVD risk factors and those with existing CVD.

Table 112 Distribution of lipids, biochemical measures and plasma biomarkers in South Asian men

Characteristics	South Asians asymptomatic for CVD, n=276	South Asians with CVD risk factors, n = 294	Subjects with CVD, n = 132	P value
Apolipoprotein AI (g/l)[+]	1.24 (1.21 – 1.28)	1.25 (1.22 – 1.29)	1.20 (1.14 - 1.30)	0.070
Apolipoprotein B (g/l)[*]	0.80 (0.3)	0.70 (0.3)	0.60 (0.2)	<0.001
Serum cholesterol (mmol/l)[*]	4.70 (1.3)	4.20 (1.3)	3.71 (1.20)	<0.001
HDL cholesterol (mmol/l)[*]	0.99 (0.3)	0.99 (0.3)	0.97 (0.3)	0.706
LDL cholesterol (mmol/l)[*]	2.70 (0.9)	2.20 (0.9)	1.84 (0.7)	<0.001
Triglycerides (mmol/l)[+]	2.50 (2.28 – 2.71)	2.44 (2.27-2.62)	2.30 (2.02 – 2.58)	0.196
Alkaline phosphatase (U/L)[+]	73.5 (71.0 – 76.4)	71.4 (69.0 – 74.3)	70.0 (65.3 – 74.0)	0.126
Alanine transaminase (U/L)[+]	25.0 (23.2 – 26.3)	27.0 (25.0-29.0)	23.0 (20.0-25.1)	0.003
Aspartate transaminase (U/L)[+]	22.0 (20.4-23.0)	23.0 (21.4-24.1)	22.0 (20.0 -24.0)	0.299
Creatinine (µmol/l)[+]	69.4 (57.1 – 78.0)	69.0 (59.0 -79.0)	76.0 (63.0 – 90.0)	<0.001
Gamma glutamyl transpeptidase (U/L)[+]	32.2 (30.0 -35.0)	42.2 (38.0-47.0)	40.0 (29.0-45.0)	0.002
D-dimer (µg/ml)	0.23 (0.21 -0.26)	0.33 (0.26 -0.40)	0.40 (0.30 -0.52)	0.006
P-selectin (ng/ml)	34.0 (32.0 -36.1)	34.0 (31.2-36.1)	32.0 (29.3 -35.0)	0.825
Von willebrand factor (IU/dL)	79.4 (15.4)	79.0 (21.4)	81.2 (15.3)	0.409

[*]Parametric data reported as mean (SD) – One way anova, [+] Non-parametric data reported as median (IQR)-Kruskal-Wallis test

Table 113 Distribution of lipids, biochemical measures and plasma biomarkers in South Asian women

Characteristics	South Asians asymptomatic for CVD, n =229	South Asians with CVD risk factors, n ==225	Subjects with CVD, n=66	P value
Apolipoprotein AI (g/l)[+]	1.34 (1.30-1.40)	1.30 (1.25-1.40)	1.30 (1.20 - 1.34)	0.060
Apolipoprotein B (g/l)[*]	0.76 (0.25)	0.67 (0.25)	0.60 (0.22)	<0.001
Serum cholesterol (mmol/l)[*]	4.80 (1.30)	4.10 (1.23)	4.00 (1.30)	<0.001
HDL cholesterol (mmol/l)[*]	1.20 (0.40)	1.10 (0.40)	1.04 (0.40)	0.012
LDL cholesterol (mmol/l)[*]	2.70 (0.90)	2.14 (0.82)	1.95 (0.80)	<0.001
Triglycerides (mmol/l)[+]	2.13 (1.98 -2.2.9)	2.22 (2.04-2.41)	2.34 (1.98-2.70)	0.511
Alkaline phosphatase (U/L)[+]	77.0 (73.2 -80.2)	78.0 (74.2 -81.3)	78.0 (70.0 -85.3)	0.875
Alanine transaminase (U/L)[+]	19.0 (17.2 -20.3)	18.0 (17.0 -19.0)	21.0 (17.2 -25.0)	0.868
Aspartate transaminase (U/L)[+]	20.0 (19.0 -21.0)	19.0 (17.5 -20.0)	21.0 (18.0 -24.2)	0.139
Creatinine (µmol/l)[+]	52.0 (46.0 -61.0)	53.0 (43.0 -63.0)	55.0 (48.3 -70.0)	0.125
Gamma glutamyl transpeptidase (U/L)[+]	25.2 (22.0 -29.0)	25.0 (22.0 -28.0)	37.1 (22.1 -52.3)	0.243
D-dimer (µg/ml)	0.40 (0.33 – 0.50)	0.40 (0.30 – 0.44)	0.40 (0.26 -0.55)	0.815
P-selectin (ng/ml)	34.0 (32.0 – 36.4)	33.0 (31.0 -35.0)	31.0 (28.0 -34.3)	0.716
Von willebrand factor (IU/dL)	77.0 (15.0)	80.0 (22.0)	80.0 (16.2)	0.138

[*]Parametric data reported as mean (SD) – One way anova, [+] Non-parametric data reported as median (IQR)-Kruskal-Wallis test

6.1.3.1 The association between D-dimer, P-selectin, von willebrand factor and cardiovascular risk factors in South Asians

Correlation analysis was performed to determine the clinical correlates of D-dimer, P selectin and vWF in South Asians.

On correlation analysis, D-dimer was positively associated with age, gender, CRP, vWF and negatively with red blood cell count, haemoglobin, ALT, GGT and triglycerides, all $p<0.05$. Correlation analysis for D-dimer in South Asians is shown in table 114.

Table 114 Correlation analysis for D-dimer in South Asians

Variables	South Asians , total n =1222 (South Asians asymptomatic for CVD =505, South Asians with modifiable CVD risk factors =519, South Asians with existing CVD =198	
	Spearman's correlation coefficient, r	P value
Age (years)	0.159	<0.001
Gender	0.162	<0.001
High sensitivity C-reactive protein (mg/L)	0.142	<0.001
Red blood cell count	-0.173	<0.001
Haemoglobin (g/dL)	-0.168	<0.001
Alanine transaminase (U/L)	-0.159	<0.001
Gamma glutamyl transpeptidase (U/L)	-0.084	0.005
Triglycerides (mmol/l)	-0.113	<0.001
Von willebrand factor (IU/dL)	0.090	0.002

Variables included in the analysis were waist circumference, BMI, systolic blood pressure, diastolic blood pressure, white blood cell count, HbA1c, alkaline phosphatase, AST, gamma glutamyl transpeptidase, creatnine, ApoAI, ApoB, total cholesterol, LDL-c, HDL-c, triglycerides and P selectin

P-selectin was positively associated with waist circumference, diastolic blood pressure, CRP, white blood cell count, red blood cell count, haemoglobin, alkaline phosphatase, ALT, ApoB, AST, total cholesterol, GGT, creatinine, LDL-c and triglycerides, all $p<0.05$. Correlation analysis for P-selectin in South Asians is shown in table 115.

Table 115 Correlation analysis for P selectin in South Asians

Variables	South Asians , total n =1222 (South Asians asymptomatic for CVD =505, South Asians with modifiable CVD risk factors =519, South Asians with existing CVD =198	
	Spearman's correlation coefficient, r	P value
Waist circumference (cm)	0.060	0.040
Diastolic blood pressure (mmHg)	0.086	0.003
High sensitivity C-reactive protein (mg/L)	0.082	0.005
White blood cell count	0.145	<0.001
Red blood cell count	0.080	0.035
Haemoglobin (g/dL)	0.094	0.012
Alkaline phosphatase (U/L)	0.155	<0.001
Alanine transaminase (U/L)	0.078	0.009
Apolipoprotein B (g/dL)	0.111	<0.001

Aspartate transaminase (U/L)	0.087	0.003
Total cholesterol (mmol/l)	0.117	<0.001
Gamma glutamyl transpeptidase (U/L)	0.106	<0.001
Creatnine	0.072	0.015
LDL cholesterol (mmol/l)	0.085	0.004
Triglycerides (mmol/l)	0.138	<0.001

Variables included in the analysis were age, gender,BMI, systolic blood pressure, HbA1c, ApoAI, ApoB, HDL-c and vWF.

vWF levels were positively associated with age, systolic blood pressure, CRP, alkaline phosphatase, creatinine and D-dimer, all $p<0.05$. Correlation analysis for vWF in South Asians is shown in table 116.

Table 116 Correlation analysis for von willebrand factor in South Asians

Variables	South Asians , total n =1222 (South Asians asymptomatic for CVD =505, South Asians with modifiable CVD risk factors =519, South Asians with existing CVD =198	
	Pearson's correlation coefficient, r	P value
Age (years)	0.210	<0.001
Systolic blood pressure (mmHg)	0.062	0.033
	Spearman's correlation coefficient, r	P value
High sensitivity C-reactive protein (mg/L)	0.097	0.001
Alkaline phosphatase (U/L)	0.078	0.009
Creatnine (µmol/l)	0.163	<0.001
D-dimer (µg/ml)	0.090	0.002

Variables included in the analysis were gender, waist circumference, BMI, diastolic blood pressure, Red blood cell count, white blood cell count, HbA1c, hemoglobin, ALT, AST, GGT, ApoAI, ApoB, total cholesterol, LDL-c, HDL-c, triglycerides and P selectin

6.1.3.2 Clinical correlates of D-dimer, P selectin and von willebrand factor in South Asians: multiple regression models

Multiple regression models were developed with variables associated with D-dimer, P selectin and vWF respectively. The models enabled to determine clinical correlates that independently contributed to the variation in the above mentioned plasma biomarkers.

On multiple regression models, red blood cell count, CRP, age and triglycerides explained 7% of the variation in D-dimer levels in South Asians. Table 117 shows the clinical correlates of D-dimer in South Asians.

Table 117 Clinical correlates of D-dimer in South Asians: multiple linear regression model

Predictor variables	Beta coefficients	95% Confidence Interval	P value	Adjusted R^2 (simple linear regression)
Red blood cell count	-0.084	-0.128 - -0.040	<0.001	0.020
CRP (mg/L)	0.011	0.002 – 0.019	0.012	0.003
Age (years)	0.003	0.001 – 0.006	0.020	0.023
Triglycerides (mmol/l)	-0.018	-0.035 - -0.001	0.043	0.003

Overall adjusted R^2 for the model -0.072
Variables included in the model- age, gender, CRP, red blood cell count, haemoglobin, alanine transaminase, GGT, BNP, vWF and triglycerides

Total cholesterol, white blood cell count, creatinine and BNP explained 6% of the variations in P-selectin in South Asians. Table 118 shows the clinical correlates of P- selectin in South Asians.

Table 118 Clinical correlates of P-selectin in South Asians: multiple linear regression model

Predictor variables	Beta coefficients	95% Confidence Interval	P value	Adjusted R^2 (simple linear regression)
Total cholesterol (mmol/l)	1.763	0.545 – 2.981	0.005	0.011
White blood cell count	1.277	0.350 – 2.205	0.007	0.009
Creatinine (µmol/l)	0.116	0.033 – 0.199	0.006	0.006
BNP (pmol/L)	-0.115	-0.226 - -0.004	0.042	0.003

Overall adjusted R^2 for the model -0.056
Variables included in the model- age, gender, waist circumference, diastolic blood pressure, CRP, white blood cell count, red blood cell count, haemoglobin, alkaline phosphatase, ALT, AST, ApoB, total cholesterol, creatinine, GGT, LDL-c BNP and triglycerides

Age, alkaline phosphatase and creatinine explained 7% of the variation in vWF levels in South Asians. Table 119 shows the clinical correlates of von willebrand factor in South Asians.

Table 119 Clinical correlates of von willebrand factor in South Asians: multiple linear regression model

Predictor variables	Beta coefficients	95% Confidence Interval	P value	Adjusted R^2 (simple linear regression)
Age (years)	0.365	0.221 – 0.508	<0.001	0.043
Alkaline phosphatase (U/L)	0.111	0.059 – 0.163	<0.001	0.013
Creatinine (µmol/l)	0.073	0.016 – 0.129	0.012	0.015

Overall adjusted R^2 for the model -0.065
Variables included in the model- age, systolic blood pressure, CRP or alkaline phosphatase, creatinine, BNP and D-dimer

6.1.3.3 The performance of D-dimer, P selectin and von willebrand factor in the prediction of cardiovascular risk in South Asians

ROC analysis was done to evaluate the performance of D-dimer, P selectin and vWF as discriminators of CVD risk in South Asians i) South Asians asymptomatic for CVD ii) South Asians with CVD risk factors and iii) South Asians with existing CVD.

On ROC analysis, vWF significantly discriminated South Asians with existing CVD from South Asians asymptomatic for CVD, AUC 0.555 (95% CI, 0.507 – 0.603), p =0.030. D-dimer and P-selectin did not discriminate South Asians with CVD from those asymptomatic for CVD. Table 120 shows plasma markers as discriminators of CVD in a cohort which consisted of South Asians with existing CVD (n=198) versus South Asians asymptomatic for CVD, (n=506).

Table 120 Plasma biomarkers as discriminators of CVD in a cohort which consisted of South Asians with existing CVD (n=198) versus South Asians asymptomatic for CVD, (n=506)

Biomarkers	P value	AUC	95% CI
vWF (IU/dL)	0.030	0.555	0.507-0.603
D-dimer (µg/ml)	0.066	0.545	0.498-0.592
P-selectin (ng/ml)	0.854	0.495	0.447-0.544

vWF, D-dimer and P-selectin did not discriminate South Asians with existing CVD and CVD risk factors (combined n=198 & n=519 respectively) from those asymptomatic for CVD, n =506.

Table 121 shows plasma markers as discriminators of CVD in a cohort which consisted of South Asians with existing CVD (n=198) & CVD risk factors (n=519) versus South Asians asymptomatic for CVD, (n=506).

Table 121 Plasma markers as discriminators of CVD in a cohort which consisted of South Asians with existing CVD (n=198) & CVD risk factors (n=519) versus South Asians asymptomatic for CVD, (n=506).

Biomarkers	P value	AUC	95% CI
vWF (IU/dL)	0.483	0.512	0.478 - 0.545
D-dimer (µg/ml)	0.199	0.522	0.489 -0.555
P-selectin (ng/ml)	0.689	0.507	0.474 -0.540

vWF, D-dimer and P-selectin did not discriminate South Asians with CVD risk factors, n=519 from those asymptomatic for CVD, n =506. Table 122 shows plasma markers as discriminators of CVD in a cohort which consisted of South Asians with CVD risk factors (n=519) versus South Asians asymptomatic for CVD, (n=506).

Table 122 Plasma markers as discriminators of CVD in a cohort which consisted of South Asians with CVD risk factors (n=519) versus South Asians asymptomatic for CVD, (n=506).

Biomarkers	P value	AUC	95% CI
vWF (IU/dL)	0.782	0.495	0.459 - 0.531
D-dimer (µg/ml)	0.483	0.513	0.477 -0.549
P-selecting (mg/ml)	0.559	0.511	0.475 -0.547

6.1.4 Discussion

In South Asians asymptomatic for CVD, D-dimer and vWF significantly discriminated South Asians with central obesity from healthy South Asians. Moreover, vWF discriminated South Asians with increased CVD risk from healthy South Asians.

Elevated levels of D-dimer reflect increased fibrinolytic activity and have been associated with varying degrees of atherosclerosis (Panchenko et al., 1995; Tataru et al., 1999). Previous studies have associated D-dimer and vWF with obesity. Ferguson et al., (1998) reported elevated levels of D-dimer in obese Black children whereby D-dimer was positively associated with percentage

body fat, subcutaneous abdominal adipose tissue and BMI. In the Framingham offspring study, BMI and waist-to-hip ratio were associated with haemostatic factors -fibrinogen, factor VII, plasminogen activator inhibitor antigen, tissue plasminogen activator and vWF (Rosito et al., 2004). Mills et al., (2008) reported in healthy young adults of diverse ethnic background that elevated levels of D-dimer were associated with higher BMI irrespective of ethnicity.

Pomp et al., (2007) reported a 2.4 fold increase in the risk of venous thrombosis in subjects with BMI >30 kg/m^2 compared to non-obese subjects. Obesity is an established risk factor for CVD (Hubert et al., 1983) and its effect is mediated through enhanced inflammation (Visser et al., 1999), platelet activation (Anfossi et al., 2009; Anfossi et al., 2010), increased levels of coagulation factors (Rosito, 2004), impaired fibrinolysis (Lijnen, 2009) and endothelial dysfunction (Perticone et al., 2001). In our cohort, the high prevalence of obesity (51.0%) and increased waist circumference (79.4%) amongst South Asians asymptomatic for CVD along with elevated levels of D-dimer and vWF reflect the adverse risk profile associated with body fat in South Asians.

Adhesion molecule, P-selectin significantly discriminated South Asians with elevated triglycerides from healthy subjects. Kavazarakis et al., (2002) demonstrated in healthy children that elevated levels of triglycerides increased the risk of endothelial dysfunction and reported an association between triglycerides and P-selectin levels. Broijersen et al., (1998) demonstrated in normolipidemic subjects after a fatty meal, postprandial increase in triglycerides sensitized platelets resulting in enhanced platelet P-selectin expression.

Adhesion molecules are involved in the early stages of atherosclerosis and up regulation of these molecules in the endothelium reflect platelet activation and endothelial dysfunction (Varughese et al., 2007). Elevated levels of P-selectin have been associated with worse CVD outcomes and

recurrent CV events in subjects with MI (Blann et al., 1997; Ridker, 2001b). In the ASCOT trial, P-selectin was a significant predictor of MI in hypertensive subjects (Varughese, 2007). In our cohort elevated levels of P-selectin in asymptomatic South Asians with abnormal triglycerides suggest an adverse risk for the development of CVD.

Of all the plasma biomarkers measured in this cohort, vWF alone significantly discriminated South Asians with existing CVD from those asymptomatic for CVD. Elevated levels of vWF have been associated with incident CVD (Wennberg, 2011).

The main limitations of the study are the cross sectional study design, generalization of the South Asian ethnic groups and inclusion of subjects above the age of 45 years. Hence our finding might not be applicable to individual ethnic groups and to young South Asians. Bloods were obtained for the measurement of plasma biomarkers at a single point of time and hence the diurnal variability of plasma biomarkers was not addressed. The strength of the study was the ability to determine the performance of plasma biomarkers of CVD in South Asians asymptomatic for CVD who were not under the influence of any medication for modifiable CVD risk factors.

6.1.4.1 Conclusion

The performance of D-dimer, P-selectin and vWF as biomarkers of CVD risk in South Asians was modest, with only vWF discriminating South Asians with increased risk of CVD and existing CVD.

6.2 The performance of brain natriuretic peptide as a discriminator of left ventricular systolic dysfunction in South Asians in the UK

6.2.1 Aims and hypothesis

Left ventricular systolic dysfunction if left untreated can lead to chronic heart failure which has poor outcomes. Early identification of asymptomatic LVSD enables clinicians in the prevention and better management of heart failure. BNP has shown to be a useful marker for the screening of LVSD in the general population. South Asians have an increased risk of CVD which might predispose this ethnic group to LVSD but the performance of BNP as a discriminator of LVSD has not been explored in this ethnic group. The aim of this study was to evaluate the discriminative value for BNP in the identification of South Asians with LVSD defined as LVEF <50%.

6.2.2 The performance of brain natriuretic peptide as a discriminator of left ventricular systolic dysfunction is South Asians in the UK

6.2.2.1 Characteristics of the study population

Of 638 South Asian subjects, 629 subjects had normal LV function and 9 had LVSD defined as LVEF <50%. The prevalence of LVSD defined as LVEF < 50% in our study cohort was 1.4%. South Asian patients with LVSD were much older compared to those with normal LV function, 68 years vs. 57.4 years, p=0.001. BMI, waist circumference, hypertension were comparable in South Asians with normal LV function and in those with LVSD. Pulse pressure were higher in South Asian patients with LVSD compared to those with normal LV function, p=0.007. The

prevalence of HF, MI, angina and diabetes were higher amongst South Asian patients with LVSD compared to those with normal LV function, all p<0.05. Compared to South Asians with normal LV function, those with LVSD were significantly on ACE inhibitors, 19.1% vs. 68.0%, p=0.003. Differences in demographic details between South Asians with normal LV function and those with LVSD are shown in Table 123.

Table 123 Differences in demographic details between South Asians with normal LV function and those with LVSD

Characteristics	South Asians with normal LV function (LVEF >50%) n=629	South Asians with LVSD (defined as LVEF <50%) n =9	P value
Mean age (years)	57.4 (9.2)	68.0 (11.0)	0.001
Male (%)	350 (56.0)	6 (67.0)	0.379
Current smokers (%)	52 (8.3)	0 (0.0)	0.463
Body mass index (kg/m^2)	28.1 (5.0)	26.2 (3.2)	0.246
Waist circumference (cm)	97.0 (12.0)	97.0 (7.0)	0.965
Systolic blood pressure (mmHg)	140.0 (19.1)	150.1 (38.2)	0.125
Diastolic blood pressure (mmHg)	82.1 (11.1)	78.2 (14.0)	0.311
Pulse pressure (mmHg)	58.0 (15.0)	72.0 (33.0)	0.007
Obesity (kg/m^2), (%)	357 (57.0)	3 (33.3)	0.143
Increased waist circumference (cm) (%)	539 (86.0)	9 (100)	0.252
Past medical history			
Previous HF (%)	3 (0.5)	1 (11.1)	0.055
Previous MI (%)	41 (6.5)	3 (33.3)	0.019
Angina (%)	48 (8.0)	4 (44.4)	0.004
Hypertension (%)	491 (78.1)	9 (100)	0.110
Diabetes (%)	216 (34.3)	6 (67.0)	0.051
Drug therapy (%)			
ACE inhibitors	120 (19.1)	6 (68.0)	0.003
Beta-blockers	73 (12.0)	3 (33.3)	0.080
Calcium blockers	85 (14.0)	2 (22.2)	0.353
Diuretics	84 (13.4)	2 (22.2)	0.348
Anti-platelet therapy	56 (10.0)	2 (22.2)	0.193

*Parametric data reported as mean (SD) – Independent T-test & n (%)-chi-square test.

Differences in haematological and lipid profiles between South Asians with normal LV function and those with LVSD are shown in Table 124. Levels of ApoAI, ApoB, total cholesterol, LDL-c, HDL-c, triglycerides, haemoglobin, MCV and creatinine were comparable in both groups.

Table 124 Differences in haematological and lipid profiles between South Asians with normal LV function and those with LVSD

Characteristics	South Asians with normal LV function (LVEF >50%) n=601	South Asians with LVSD (defined as LVEF <50%) n =9	P value
Apolipoprotein AI (g/dL)[+]	1.27 (1.11 – 1.46)	1.30 (1.20 – 1.34)	0.858
Apolipoprotein B (g/dL)[*]	0.70 (0.2)	0.65 (0.2)	0.653
Total cholesterol (mmol/l)[*]	4.40 (1.4)	4.00 (0.7)	0.333
LDL cholesterol (mmol/l)[*]	2.34 (1.0)	2.01 (0.5)	0.303
HDL cholesterol (mmol/l)[*]	1.04 (0.4)	1.10 (0.3)	0.729
Triglycerides (mmol/l)[+]	1.98 (1.46 – 2.94)	1.90 (1.25 – 2.61)	0.525
Haemoglobin (g/dL)[*]	14.0 (2.0)	14.3 (1.0)	0.617
Mean corpuscular volume (fL)[*]	87.4 (6.5)	83.3 (2.3)	0.275
Creatinine (µmol/l)[*]	65.2 (25.0)	68.2 (19.0)	0.715

[*]Parametric data reported as mean (SD) –Independent T-test, [+] Non-parametric data reported as median (IQR)- Mann-Whitney test
Haematological factors were available only for 380 subjects (Subjects with LVSD=3, without LVSD=377)

6.2.2.2 Brain natriuretic peptide and left ventricular systolic dysfunction

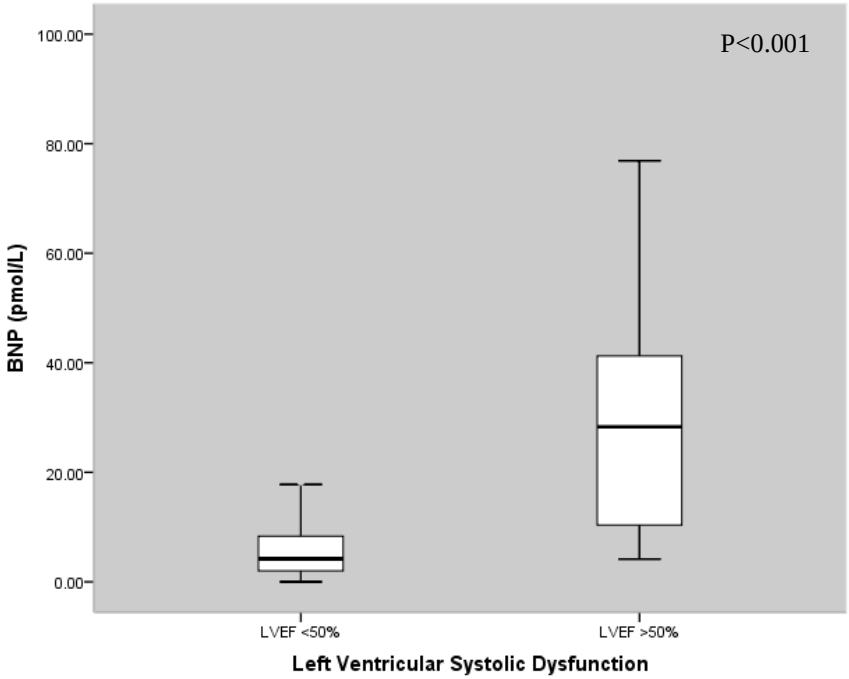
BNP levels were determined in South Asians with LVSD and in those with normal LV function. Levels of BNP were significantly higher in South Asian patients with LVSD compared to those with normal LV function, median (IQR), 3.93 pmol/l (2.00 -8.12) vs. 28.30 pmol/l (9.30 -45.10), p<0.001. Figure 39 shows levels of BNP in South Asians with normal LV function and those with LVSD.

Table 125 Levels of brain natriuretic peptide in South Asians with and without left ventricular systolic dysfunction in South Asians

	South Asians without LVSD (defined as LVEF >50%), n=629	South Asians with LVSD (LVEF < 50%), n=9	P value
Brain natriuretic peptides (pmol/l)	3.93 (2.00 – 8.12)	28.30 (9.30 – 45.10)	<0.001

Reported as median (IQR) –Mann-Whitney test

figure 39 Levels of brain natriuretic peptides in South Asians with normal LV function and those with left ventricular systolic dysfunction



6.2.2.3 Correlation and multiple regression analysis for brain natriuretic peptide in South Asians

Correlation analysis was performed to determine clinical correlates of BNP in South Asians. Multiple regression models were then developed with variables associated with BNP in order to determine independent predictors of BNP levels in South Asians.

BNP was positively associated with age (r=0.308), pulse pressure (r=0.218), HbA1c (r=0.115), vWF (r=0.122), D-dimer (r=0.138), and negatively associated with diastolic blood pressure (r=-0.161), red blood cell count (r=-0.275), haemoglobin (r=-0.317), ApoB (r=-0.105), cholesterol (r=-0.123), LDL-c (r=-0.101), triglycerides (r=-0.162) and P-selectin (r=-0.119). Correlation analyses for BNP in South Asians are shown in table 126.

Table 126 Correlation analyses for brain natriuretic peptides in South Asians

Variables	South Asian men	
	Spearman's correlation coefficient, r	P value
Age (years)	0.308	<0.001
Diastolic blood pressure (mmHg)	-0.161	<0.001
Pulse pressure (mmHg)	0.218	<0.001
HbA1c (%)	0.115	0.005
Red blood cell count	-0.275	<0.001
Haemoglobin (g/dL)	-0.317	<0.001
Apolipoprotein B (g/dL)	-0.105	0.009
Total Cholesterol (mmol/l)	-0.123	0.002
LDL cholesterol (mmol/l)	-0.101	0.012
Triglycerides (mmol/l)	-0.162	<0.001
vWF (IU/dL)	0.122	0.002
D-dimer (µg/ml)	0.138	0.001
P selectin (ng/ml)	-0.119	0.003

Other variables in the analysis- waist circumference, BMI, systolic blood pressure, CRP, white blood cell count, MCV, ApoAI, HDL-c, creatnine, LVEF

On multiple linear regression, age, haemoglobin, pulse pressure, D-dimer and triglycerides explained 14.5% of the variations in BNP levels in South Asians. Table 127 shows the clinical correlates of brain natriuretic peptides in South Asians.

Table 127 Clinical correlates of brain natriuretic peptides in South Asians: multiple regression model

Predictor variables	Beta coefficients	95% Confidence Interval	P value	Adjusted R^2 (simple linear regression)
Age (years)	0.146	0.031 -0.261	0.013	0.093
Haemoglobin (g/dL)	-0.802	-1.346 - -0.257	0.004	0.050
Pulse pressure (mmHg)	0.112	0.047 – 0.177	0.001	0.070
D-dimer (µg/ml)	4.103	0.831 – 7.376	0.014	0.020
Triglycerides (mmol/l)	-0.765	-1.378 - -0.153	0.014	0.030

Overall adjusted R^2= 0.145

Variables included in the model-gender, diastolic blood pressure, HbA1c, Red blood cell count, ApoB/total cholesterol/ LDL cholesterol, P-selectin

BNP were not associated with LVEF (r=-0.072, p=0.068) but was associated with pulse pressure (r = 0.281, p<0.001). Relationship between BNP and ejection fractions is shown in figure 40. Figure 41 shows the relationship between BNP and pulse pressure.

figure 40 *Relationship between brain natriuretic peptide and left ventricular ejection fraction in South Asians*

Simple linear regression

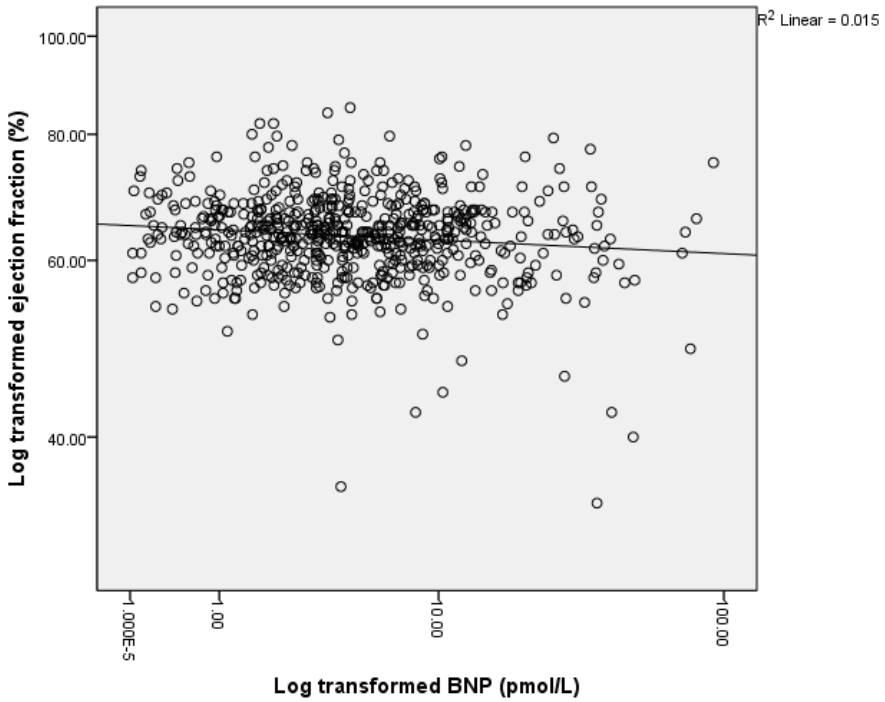
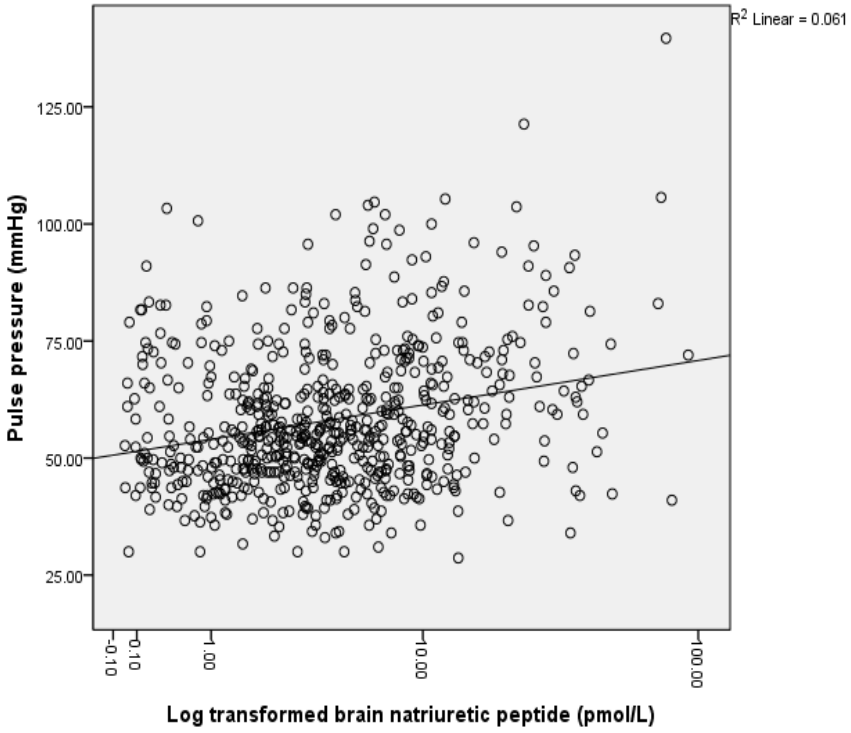


figure 41 *Relationship between brain natriuretic peptide and pulse pressure in South Asians*

Simple linear regression



6.2.2.4 Brain natriuretic peptide and haemoglobin levels

On correlation analysis, BNP were negatively associated with haemoglobin levels (r=-0.317, p<0.001). Figure 42 shows the relationship between natriuretic peptide and haemoglobin levels. Levels of BNP were highest in subjects with lowest haemoglobin levels and vice versa. Levels of brain natriuretic peptide across haemoglobin quartiles are shown in Table 128.

figure 42 Relationship between brain natriuretic peptide and haemoglobin levels

Simple linear regression

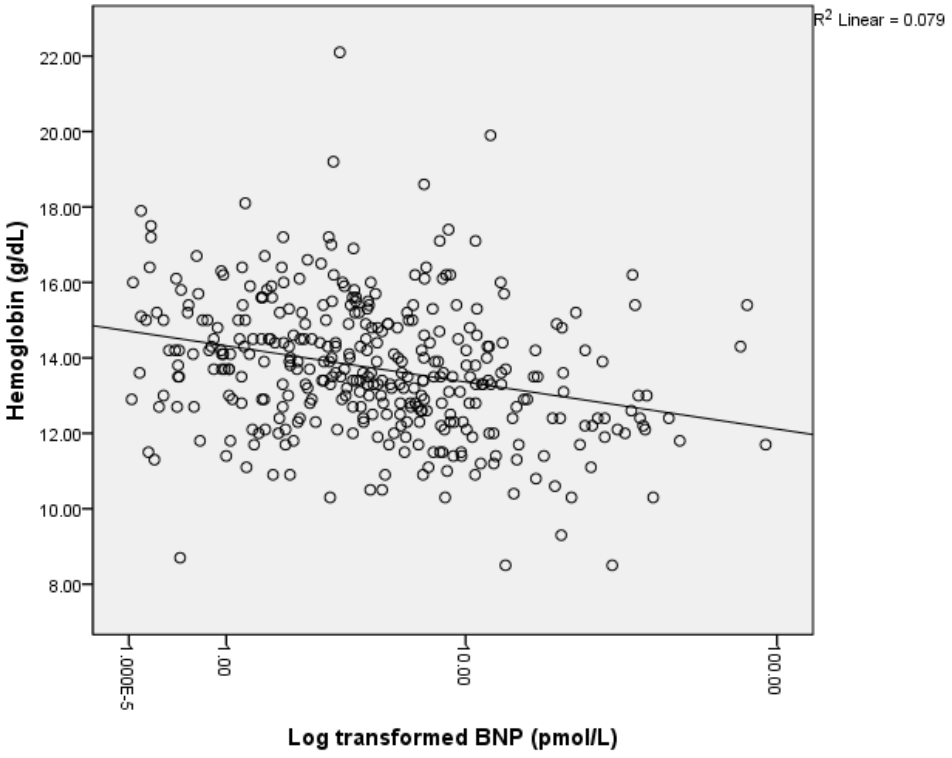


Table 128 Levels of brain natriuretic peptide across haemoglobin quartiles

Haemoglobin quartiles	Haemoglobin levels			
	<12.57g/Dl (n=94)	12.58 – 13.63 g/dL (n=97)	13.64 -14.92 g/dL (n=96)	>14.93 g/dL (n=93)
BNP (pmol/l)	8.40 (3.80-17.42)	5.02 (2.60-8.34)	3.10 (1.30-6.80)	2.99 (1.06-6.29)

Kruskal-wallis test, p value <0.001
Haemoglobin levels were available only 380 subjects

6.2.2.5 The performance of brain natriuretic peptide as a discriminator of left ventricular dysfunction in South Asians in the UK

The purpose of this section was to evaluate the performance of BNP as a discriminator of LVSD in South Asians. The discriminative measure was assessed by sensitivity and specificity and the area under the ROC curves.

On ROC analysis, BNP significantly discriminated South Asians with LVSD from those with normal LV function, AUC -0.876 (95% CI, 0.777 – 0.976), p<0.001. Table 129 shows brain natriuretic peptide as a discriminator of left ventricular systolic dysfunction in South Asians.

Table 129 Brain natriuretic peptide as a discriminator of left ventricular systolic dysfunction in South Asians

	Left ventricular systolic dysfunction (defined as LVEF < 50%), Positive n=9, negative =629		
Brain natriuretic peptide (pmol/l)	P value	AUC	95% CI
	<0.001	0.876	0.777 – 0.976
Cut off value	8.865 pmol/l or 30.8 pg/ml		

BNP concentrations were expressed in pmol/l, to convert to pg/ml levels were multiplied by 3.47

Since there were only 9 subjects with LVEF <50% in our study cohort which did not give us enough power for ROC analysis, 46 South Asian HF subjects from a previous study conducted at the Centre for Cardiovascular Sciences, City Hospital were added and the ROC analysis for BNP was repeated. These South Asian HF patients were recruited from the specialist heart failure clinics at City and Sandwell Hospitals. HF was defined as those with typical HF symptoms associated with LVSD (ejection fraction <40%) documented by echocardiography. The study was approved by the West Midlands research ethics committee and all patients gave written informed consent prior to participation (Sosin et al., 2008). The characteristics of these 46 South Asian HF patients are shown in Table 130.

Table 130 Characteristics of South Asian heart failure patients

Characteristics	South Asian heart failure patients (defined as LVEF <40%) n =46
Mean age (years)	62.0 (13.2)
Male (%)	31 (67.4)
Current smokers (%)	3 (6.5)
Systolic blood pressure (mmHg)	138.4 (26.0)
Diastolic blood pressure (mmHg)	82.4 (13.0)
Past medical history Previous MI (%) Angina (%) Hypertension (%) Diabetes (%)	 31 (67.4) 25 (54.3) 21 (45.7) 21 (45.7)
Drug therapy (%) ACE inhibitors Beta-blockers Diuretics	 39 (85.0) 24 (52.2) 29 (63.0)
Total cholesterol (mmol/l)*	4.60 (1.1)
HDL cholesterol (mmol/l)*	1.30 (0.3)
Triglycerides (mmol/l)+	1.53 (1.20 – 2.24)
Brain natriurectic peptide (pmol/l)	4.50 (2.14 – 10.0)

*Parametric data reported as mean (SD) – One sample T-test & n (%)-chi-square test.

On ROC analysis, BNP significantly discriminated South Asians with LVSD from those without, AUC -0.896 (95% CI, 0.852 – 0.940), p<0.001. Table 131 shows brain natriuretic peptide as a discriminator of left ventricular systolic dysfunction in South Asians. Table 132 shows the performance of BNP in identifying LVSD in South Asians.

Table 131 Brain natriuretic peptide as a discriminator of left ventricular systolic dysfunction in South Asians

	Left ventricular systolic dysfunction (defined as LVEF < 50%) Positive n= 55, negative = 629		
Brain natriuretic peptide (pmol/l)	P value	AUC	95% CI
	<0.001	0.896	0.852 – 0.940
Cut off value	10.5 pmol/l or 36.4 pg/ml		

BNP concentrations were expressed in pmol/l, to convert to pg/ml levels were multiplied by 3.47

Table 132 Performance of BNP in identifying LVSD in South Asians

Characteristics	South Asians with LVSD <50%
Sensitivity	80.0%
Specificity	82.8%
Positive predictive value	28.9%
Negative predictive value	97.9%

True positive=44, false positive=108
True negative=521, false negative=11

6.2.3 Discussion

The main finding of this study was that BNP significantly discriminated South Asians with LVSD from those with normal LV function with a discriminative value of 36.4 pg/ml in combined community based population and HF patients from a clinic setting with a high negative predictive value of 97.9%. Our findings add to the already existing evidence of high predictive value for natriuretic peptide in HF settings. There is no clear consensus on the best discriminative value for BNP in the identification of LVSD or assay method (Thygesen, 2011).The performance of Bayer ADVIA Centaur BNP assay used in this study is equivalent to other methods for determining BNP such as Triage BNP and ShionoRIA assay (Wu et al., 2004).

In the Framingham Heart study, BNP values of 45 pg/ml in men and 50 pg/ml in women) showed 95% specificity for LVSD (Vasan et al., 2002). While values of 100 pg/ml has shown to increase the diagnostic accuracy of BNP in the emergency diagnosis of HF (Maisel, 2002; Mueller et al., 2004). McDonagh (1998) reported BNP concentration of 17.9 pg/ml or more in the identification of LVSD in the general population. Our discriminative values were closer to the ones reported in the Framingham heart study.

The prevalence of LVSD in South Asians in our study cohort was 1.4% The prevalence rate for LVSD was similar to those reported in non-Asian populations, in the Echocardiographic Heart of England Screening study, the prevalence rate for LVSD was 1.8% (Davies, 2001), in the

Rotterdam study the prevalence for LVSD was 3.7% (Mosterd et al., 1999) and McDonagh et al., (1997) reported a prevalence rate of 2.9% for LVSD in an Urban UK population. In this cohort the prevalence of LVSD was mainly in those with concomitant diabetes or MI and this finding has been reported in previous studies (Lip, 1997; Galasko, 2005).

Even though there was a significant difference in the levels of BNP between South Asian with LVSD and those with normal LV function, BNP levels did overlap in both groups. This could be due to concomitant diuretics and angiotensin-converting enzyme inhibitor therapy which can lower BNP levels (Bhatia et al., 2006). This limits the usefulness of BNP as a screening biomarker for LVSD in South Asians already on cardiovascular medications.

In our South Asian cohort, BNP was associated with pulse pressure and not with LVEF. This suggests the classic pathway where BNP is secreted in response to volume and pressure overload in HF patients (Maisel, 2002). Pulse pressure was significantly higher in South Asians with LVSD compared to those with normal LV function. The visual assessment of LVSD provides valuable diagnostic and prognostic information to clinicians (Devereux et al., 1986; Watanabe et al., 2001). The lack of association between BNP and LVSD could be due to skewed distribution.

BNP was also shown to be associated with haemoglobin levels in South Asians. Anemia in HF patients is associated with poor outcomes and increased risk of mortality (Horwich et al., 2002; Ezekowitz et al., 2003). The relationship between anemia, HF and poor outcomes is not well understood but several mechanisms have been proposed. Enhanced inflammation in HF can affect bone marrow function and predispose HF patients to anemia (Levine et al., 1990; Iversen et al., 2002). The presence of anemia and reduced kidney function itself can predispose HF patients to worse outcomes (Al-Ahmad et al., 2001; Horwich, 2002)

Ralli et al., (2005) demonstrated an increased risk of mortality in HF when elevated levels of BNP were accompanied by anemia. Reduced hemoglobin levels suggest an increase in cardiac output secondary to a higher myocardial oxygen demand and this can lead to ventricular dilation and left ventricular hypertrophy (Ueno et al., 2008). In our cohort, hemoglobin levels were similar in South Asians with LVSD and in those with normal LV function but levels of BNP were highest in subjects with lowest hemoglobin levels and vice versa.

Possible mechanisms for the link between BNP and hemoglobin levels in South Asians could be independent of hemodynamic parameters and due to endothelin-1; a direct stimulator of BNP in HF. Elevated levels of endothelin-1 has been linked to anemia and hypoxia (Helfman and Falanga, 1993). Over expression of endothelin-1 in cardiocytes and smooth muscle cells lead to myocardial hypertrophy and cardiotoxicity (Sakai et al., 1996) and BNP can be an indicator of endothelin-1 induced myocardial injury (Nakagawa et al., 1995; Ralli, 2005). South Asian patients with LVSD had a higher prevalence of prior MI and diabetes mellitus which could lead to ischemia induced endothelin-1.

Another possible reason for the release of endothelin-1 in migrant South Asians could be the migration from low altitudes within the Indian sub-continent to high altitudes in the UK which could have resulted in high altitude induced hypoxia. Prior studies have shown that subjects who live permanently in a high altitude might have adapted to high altitude hypoxia and hence have lower pulmonary pressure, higher plasma concentration of nitric oxide compared to migrants (Erzurum et al., 2007). Elevated levels of endothelin-1 have also been reported in infectious diseases such as malaria (Basilico et al., 2002; Dietmann et al., 2008). Further studies are warranted to prove this relationship between anaemia and secretion of BNP in South Asians.

The limitations are the cross sectional design and relatively small numbers due to the lower prevalence of LVSD in South Asian community population. South Asians are a heterogeneous group and our study has reported Indians, Pakistanis and Bangladeshi as a combined group. Even though the study population was community based and was expected to have only asymptomatic subjects for LVSD, 4 subjects were known to have HF and only one subjects had LVEF less than 50%. In order to confirm the discriminative value obtained for BNP in the South Asian community samples we combined South Asian HF patients recruited from a clinical setting with South Asian recruited from General Practices who might be asymptomatic or have less severe disease. However the discriminative values for BNP in the identification of LVSD in the community population was close to the combined clinic and community populations.

6.2.3.1 Conclusion

BNP significantly discriminated South Asians with LVSD from those with normal LV function with a high negative predictive value.

CHAPTER 7 RESULTS 4

7.1 Haemoglobin abnormalities and the risk of cardiovascular disease in South Asians

7.1.1 Aims and hypothesis

Haemoglobinopathies such as beta thalassemia are blood born disorders that are particularly prevalent amongst populations originating from the Indian subcontinent. It is estimated that the carrier rate for beta thalassemia amongst South Asians are 1 in 20 (Lahiry et al., 2008). Bartels et al., (2006) demonstrated that there is accelerated erythropoiesis in subjects with beta thalassemia trait, evidence to support 'intravascular haemolysis – an increased release of iron from these red blood cells, which is implicated in the progression of CHD (Rother et al., 2005; Levy et al., 2007). However studies in non-Asian populations have shown beta thalassemia trait to confer a protective role against CVD (Crowley, 1987; Wang and Schilling, 1995). This is mainly considered due to its favourable impact on cholesterol metabolism (Shalev et al., 2007) whereby there is an increased uptake of LDL by the bone marrow to provide cholesterol for the production of red blood cells (Calandra et al., 2004). Less is known about the cardiovascular implications of haemoglobin abnormalities in South Asian populations. The aim of this case control study was to analyse haemoglobin abnormalities by ESI-MS and determine whether they were more common amongst South Asians with CVD than those without CVD.

7.1.1.1 Study population

The study population were from two main studies involving South Asian population in the department, namely E-ECHOES and NIASTAR trial.

7.1.1.2 Power calculation

The pilot study was designed to use groups of n=100 of controls and patients, where 67 subjects were needed for 80% power to be able to detect a 5% difference in rates of haemoglobin disorders at 5% alpha.

7.1.2 Haemoglobin abnormalities and the risk of cardiovascular disease in South Asians in the UK

7.1.2.1 Characteristics of the study cohort

A total of 156 South Asians were included in this pilot study, of whom 84 were control subjects (without any documented evidence of CVD) and 72 were CVD patients. The characteristics of South Asian cardiovascular patients and control subjects are shown in Table 133. The CVD patients were older, and comparable with control subjects for gender, BMI and blood pressure. Lipid lowering therapy was mainly statin therapy in controls, all CVD patients were on statin and anti-platelet therapy.

Table 133 Characteristics of South Asian cardiovascular disease patients and control subjects

Characteristics	Subjects without CVD (n= 84)	Subjects with CVD (n = 72)	P value
Age (years)*	55.5 (7.9)	62.6 (13.1)	<0.001
Male gender, n (%)	47 (60.3)	51 (71.8)	0.14
Body mass index (kg/m2)*	28.7 (7.1)	27.5 (4.4)	0.28
Ischaemic stroke, n (%)	0	72 (100)	-
Coronary heart disease, n (%)	0	18 (25.0)	-
Peripheral vascular disease, n (%)	0	2 (2.7)	-
Hypertension, n (%)	44 (52.4)	65 (90.1)	<0.05
Diabetes mellitus, n (%)	25 (29.8)	33 (45.8)	0.08
Systolic blood pressure (mmHg)*	140 (15)	145 (22)	0.09
Diastolic blood pressure (mmHg)*	91.0 (48.9)	81.0 (12.3)	0.10
Anti-platelet therapy, n (%)	14 (16.7)	72 (100)	<0.001
Lipid lowering therapy, n (%)	29 (34.5)	72 (100)	<0.001
Ever smoked, n (%)	84 (28.6)	19 (26.4)	0.55

*Parametric data reported as mean (SD)

With regards to lipids and lipoproteins, CVD patients had lower apolipoproteins and LDL-c compared to control subjects, all p<0.001 except HDL-c (NS). Table 134 shows the distribution of lipids and lipoproteins in South Asian cardiovascular disease patients and control subjects.

Table 134 Distribution of lipids and lipoproteins in South Asian cardiovascular disease patients and control subjects

Characteristics	Subjects without CVD (n= 84)	Subjects with CVD (n = 72)	P value
Apolipoprotein AI (g/l)*	1.44 (0.29)	1.20 (0.41)	<0.001
Apolipoprotein B (g/l)*	0.73 (0.22)	0.47 (0.16)	<0.001
Serum cholesterol (mmol/l)*	4.80 (1.08)	2.52 (3.08)	<0.001
HDL cholesterol (mmol/l)*	1.20 (0.40)	1.06 (0.49)	0.07
LDL cholesterol (mmol/l)*	2.56 (0.93)	1.58 (0.75)	<0.001

*Parametric data reported as mean (SD)

7.1.2.2 Haemoglobin abnormalities in South Asian cardiovascular disease patients and controls

On comparison of haematological factors, haemoglobin levels, red blood cell counts and MCV were comparable between CVD patients and control subjects. Haemoglobin abnormalities were significantly more common in CVD patients compared to control subjects, p=0.002. Elevation of delta chains within the haemoglobin was significantly more common amongst CVD patients compared to control subjects, p=0.005. Lipoprotein subfraction concentration of iron were significantly higher amongst CVD patients compared to control subjects, all p<0.05. Haematological factors and abnormalities of the haemoglobin in South Asian cardiovascular patients and controls are shown in Table 135.

Table 135 Haematological factors and abnormalities of the haemoglobin in South Asian cardiovascular disease patients and controls

	Without CVD (n=84)	With CVD (n=72)	P value
Haemoglobin (g/dL)+	13.6 (12.5-14.6)	13.7 (12.6-14.7)	0.72
Red blood cell count (×1000,000 cells/µl)+	5.01 (4.68-5.31)	4.93 (4.53-5.30)	0.40
Mean corpuscular volume (fL)+	86.8 (83.2-90.1)	87.1 (84.0-89.6)	0.85
Platelet count (×1000 cells/µl)+	292 (238-348)	256 (220-294)	0.009

All haemoglobin disorders, n (%)	12 (14.3)	25 (34.7)	0.002
Elevated delta chain level, n (%)	4 (4.8)	14 (19.4)	0.005
High levels of alpha/beta chain Glycation, n (%)	6 (7.1)	12 (16.7)	0.08
Beta chain variants, n (%)	2 (2.4)	0	-
VLDL iron	0.30 (0.00-0.90)	0.60 (0.20-1.10)	0.031
LDL iron	0.30 (0.00-0.80)	0.70 (0.30- 1.30)	0.01
HDL_3 iron	0.70 (0.00 – 1.10)	1.90 (1.25-2.80)	<0.001
HDL_2 iron	0.30 (0.00 – 1.00)	0.60 (0.35-1.15)	0.01

Data reported as median (IQR) –Mann-Whitney test, number (%)- chi-square test

Amongst CVD patients, carotid intima media thickness (CIMT) was higher and HDL c lower in those with elevated delta chain levels compared to CVD patients with no anomaly detected, p <0.05.

Differences in lipids, lipoproteins and carotid intima media thickness in South Asian cardiovascular disease patients by elevated delta chain levels are shown in Table 136.

Table 136 Differences in lipids, lipoproteins and carotid intima media thickness in South Asian cardiovascular disease patients by elevated delta chain levels

	Patient with no anomaly detected (n=47)	Patient with elevated delta chain levels (n=14)	P value
Age (years)*	60.7	63.9	0.46
Apolipoprotein AI (g/l)*	1.22 (0.42)	1.12 (0.44)	0.71
Apolipoprotein B (g/l)*	0.47 (0.15)	0.44 (0.21)	0.67
Serum cholesterol (mmol/l)*	2.41 (3.22)	3.21 (1.37)	0.21
HDL cholesterol (mmol/l)*	1.03 (0.37)	0.78 (0.23)	0.003
LDL cholesterol (mmol/l)*	1.61 (0.82)	1.51 (0.65)	0.66
Carotid intima media thickness (mm)*	0.65 (0.17)	0.75 (0.22)	0.008

*Parametric data reported as mean (SD)- Independent T-test, available only in CVD patients

On logistic regression across patients and controls combined, the presence of any Haemoglobin anomalies were independently associated with control vs. patient status (β = -1.7, p = 0.002) in a model that included age, gender, diabetes, haemoglobin concentration and LDL c.

7.1.3 Discussion

The main finding of this pilot study was that haemoglobin disorders are more common amongst South Asians with CVD and that this is associated with low levels of HDL-C. Of all the haemoglobin abnormalities, elevated delta chain appeared to be more common amongst South Asian CVD patients. Moreover, patients with elevated delta chain levels had greater vascular damage reflected by greater CIMT and lower HDL-c than those with normal haemoglobin. These preliminary findings, taken together with the observation of higher levels of iron measured within sub fractions of lipids amongst South Asian CVD patients suggest an interaction between haemoglobin disorders and lipid metabolism in South Asians that may contribute to a higher risk of CVD (Patel et al., 2012).

Despite lower levels of LDL and HDL particles amongst CVD patients, levels of iron in the lipid sub fractions of these patients were much higher than in controls. Camejo (1998) suggested a model for the interaction between hemin and apolipoproteins. There is a high affinity between the carboxylic groups of the hemin and positively charged segments of ApoB rich in lysine and arginine on the surface of LDL monolayer whereby the porphyrin ring is interacting with fatty acid chains of phospholipids and free cholesterol and the tetrapyrrole ring is embedded in the hydrophobic core of LDL. The alteration in the charge and structure of the LDL particle after hemin oxidation leads to increase uptake and degradation of oxidised LDL by macrophages, thus indicating a pathological significance. Similar interaction have been reported for HDL whereby proteins within its structure interact with iron binding protein such as hemopexin in sequestrating excess free heme in the circulation (Vaisar et al., 2007). In CVD patients, high levels of iron were observed in HDL sub fractions, it is still unclear whether this interaction is of physiological or pathological nature.

‘The majority of patients identified with abnormal haemoglobin in this study had normal blood profiles, and whilst low levels of mean corpuscular volume are reported amongst patients with elevated delta chain haemoglobin, such abnormalities were not observed here. The older age of our CVD patients could explain the higher levels of iron measured within lipoprotein sub fractions. Moreover, while a positive association between carotid intima media thickness (CIMT) with HDL cholesterol, and a negative association between HDL cholesterol with iron measured within HDL subfractions were observed amongst those with haemoglobin abnormalities, the study is underpowered to confirm such observations. Further research is needed amongst large cohorts assessing the cardiovascular impact of haemoglobin disorders in this and other populations’(Patel et al., 2012b).

7.1.3.1 Conclusion

Haemoglobin disorders particularly elevation of delta chain levels were more common amongst South Asians with CVD and was associated with greater vascular damage and lower levels of HDL-c. Furthermore, the higher levels of iron on sub fractions of lipids amongst South Asian CVD patients suggest an interaction between haemoglobin disorders and lipid metabolism which might contribute to a higher risk of CVD, further research is needed.

CHAPTER 8 GENERAL DISCUSSION

South Asians have an increased risk of CVD compared to the general population in the UK; the underlying pathophysiology for this is unclear. Traditional risk factors such as diabetes mellitus and triglycerides do reflect an increased risk of CVD in South Asians. CVD events in South Asians occur much earlier in life and are often accompanied by normal serum lipids and blood pressure measurements (Patel, 2012). Hence, the focus of this thesis was to meet the need to establish biomarkers that can identify South Asians at increased risk of CVD early.

To do this properly, a large prospective study of South Asians without CVD is required to look at how well various biomarkers predict incident disease. However the logistics for setting up such a study is difficult, for instance, the cost involved, the duration of such a study, the selection of biomarkers of interest, the constantly changing primary prevention strategies which might introduce confounding factors such as effect of intervention on biomarkers.

The study design for this thesis was cross-sectional and subjects were randomly selected from a large community based sample of South Asians recruited through population and hospital based studies. The limitations of the study are its cross-sectional nature and the generalization with the wider South Asian diaspora. Subjects were recruited for the E-ECHOES study on a consecutive basis, and as such were not age-gender matched nor case-control matched. The main findings of this thesis were as follows.

Performance of circulating biomarkers in South Asians

This thesis looked at cardiovascular biomarkers that have been extensively studied in European population but not in South Asian population and found that these biomarkers do operate in

South Asians, but maybe at a lower discriminative range for instance in the performance of CRP as a discriminator of CVD risk in South Asians. High levels of CRP are not fully explained by traditional CVD risk factors, suggesting the involvement of environmental or genetic factors (Albert, 2004). This could also be consistent with the hypothesis that people with ancestral origins in hostile tropical environments exhibit increased pro-inflammatory responses compared to those living in a temperate environment. This genetic selection is important for their defence mechanisms and survival in a pathogenic tropical environment. However, when migrating to temperate environment the balance between the benefit and the risk of these increased pro inflammatory responses is altered, resulting in an increased incidence of inflammatory related diseases (Le Souef et al., 2000).

Of all the biomarkers, CRP was a comprehensive marker of CVD risk where a range of 1.43-2.30 mg/L maximised sensitivity and specificity. This level is lower than that seen in White populations. This was established in South Asian cohorts asymptomatic for CVD, not on any prior medications such as statin therapy which can have an impact on CRP levels. Our findings were replicated in further two South Asian cohorts i) a homogeneous Indian community in the UK ii) a homogeneous Indian community in India. However, atherosclerosis is an inflammatory disease with a prolonged asymptomatic phase, and subjects in this study were not screened for the presence of subclinical atherosclerosis using imaging techniques. Another limitation is it has already been established that traditional CVD risk assessment underestimate CVD risk in South Asians but Framingham risk score was used to establish 10-year CVD risk in this study. Hence in order to validate the performance of all the blood biomarkers studied in this thesis in the prediction of incident disease in South Asians, a prospective longitudinal study needs to be

conducted. Again a major issue will be statin therapy as it is widely used both in primary and secondary settings in the management of abnormal lipid levels and prevention of CVD.

Non-fasting triglycerides was another marker that discriminated South Asians at increased risk of CVD in this thesis and can be used as an alternative for fasting triglyceride measurements in routine clinical practice. Fasting triglycerides are better discriminator of CVD risk in South Asian population compared to other lipid and lipoprotein measures (Patel, 2010). Due to the logistic issue in obtaining bloods in fasting state, fasting triglycerides are not often measured in primary settings. Recent studies have shown non-fasting triglycerides as an independent predictor of incident CVD compared to fasting levels of triglyceride (Jeppesen et al., 1995; Bansal et al., 2007; Nordestgaard et al., 2007). Our findings support the hypothesis that atherosclerosis is a postprandial phenomenon and this needs to be validated in a larger South Asian population.

The performance of D-dimer, P-selectin and vWF as biomarkers of CVD risk in South Asians was modest in comparison to CRP and non-fasting triglycerides. But before ruling them out as poor markers, a prospective follow up study needs to be conducted to associate these markers with incident CVD in South Asians.

BNP, an established marker of LVSD was also studied in this thesis mainly because the increased risk of CHD observed in South Asians might predispose them to higher prevalence of LVSD and HF (Blackledge, 2003; Patel, 2008a). BNP significantly discriminated South Asians with LVSD from those with normal LV function with a discriminative value of 36.4 pg/ml and high negative predictive value. The findings were similar to the ESC guidelines which recommend a cut off value of 35 pg/mL for BNP for ruling out HF in patients in non-acute setting. Since the study population was community based, the prevalence of LVSD in this study cohort was only 1.4%. To determine the performance of BNP as a marker of LVSD in South Asians, the community

based samples were combined with South Asian heart failure patients recruited from heart failure clinics who might have be more symptomatic. In order to confirm the discriminative value of BNP in the identification of LVSD in South Asians, our finding needs to be replicated in a larger South Asian population.

Genetic-association studies in South Asians

The thesis also looked at candidate gene approach and SNPs that have been extensively studied in European population. The aim was to dissect the underlying pathophysiology for increased CVD risk observed amongst the South Asian population by studying SNPs that contribute to the variation in inflammatory and metabolic phenotypes involved in the development of CVD. Even though the SNPs of interest were associated with their phenotypes, their lack of association with CVD makes them unlikely to have any clinical implications.

The *CRP* promoter polymorphism rs3091244 contributed minimally to variation in CRP levels in South Asians and were not associated with clinical CVD. Genetic studies so far argue against a causal relationship between CRP and CVD and show this marker as a mere bystander with no active role in the development of CVD (Kardys, 2006; Elliot et al., 2009). Again *APOA5* -1131T>C was an independent determinant of plasma triglyceride levels in the South Asian population and was not associated with CVD. Prior studies have associated *APOC3* promoter polymorphisms with serum triglyceride levels and CVD, but these findings were not replicated in our South Asian cohort.

There can be a number of reasons for the lack of association between the polymorphisms and CVD in our cohort. One of the major limitations was that the study subjects were recruited randomly through a population based study in the UK. The overall prevalence of CVD was only 24.0% and the low numbers may have failed to detect a significant association. Apart from this,

CVD is a multi-factorial disease with other multiple rare variants that could be associated with CVD. Clinical correlates can also influence the relationship between genetic variants and CVD.

Novel biomarkers in South Asians

This thesis explored novel biomarkers in South Asians targeting putative mechanisms of disease. In a pilot case-control study, haemoglobinopathies were linked to CVD in South Asians which requires further exploration using a larger cohort. Aberrant erythropoiesis is associated with haemolytic diseases and this could increase iron availability in the circulation (Rother, 2005: Levy, 2006) and potentially lead to iron transport on lipoproteins but this concept needs further investigation.

Increased body iron stores have been implicated in the pathogenesis of CVD and diabetes mellitus (Sullivan, 1981; Jiang, 2004). A novel in-house ELISA for ferritin bound to apolipoprotein B was developed which showed elevated levels of ferritin bound to ApoB in non-diabetic South Asians compared to non-diabetic Caucasians. Whether this interaction is of pathological significance needs to be further investigated (Main findings of this thesis are shown in table 137).

Diversity in the South Asian population

In our study population, 49% of South Asians were of Indian descent, 35% of Pakistani origin and hence the observation may not be applicable to other ethnic groups among whom the CVD risk profile varies. Smoking was significantly higher amongst Bangladeshi men compared to Pakistani and Indian men. This is consistent with the findings from Bush (2003) community based study in New Castle. The prevalence of diabetes mellitus was higher in Pakistani men compared to Bangladeshi and Indian men. The prevalence of obesity and central obesity was also higher in Pakistani men compared to Bangladeshi and Indian men. In our cohort, the higher rates

of diabetes mellitus observed among Pakistani men could be attributed to their higher prevalence of central obesity. Bhopal (1999) demonstrated in a cross sectional study in Newcastle upon Tyne that the prevalence of diabetes mellitus was significantly higher among Pakistani and Bangladeshi men compared to Indian men. There were no significant differences in the demographic details for women belonging to Indian, Pakistani and Bangladeshi origin. The rates of diabetes and hypertension in this study cohort were similar to those rates reported from South Asian community groups living in the Birmingham area (Patel, 2007).

This thesis provides a platform for future studies

i. The population based study, E-ECHOES enables to establish screening biomarkers in South Asians for example in this thesis the performance of CRP as a discriminator of CVD and BNP as a discriminator of LVSD in South Asians was evaluated.

ii. Prospective longitudinal study - The performance of CRP, calculated & non-fasting triglycerides, vWF and BNP needs to validate in prospective longitudinal studies in order to look at hard outcomes such as acute coronary syndromes, LVSD or heart failure.

iii. Exploring novel biomarkers in South Asians- a novel ELISA was developed for ferritin bound to apolipoprotein B. There is a need to further explore the association between ferritin bound to apolipoprotein B and the disease of interest i.e. diabetes mellitus and also establish reference limits in a cross-sectional study. The structural interaction between ferritin and apolipoprotein B needs to be explored by proteomic techniques.

iv. Haemoglobinopathies were linked to CVD in South Asians, potentially through iron transportation on lipoproteins but this link needs to be further investigated in a larger cohort.

Table 137 Main findings of this thesis

Performance of circulating biomarkers in South Asians	• CRP levels in the range of 1.43-2.30 mg/L discriminated South Asians at increased risk of CVD. This level is lower than that seen in white populations. IL-6 levels did not discriminate South Asians at increased risk of CVD. • Calculated triglycerides and non-fasting triglycerides discriminated South Asians with increased risk of CVD and hence can be used as an alternative for fasting triglyceride measurements in routine clinical practice. • The performance of D-dimer, P-selectin and vWF as biomarkers of CVD risk in South Asians was modest, with only vWF discriminating South Asians with increased risk of CVD and existing CVD. • BNP significantly discriminated South Asians with LVSD from those with normal LV function with a discriminative value of 36.4 pg/ml and high negative predictive value.
Genetic-association studies in South Asians	• The *CRP* promoter polymorphism rs3091244 contributed minimally to variation in CRP levels in South Asians and were not associated with clinical CVD. • *APOA5* -1131T>C is a determinant of plasma triglyceride levels in the South Asian population; however this polymorphism was not associated with CVD. *APOC3* promoter polymorphisms are associated with serum triglyceride levels and CVD, but these findings were not replicated in our South Asian cohort.
Novel biomarkers in South Asians	• Haemoglobin disorders are common amongst South Asians with CVD and were associated with greater vascular damage and lower levels of HDL-c (Pilot study). • Development of a novel enzyme linked immunoassay-Ferritin bound to Apolipoprotein B

APPENDICES

9.1 Appendix A

9.1.1 Ethnic proportion of the UK

Table 138 Proportion of ethnic minority population in the West Midlands

West Midlands Population By Ethnic Group		
Ethnic Group	Number of People	Percentage of West Midlands population (86.2% of West Midlands Population is White British)
White British	4,537,892	86.2%
White Irish	73,136	1.4%
White Others	63,268	1.2%
White/Caribbean	39,782	0.8%
White/African	3,680	0.1%
White/Asian	18,160	0.3%
Other Mixed	11,600	0.2%
Indian	178,691	3.4%
Pakistani	154,550	2.9%
Bangladeshi	31,401	0.6%
Other Asian	20,931	0.4%
Caribbean	82,282	1.6%
African	11,983	0.2%
Other Black	9,765	0.2%
Chinese	16,099	0.3%
Others	14,083	0.3%
Total	5,267,303	100%

Table 139 Ethnic minority population in Birmingham

Proportion of Ethnic Minority Population in Birmingham	
Ethnic Minority group	Percentage living in Birmingham
Mixed	38%
Indian	31%
Pakistani	67%
Bangladeshi	66%
Other Asian	48%
Caribbean	58%
African	52%
Other Black	59%
Chinese	32%
Others	43%

Reproduced from Race for opportunity, regional factsheet, 2010

9.2 Appendix B

9.2.1 Isolation of deoxyribonucleic acid from human blood and Taqman single nucleotide polymorphism genotyping

9.2.1.1 Isolation of deoxyribonucleic acid from human whole blood using Taqman sample to SNP kit

Taqman sample to SNP kit was also used to isolate DNA from whole blood. The kit is composed of two reagents, lysis solution and DNA stabilizing solution. Preparation of sample lysate was performed according to table 140. The lysates were then stored at -20^{0}C. When performing genotying assay on lysates, taqman GTXpress master mix was used, reaction mix and cycling stage for the PCR are shown in table 141 & 142 respectively.

Table 140 Preparation of sample lysate

Sample type	Sample input	Volume of lysis solution (µl)	Incubation temperature for 3 minutes (^{0}C)	Volume of DNA stabilizing solution (µl)
Blood	2µl	20	Room temperature	20

Table 141 Reaction mix for lysate

Reagents	Volume (µl/well)
Taqman GTXpress Master mix	5.0
Taqman genotyping assay mix	0.5
DNase-free water	2.5
DNA	2.0
Total	10.0

Table 142 Cycling stage for the PCR

	Thermal cycling		
Stage	Holding stage	Cycling (40 cycles)	
		Denature	Anneal/ extend
Temperature	95^{0}C	95^{0}C	60^{0}C
Time (mm:ss) (StepOne Plus)	00.20	00:03	00:20
Time (mm:ss) (Fast 7500)	00.20	00.03	00.30

9.2.1.2 Custom designed taqman single nucleotide polymorphism assays

figure 43 Overview of the custom designed taqman SNP for rs1800795

Target sequence was obtained from the dbSNP database

at http://www.ncbi.nlm.nih.gov/SNP

dbSNP-rs1800795
ACTCAGTTCAGAACATCTTTGGTTTTTACAAATACAAATTAACTGGAACGCTAAATTCTA
GCCTGTTAATCTGGTCACTGAAAAAAAATTTTTTTTTTTTCAAAAAACATAGCTTTAGCT
TATTTTTTTTCTCTTTGTAAAACTTCGTGCATGACTTCAGCTTTACTCTTTGTCAAGACA
TGCCAAAGTGCTGAGTCACTAATAAAAGAAAAAAAGAAAGTAAAGGAAGAGTGGTTCT
GC TTCTTAGCGCTAGCCTCAATGACGACCTAAGCTGCACTTTTCCCCCTAGTTGTGTCTTGC
[G/C]ATGCTAAAGGACGTCACATTGCACAATCTTAATAAGGTTTCCAATCAGCCCCACCCGC
TCTGGCCCCACCCTCACCCTCCAACAAAGATTTATCAAATGTGGGATTTTCCCATGAGTCTC
AATATTAGAGTCTCAACCCCCAATAAATATAGGACTGGAGATGTCTGAGGCTCATTCTGC
CCTCGAGCCCACCGGGAACGAAAGAGAAGCTCTATCTCCCCTCCAGGAGCCCAGCTATGA
ACTCCTTCTCCACAAGTAAGTGCAGGAAATCCTTAGCCCTGGAACTGCCAGCGGCGGTCG

Assessing the quality of the sequence

1) The uniqueness of the sequences was established by BLAST (Basic Local Alignment Search Tool) at http://www.ncbi.nlm.nih.gov/BLAST assessed on 08.02.2010

Sequence producing significant alignment; Locus- NT 007819.17-Homo sapiens chromosome 7 genomic contig, score -588, E-value = 1e-165

2) There were no repetitive elements detected in this sequence after analysing the sequence through the program Repeat masker (http://repeatmasker.genome.washington.edu)

3) Specialized BLAST was performed to identify SNPs not of interest and was masked with a N.

ACTCAGTTCAGAACATCTTTGGTTTTTACAAATACAAATTAACTGGAACGCTAAATTCTA
GCCTGTTAATCTGGTCACTNAAAAAAAAANTTNTTTTTTTNCAAAAANCATAGCTTTAGCT
TATTTTTTTTCTCTTTGTAAAACTTCGTGCATGACTTCAGCTTTACTCTTTGTCAAGACA
TGCCAAAGTGCTGAGTCACTAATAAAAGAAAAAAAGAANGTAAAGGAAGAGTGGTTCTGC
TTCTTAGCGCTAGCCTCAATGACGACCTAAGCTGCACTTTTNCCCNCTAGTTGTGTCTTGC
[G/C]ATGCTAAAGGACGTCACATTGCACAATCTTAATAAGGTTTCCAATCAGCCCCACCCGCTC
TGNCCCCACCCTCACCCTCCAACAAAGATTTATCAAATGTGGGATTTTCCNATGAGTCTC
AATATTAGAGTCTCAACCCCCAATAAATATAGGACTGGAGATGTCTGAGGCTCATTCTGC
CCTCGANCNCACCGGGAACGAAANAGAAGCTCTATCTCCCCTCCAGGAGCCCAGCTATGA
ACTCCTTCTCCACAAGTAAGTGCAGGAAATCCTTAGCCCTGGAACTGCCAGCGGCGGTCG

4) The masked sequence with SNP of our interest was then submitted to Applied Biosytems for taqman SNP genotyping assay

figure 44 *Overview of the custom designed taqman SNP for rs2854116*

Target sequence was obtained from the dbSNP database at

http://www.ncbi.nlm.nih.gov/SNP

dbSNP-rs2854116
ATCCCTAGGAGACTGAGTCCACGCTGCTGTCCCGCCAGCCCTGCAGCCCAGATGAGCTCA
GGAACTGGGGGTGGGCCTGGGGAGCCCTCATTCCTTCCTAGCTGACTGGCTCCCCAGGGA
GAGGCTGGTGAGAGGGGAAATGGCTTGGACATAGGCCAGGGGCCTCGGGCCCATCTCAG
C CTTTCACACTGGAATTTCAGGCCCCTCCCTCCACCAGCCCCAAGCCCTGAACACAGCCTG
GAGTAGAGGGGTGAGGGGCTTCTTCAGACTTGAGAACAAGTGGGTGGCTTGGGCTGGGGG
[A/G]TGTTTGGAGTAAAGGCACAGAAGACCAGGCATCAGTGGCTCCGGCAAGGCCTGGTTA
CAGGGCTGAGCTCTCACAGCCCCTCCCAGCACCTCCATCTCTGGGTTTCAATCCAGGCAGGC
GAGTGCTGCTCACCCAGCAGCCCCCCACCCGCCCCCACCCTGTGTGCCCCCCGCTGCCTCC
CCCTTAGTGTAGGGCAGGGGTTGGTGGAGAAGGGCCAAGGCCGCTCAGAGCCCGAGGCCT
TTGCCCCTCCCTCCACCAGGCTCCCTATTTTGCCCTCTGGACCCACTGATAACATCCCCT

Assessing the quality of the sequence

1) The uniqueness of the sequences was established by BLAST (Basic Local Alignment Search Tool) at http://www.ncbi.nlm.nih.gov/BLAST assessed on 20.02.2010

Sequence producing significant alignment; Locus- NT 033899.8 -Homo sapiens chromosome 11 genomic contig, score – 1105, E value = 0.0

2) There were no repetitive elements detected in this sequence after analysing the sequence through the program Repeat masker (http://repeatmasker.genome.washington.edu)

3) Specialized BLAST was performed to identify SNPs not of interest and was masked with a N.

ATCCCTAGGAGACTGAGTCCACGCTGCTGTCCCGCCAGCCCTGCAGCCCAGATGAGCTCAG
GAACTNGGGGTGGGCCTGGGGAGCCCTCATTCCTTCCTAGCTGACTGGCTCCCCAGGGAGA
GGCTGGTGAGAGGGGAAATGGCTTGGACATAGGCCAGGNGCCTCGGGCCCATCTCAGCCTT
TCACACTGGAATTTCAGGCCCCTCCCTCCACCAGCCCCAAGCCCTGAACACAGCCTGGAGTA
GAGGGGTGAGGGGCTTCTTCAGACTTGAGANCAAGTGGGTGGCTTGGGCTGGGGG**[A/G]**TGT
TTGGAGTAAAGGCACAGAAGACCNGGCATCAGTGGCTCCGGCAAGGCCTGGTTACAGGGCTG
AGCTCTCACAGCCCCTCCCAGCACCTCCATCTCTGGGTTTCAATCCAGGCAGGCGAGTGCTGC
TCACCCAGCAGCCCCCCACCCGCCCCCACCCTGTGTGCCCCNCGCNGCCTCCCCCTNAGTGTA
GGGCAGGGGTTGGTGGAGAAGGGCCAAGGCCGCTCAGAGCCCGAGGCCTTTGCCCCTCCCTC
CACCAGGCTCCCTATTTTGCCCTCTGGACCCACTGATAACATCCCCT

4) The masked sequence with SNP of our interest was then submitted to Applied Biosytems for taqman SNP genotyping assay

figure 45 *Overview of the custom designed taqman SNP for rs2854117*

Target sequence was obtained from the dbSNP database at
http://www.ncbi.nlm.nih.gov/SNP

dbSNP-rs2854117
TGTCCCGCCAGCCCTGCAGCCCAGATGAGCTCAGGAACTGGGGGTGGGCCTGGGGAGCCC
TCATTCCTTCCTAGCTGACTGGCTCCCCAGGGAGAGGCTGGTGAGAGGGGAAATGGCTTG
GACATAGGCCAGGGGCCTCGGGCCCATCTCAGCCTTTCACACTGGAATTTCAGGCCCCTC
CCTCCACCAGCCCCAAGCCCTGAACACAGCCTGGAGTAGAGGGGTGAGGGGCTTCTTCAG
ACTTGAGAACAAGTGGGTGGCTTGGGCTGGGGGGTGTTTGGAGTAAAGGCACAGAAGACC
[G/A]GGCATCAGTGGCTCCGGCAAGGCCTGGTTACAGGGCTGAGCTCTCACAGCCCCTCCC
AGCACCTCCATCTCTGGGTTTCAATCCAGGCAGGCGAGTGCTGCTCACCCAGCAGCCCCCC
AC CCGCCCCCACCCTGTGTGCCCCCCGCTGCCTCCCCCTTAGTGTAGGGCAGGGGTTGGTGG
AGAAGGGCCAAGGCCGCTCAGAGCCCGAGGCCTTTGCCCCTCCCTCCACCAGGCTCCCTA
TTTTGCCCTCTGGACCCACTGATAACATCCCCTGGGGGAGGAGCTGGTCTCATGTCTGGG

Assessing the quality of the sequence
1) The uniqueness of the sequences was established by BLAST (Basic Local Alignment Search Tool) at
http://www.ncbi.nlm.nih.gov/BLAST assessed on 19.03.2010

Sequence producing significant alignment; Locus- NT 033899.8 -Homo sapiens chromosome 11 genomic contig, score – 1105, E value = 0.0

2) There were no repetitive elements detected in this sequence after analysing the sequence through the program Repeat masker. (http://repeatmasker.genome.washington.edu)

3) Specialized BLAST was performed to identify SNPs not of interest and was masked with a N.

TGTCCCGCCAGCCCTGCAGCCCAGATGAGCTCAGGAACTNGGGGTGGGCCTGGGGAGCCC
TCATTCCTTCCTAGCTGACTGGCTCCCCAGGGAGAGGCTGGTGAGAGGGGAAATGGCTTG
GACATAGGCCAGGNGCCTCGGGCCCATCTCAGCCTTTCACACTGGAATTTCAGGCCCCTC
CCTCCACCAGCCCCAAGCCCTGAACACAGCCTGGAGTAGAGGGGTGAGGGGCTTCTTCAG
ACTTGAGANCAAGTGGGTGGCTTGGGCTGGGGGNTGTTTGGAGTAAAGGCACAGAAGACC
[G/A]GGCATCAGTGGCTCCGGCAAGGCCTGGTTACAGGGCTGAGCTCTCACAGCCCCTCCC
AGCACCTCCATCTCTGGGTTTCAATCCAGGCAGGCGAGTGCTGCTCACCCAGCAGCCCCCC
AC CCGCCCCCACCCTGTGTGCCCCNCGCNGCCTCCCCCTNAGTGTAGGGCAGGGGTTGGTG
GAGAAGGGCCAAGGCCGCTCAGAGCCCGAGGCCTTTGCCCCTCCCTCCACCAGGCTCCCTA
TTTTGCCCTCTGGACCCACTGATAACATCCCCTGGGGGAGGAGCTGGTCTCATGTCTGGG

4) The masked sequence with SNP of our interest was then submitted to Applied Biosytems for taqman SNP genotyping assay

Genomic wet DNA was used for genotyping experiments. Negative controls (sterile water) and two positive controls for the SNPs rs1800795, rs2854116, rs2854117 were analysed on all plates to ensure there was no contamination. The reaction volume for each well was set to 20 µl (Table 143) and number of cycles to 50 (Table 144).

Table 143 Reaction mix

Reagents	Volume (µl/ 96 reactions)
2 × Taqman SNP genotyping mastermix	1008.00
20 × Taqman SNP genotyping assay	50.40
DNAse-free water	756
Genomic DNA template	2 µl /well
Total volume	20.00/ well

Table 144 Cycling stage for the PCR

	Pre-PCR read	Thermal cycling			Post-PCR read
Stage	Holding stage	Holding stage	Cycling (50 cycles)		Holding stage
			Denature	Anneal/ extend	
Temperature	60^0C	95^0C	92^0C	60^0C	60^0C
Time (mm:ss)	00:30	10:00	00:15	01:00	00:30

9.2.2 Allele, genotype frequencies and hardy Weinberg equilibrium

The following equations were used for calculating allele, genotype frequencies and to determine whether the study population met Hardy Weinberg's theory (Hardy-Weinberg and population genetics calculation, online resource).

Allele frequencies were calculated using the following equations;

For example major allele is depicted as A and minor allele as a.

The frequency of the major allele = (total number of major alleles in the population/ total number of alleles in population for locus), where total number of major alleles in the population = no of homozygotes for A allele (AA*2) + no of heterozygotes (Aa) & total number of alleles in population for locus in diploid individuals = total number of individuals*2 (2 alleles at locus per individual)

The frequency of the minor allele = (total number of minor allele in the population/ total number of alleles in population for locus), where total number of minor allele in the population = no of homozygotes for minor allele, a (aa*2) + no of heterozygotes (Aa).

Genotype frequencies were calculated as follows;

Frequency of AA genotype (homozygotes for major allele) = number of AA individuals / population size

Frequency of Aa genotype (heterozygotes) = number of Aa individuals / population size

Frequency of aa genotype (homozygotes for mutant allele = number of aa individuals / population size

For the population to meet the assumptions of the Hardys Weinberg, the observed genotype frequencies calculated from the real population should agree with the expected frequencies

predicted by the Hardy-Weinberg Theory, the following equation derived from Hardy-Weinberg Equilibrium Theory was used;

p2 + 2pq + q2 = 1, where p^2 = Frequency of genotype A/A, 2pq = Frequency of genotype A/a., q = Frequency of genotype a/a

9.2.3 Zonal density gradient ultracentrifugation

EDTA treated blood samples were used to separate very low density lipoprotein (VLDL), LDL, HDL_2 and HDL_3 sub fractions using the Optima TLX Ultracentrifuge (Beckman Coulter, High Wycombe UK). Plasma from these samples were analysed within 4 days of collection. A sodium chloride/potassium bromide (NaCl/KBr) density gradient solution (density 1.30g/ml) was used to adjust the density of the plasma (density 1.006g/ml) to 1.063g/ml (density of LDL). The following equation was used to calculate the volume of density gradient solution to be added to plasma.

Volume of density gradient solution to be added = 2.1 ml (volume of plasma) × (1.063 – 1.006) / (1.30 -1.063)

The same equation was used to calculate the volume of density gradient solution to be added to prepare different density salt solutions.

The Beckman tubes were layered as follows; 1ml of 1.21 of density salt solution was added using a syringe to the bottom of the tube, followed by 1ml of 1.125 density salt solution using a dispenser pump (pump flow rate:0.17ml/min, volume:1ml), then 2.1ml of 1.063g/ml of plasma was applied using the dispenser pump (pump flow rate: 0.15ml/min, volume flow:2.1ml) and finally 1ml of saline (density 1.006g/ml) was added over the plasma layer using the dispenser pump (flow rate:0.17 ml/min, volume:1ml). The dispenser pump controls the flow rate of liquid

accurately and can minimise the mixing of the different layers when preparing the tubes. The prepared tubes were then sealed with a heat sealer and immediately placed in vertical rotors in the Optima TLX Ultracentrifuge at 4^0C and spun at 110,000 rpm for 7.38 hours.

At the end of each run, lipoprotein fractions were collected using a fractional recovery system and dispenser pump. Before cutting the top of the Beckman tube, approximately 0.1ml of VLDL layer was collected using a syringe and needle. The tubes were then placed into the fractional recovery system which is connected to the dispenser pump. The layers were then collected as follows, 1ml of VLDL (density 1.006g/ml) from the top of Beckman tube was transferred to VLDL tube and made up to 2ml using saline, followed by 2.1 ml of LDL (density 1.006-1.063 g/ml) in the upper middle into LDL tube, then 1ml of HDL_2 (density 1.063 -1.125 g/ml) layer in the lower upper middle into the HDL_2 tube and finally HDL_3 (density 1.125 – 1.21 g/ml) in the bottom portion of the Beckman tube into HDL_3 tube (Winocour *et al*, 1991). Levels of iron were measured on separated lipoprotein sub fractions on the Cobas Integra 400 (Roche Diagnostics, UK).

9.2.4 Mass spectrometry for identification of hemoglobin variants by ESI-MS

The objective of this experiment was to assess the presence of single-point mutation haemoglobin variants, high δ-chain and high glycation levels without knowledge of their disease status in 174 South Asian samples by ESI-MS. Analysis was performed on a Xevo Triple Quandrupole (Waters Corporationm Milford, MA, USA) equipped with a TriVersa NanoMate (Advion Biosciences Ltd, Ithaca, NY, USA).

Blood samples were prepared for analysis by diluting 0.5 µl of blood in 250 µl of 50% acetonitrile 0.2% HCOOH within a 96-well plate, compatible with the TriVersa NanoMate. All samples were analysed twice and for each sample a three minute acquisition was performed in multi-channel acquisition (MCA) mode at a scan speed of two spectra/sec. BioPharmaLynx software was used for data processing. The software allows to deconvolute multiple spectral outputs and compares the results (Wild et al, 2001).

The analysis identified the masses and intensities of all peaks present within the deconvoluted spectrum. The masses for the α, β, δ and glycated globin chains were pre-defined within the software and automatically detected within each sample. All data files obtained were analysed by the BioPharmaLynx method. The mass and intensity derived for alpha, beta ,delta chains and glycated alpha and beta chains and any peaks observed in the spectrum at greater than 20% intensity was imported into Excel. Excel was then used to compare the results for α-chain measurement, δ-chain intensity and glycated α- and β-chain intensity between samples. The error in α- and β-chain measurement was used to indicate whether a α- or β-chain variant with a mass shift of 6 Dalton (Da) or less was present. The presence of any additional peaks within the spectra at greater than 20% intensity suggested the presence of a structural variant resulting from a mass shift of more than 6 Da.

The percentage of glycated haemoglobin (HbA1c) was calculated as described by Roberts *et al* (2001) from intensity information obtained for the α- and β-chain and singly glycosylated α- and β-chain species from the deconcoluted spectrum for each sample. The principle behind this approach being that the measurement of glucose addition on the β chain can be quantified by a mass increase of 162 Da to the native protein chain where a mass of 180 Da is attributed to the addition of glucose through the elimination of water (-18 Da).

The following equation was used to calculated % glycosylated haemoglobin;

% glycosylated haemoglobin = $50[\alpha_g/(\alpha+\alpha_{g)} + \beta_g/(\beta + \beta_g)]$ where α and β represent the intensities of the α- and β-chains, and α_g and β_g represent the intensities of the glycated α- and β-chains respectively. The measurement of glycosylated haemoglobin level is based on several assumptions such as there is no significant contribution to glycosylated haemoglobin from other globin chains, that all α-chain species and β-chain species have the same sensitivity.

The estimation of δ-chain levels was achieved by performing a ratio calculation of δ-chain intensity to β-chain intensity. The assumption being that the β- and δ-chains have similar sensitivities. This approach uses a δ: β ratio of the intact chains to provide a surrogate marker of HbA_2 levels.

9.3 Appendix C

9.3.1 The performance of C-reactive protein as a discriminator of cardiovascular risk in a homogeneous Indian community in India and a homogeneous Indian community in the UK

Table 145 C-reactive protein as a discriminator of metabolic and cardiovascular disease risk amongst non- migrant Gujarati Indians

C-reactive protein (mg/L)	Raised blood pressure (>130 mmHg or 85 mmHg) (84, 28.4% were hypertensive)			Body mass index (> 27kg/m^2) (29, 9.8% were obese)		
	P value	AUC	95% CI	P value	AUC	95% CI
	<0.001	0.664	0.600– 0.729	<0.001	0.786	0.716-0.856
Cut-off value for CRP (mg/L)	1.09 mg/L			1.68 mg/L		
C-reactive protein (mg/L)	Central obesity (≥ 90 cm in men and ≥ 80 cm in women) (63, 23.0% were obese)			Diabetes mellitus (Oral glucose tolerance test) (73, 27.1% had either T2DM/ glucose tolerance)		
	P value	AUC	95% CI	P value	AUC	95% CI
	<0.001	0.745	0.681-0.809	0.003	0.617	0.539-0.694
Cut-off value for CRP (mg/L)	1.31 mg/L			1.05 mg/L		
C-reactive protein (mg/L	Low HDL cholesterol (<0.9 mmol/l in men &<1.0 mmol/l in women) (78, 27.0% had low-HDL-c)			Elevated triglycerides (≥ 1.7 mmol/l) (21, 7.1% had raised triglycerides)		
	P value	AUC	95% CI	P value	AUC	95% CI
	<0.001	0.641	0.571-0.711	<0.001	0.729	0.621-0.837
Cut-off value for CRP (mg/L)	1.16 mg/L			1.43 mg/L		
C-reactive protein (mg/L)	Metabolic syndrome* (ATP III defined) (27, 9.5% had metabolic syndrome)			10 years CHD risk score (>20%) (26, 10.3% had high CVD risk)		
	P value	AUC	95% CI	P value	AUC	95% CI
	<0.001	0.758	0.680-0.837	0.001	0.691	0.603-0.779
Cut-off value for CRP (mg/L)	1.57 mg/L			1.43 mg/L		

Table 146 C-reactive protein as a discriminator of metabolic and cardiovascular disease risk amongst migrant Gujarati Indians in the UK

C-reactive protein (mg/L)	Raised blood pressure (>130 mmHg or 85 mmHg) (116, 49.1% were hypertensive)			Body mass index (> 27kg/m^2) (89, 38.2% were obese)		
	P value	AUC	95% CI	P value	AUC	95% CI
	0.001	0.628	0.557-0.698	<0.001	0.691	0.622-0.759
Cut-off value for CRP (mg/L)	1.32 mg/L			1.44 mg/L		
C-reactive protein (mg/L)	Central obesity (≥ 90 cm in men and ≥ 80 cm in women) (112, 52.0% were obese)			Diabetes mellitus (Oral glucose tolerance test) (27, 14.0% had either T2DM/ glucose tolerance)		
	P value	AUC	95% CI	P value	AUC	95% CI
	0.001	0.637	0.563-0.710	0.001	0.701	0.584-0.818
Cut-off value for CRP (mg/L)	1.30 mg/L			1.91 mg/L		
C-reactive protein (mg/L	Low HDL cholesterol (<0.9 mmol/l in men &<1.0 mmol/l in women) (30,13.0% had low-HDL-c)			Elevated triglycerides (≥ 1.7 mmol/l) (35, 15.0% had raised triglycerides)		
	P value	AUC	95% CI	P value	AUC	95% CI
	0.286	0.560	0.452-0.669	0.099	0.413	0.307–0.518
Cut-off value for CRP (mg/L)	NS			NS		
C-reactive protein (mg/L)	Metabolic syndrome* (ATP III defined) (43,19.0% had metabolic syndrome)			10 years CHD risk score (>20%) (20, 9.2% had high CVD risk)		
	P value	AUC	95% CI	P value	AUC	95% CI
	<0.001	0.720	0.631-0.810	0.006	0.686	0.568-0.803
Cut-off value for CRP (mg/L)	1.89 mg/L			2.19 mg/L		

NS- non significant

9.3.2 The performance of obesity and central obesity as discriminators of metabolic and Cardiovascular disease risk in South Asians asymptomatic for cardiovascular disease

BMI significantly discriminated those with diabetes mellitus, [AUC 0.579 (95% CI, 0.502 – 0.65), p=0.050], raised triglycerides [AUC 0.581 (95% CI 0.528 – 0.634), p=0.003] and metabolic syndrome [AUC 0.623 (95% CI 0.574 -0.671), p<0.001] from healthy South Asians. Waist circumference significantly discriminated those with raised blood pressure [AUC 0.555 (95% CI 0.502 -0.608), p=0.041], T2DM [AUC 0.618 (95% CI .543 – 0.693), p=0.003], raised triglycerides [AUC 0.626 (95% CI 0.575 – 0.677), p<0.001], metabolic syndrome [AUC 0.677 (0.631 – 0.724), p<0.001] and 10 year CVD risk score [AUC 0.582 (0.508 – 0.655), p=0.040].

Table 147 Obesity and central obesity as discriminators of metabolic and cardiovascular disease risk amongst South Asian men and women asymptomatic for cardiovascular disease

Obesity						
	Diabetes mellitus (HbA1c ≥ 6.5%)			Elevated measured triglycerides (≥ 1.7 mmol/l)		
	P value	AUC	95% CI	P value	AUC	95% CI
Body mass index (kg/m^2)	0.050	0.579	0.502-0.650	0.003	0.581	0.528-0.634
	Metabolic syndrome* (ATP III defined) (243, 48.1% had metabolic syndrome)					
	P value	AUC	95% CI			
Body mass index (kg/m^2)	<0.001	0.623	0.574-0.671			
Central obesity						
	Raised blood pressure (>130 mmHg or 85 mmHg)			Diabetes mellitus (HbA1c ≥ 6.5%)		
	P value	AUC	95% CI	P value	AUC	95% CI
Waist circumference (cm)	0.041	0.555	0.502-0.608	0.003	0.618	0.543-0.693
	Elevated measured triglycerides (≥ 1.7 mmol/l)			Metabolic syndrome* (ATP III defined)		
	P value	AUC	95% CI	P value	AUC	95% CI
Waist circumference (cm)	<0.001	0.626	0.575-0.677	<0.001	0.677	0.631-0.724
	10 years CHD risk score (>20%)					
	P value	AUC	95% CI			
Waist circumference (cm)	0.040	0.582	0.508–0.655			

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